

# Demonstration of Bacterial Cells and Glycocalyx in Biofilms on Human Tonsils

Romain E. Kania, MD, PhD; Gerda E. M. Lamers, BSc; Marcel J. Vonk, BSc; Patrice Tran Ba Huy, MD; Pieter S. Hiemstra, PhD; Guido V. Bloemberg, PhD; Jan J. Grote, MD, PhD

**Objectives:** To demonstrate mucosal biofilms in human tissue by direct visualization of bacteria and glycocalyx using confocal laser scanning microscopy with double fluorescent staining on tonsils and to compare the findings with the results of scanning electron microscopy analysis.

**Design:** Prospective study.

**Setting:** Tertiary university-based referral center.

**Patients:** Twenty-four tonsils were obtained from children with chronic or recurrent tonsillitis.

**Interventions:** Tonsils were prepared for analysis by scanning electronic microscopy and by confocal laser scanning microscopy.

**Main Outcome Measures:** Double fluorescent staining for confocal laser scanning microscopy consisted of propidium iodide staining to detect bacterial cells and fluorescein isothiocyanate concanavalin A staining to

detect the glycocalyx matrix. Images were analyzed for characteristic biofilm morphologic features by 3 investigators who evaluated the images independently in a blinded retrospective manner. Consensus of all observers was required to demonstrate the presence of a biofilm in a specimen.

**Results:** Findings from analyses using scanning electronic microscopy suggested the presence of biofilm formations on tonsils by showing bacterial cells in microcolonies. Double-staining technique using confocal laser scanning microscopy showed bacterial cells and the glycocalyx matrix, providing visual evidence for the presence of biofilms on tonsils.

**Conclusion:** Using a novel visualization approach in single sections of human mucosal tissue, the presence of biofilms was demonstrated on tonsils in most (17/24 [70.8%]) patients with tonsillitis.

*Arch Otolaryngol Head Neck Surg.* 2007;133:115-121

## Author Affiliations:

Departments of Otorhinolaryngology–Head and Neck Surgery (Drs Kania and Grote and Mr Vonk) and Pulmonology (Dr Hiemstra), Leiden University Medical Center, and Institute of Biology Leiden (Ms Lamers and Dr Bloemberg), Leiden University, Leiden, the Netherlands; and Department of Otorhinolaryngology–Head and Neck Surgery, Lariboisière Hospital, Assistance Publique–Hôpitaux de Paris and Laboratoire de Neurobiologie des Réseaux Sensorimoteurs, Paris, France (Drs Kania and Tran Ba Huy).

**I**N MANY CHRONIC INFECTIONS, bacteria are present in biofilms, which are encased communities of bacteria in a self-produced matrix (also called *glycocalyx*) and which adhere to, divide, and persist on surfaces.<sup>1-4</sup> The general process of biofilm formation comprises adhesion of free-living or planktonic bacteria to a surface, which subsequently develop into microcolonies and form a biofilm. Whereas planktonic bacteria are generally susceptible to host-defense mechanisms and to antibiotic treatments, bacteria in biofilms are substantially resistant.<sup>2,5-8</sup> Bacterial cells in biofilms can be up to 1000 times more resistant to antimicrobial treatments than planktonic bacteria of the same species.<sup>5</sup> Biofilm formations represent a serious clinical problem because it has been estimated that more than 65% of all human bacterial infections involve biofilms.<sup>4</sup>

Based on recent studies<sup>9-16</sup> that showed the presence of biofilms in common sites

of chronic infections, it has become clear that bacteria may persist on mucosal surfaces through formation of biofilms. However, the presence of biofilms on airway surfaces has been demonstrated using a limited number of techniques. Proper demonstration of mucosal biofilms is challenging because it is difficult to stain both bacteria and glycocalyx in human tissue. Light and electron microscopy techniques require a dehydration process that reduces the total volume of the matrix and alters its architecture.<sup>17</sup> For a fundamental understanding of the formation and presence of bacterial biofilms, analysis should include detection of the bacteria and the matrix. Detection of the matrix could be achieved using a double-staining technique in combination with confocal laser scanning microscopy (CLSM), which allows simultaneous imaging of the structural elements of a mucosal biofilm (ie, cells and glycocalyx).<sup>18,19</sup> To stain glycocalyx, CLSM can be used in combination

with fluorescent-labeled lectins that bind to carbohydrates of the matrix.<sup>20,21</sup> Although the use of this technique has been reported in skin specimens,<sup>17,22,23</sup> the question remains as to whether it is applicable to biofilms on a mucosal surface, especially because local conditions determine the formation, structure, and behavior of biofilms.<sup>24</sup> The bacteria, microenvironment, host constituents, and host inflammatory response also modulate the composition of the extracellular matrix. Therefore, the question exists whether mucosal biofilms can be visualized by double staining in the mucosa of the upper airways.

Whereas much is known about biofilm formation on inert surfaces under laboratory conditions, little is known about mucosal biofilms. Therefore, the objective of this study was to demonstrate mucosal biofilms by double staining in human tissue. We chose tonsil specimens for this purpose because (1) tonsillitis is a common disease of the upper airways, (2) the presence of biofilms has been reported previously using electron microscopy techniques,<sup>10</sup> and (3) tonsillectomy is one of the most frequent surgical procedures performed worldwide. In this study, we provide the first direct evidence (to our knowledge) of mucosal biofilms in humans by direct visualization of bacteria and glycocalyx in tonsils removed for chronic or recurrent tonsillitis.

## METHODS

### TISSUE COLLECTION

Tonsil specimens were obtained during routine tonsillectomy from children with chronic or recurrent tonsillitis ( $n=24$ ). The Committee of Medical Ethics, Leiden University Medical Center, Leiden, the Netherlands, approved the protocol. The specimens were washed in phosphate-buffered saline and were cut in 2 parts to be prepared for scanning electron microscopy (SEM) and for CLSM.

### SCANNING ELECTRON MICROSCOPY

The specimens for SEM were fixed in 1.5% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.4) for 24 hours at 4°C on a rotary shaker. The samples were then 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 (Polaron 5000; Polaron Equipment Ltd, Watford, Hertfordshire, England) before imaging. The specimens were examined in a scanning electron microscope (JSM-6400; JEOL Ltd, Tokyo, Japan) with digital imaging capabilities. The images were collected at an acceleration voltage of approximately 5.0 kV, a filament current of approximately  $10^{-10}$ A, and a working distance of approximately 39 mm. All images were digitized as high-resolution TIFF computer files (resolution, 635 dots per inch) and were then converted to high-quality JPEG files using commercially available software (Adobe Photoshop version 7.0; Adobe Systems Inc, San Jose, Calif).

### CONFOCAL LASER SCANNING MICROSCOPY

After removal, the specimens for CLSM were immediately snap frozen in cold isopentane on dry ice and stored at  $-80^{\circ}\text{C}$ . The

frozen tissue specimens were cut to a thickness of 10  $\mu\text{m}$  at  $-24^{\circ}\text{C}$  using a cryostat (CM 3050S; Leica Microsystems, Bensheim, Germany) and were fixed in 70% acetone. The obtained sections were then processed for double staining. They were washed 3 times in phosphate-buffered saline solution and were first stained with 15 $\mu\text{M}$  propidium iodide for 5 minutes at room temperature to detect bacterial cells in red. After being washed in phosphate-buffered saline, the sections were incubated with 50  $\mu\text{g}/\text{mL}$  of concanavalin A (Con A) fluorescein isothiocyanate (FITC) (C7642; Sigma-Aldrich Inc, St Louis, Mo) for 5 minutes at room temperature to stain the glycocalyx matrix green. The sections were then successively washed in a combined solution of phosphate-buffered saline and demineralized water and were embedded in a 3-dimensional matrix (Gelvatol-DABCO mixture, Sigma-Aldrich Inc). The sections were examined using an upright microscope (Axioplan; Carl Zeiss, Oberkochen, Germany) equipped with a scan head (MRC1024ES; BioRad, Hercules, Calif) with a krypton-argon laser for visualization of Con A FITC (number of signals acquired, 488 nm; emission 552 disconnecting filter [DF], 32 nm) and propidium iodide (number of signals acquired, 568 nm; emission 605 DF, 32 nm). Digital images of the CLSM optical sections were collected using commercially available software (Lasersharp 2000, BioRad). Merged red and green images were obtained in single TIFF format and were converted to high-quality JPEG files using Adobe Photoshop version 7.0.

## IMAGE ANALYSIS

Three investigators (R.E.K., G.E.M.L., and G.V.B.) evaluated the images independently in a blinded retrospective manner. Images were analyzed for characteristic biofilm morphologic features using the following criteria: (1) the presence of bacteria, as recognized by size, morphologic features, and red fluorescent propidium iodide staining on CLSM images, and (2) the presence of glycocalyx, as seen on CLSM images by bright green fluorescence owing to Con A FITC staining. A specimen was scored positive if more than 1 biofilm formations (based on the 2 criteria) were observed at an airway specimen interface or in crypts of the specimen. Specimens that showed artifacts of cutting for SEM and CLSM were excluded. When bacteria were identified without any matrix surrounding it, they were considered attached individual cells or bacterial microcolonies, and the specimen was not retained as containing a biofilm. No attempt was made to quantify biofilm formation. Consensus of all observers was required to demonstrate the presence of a biofilm in a specimen.

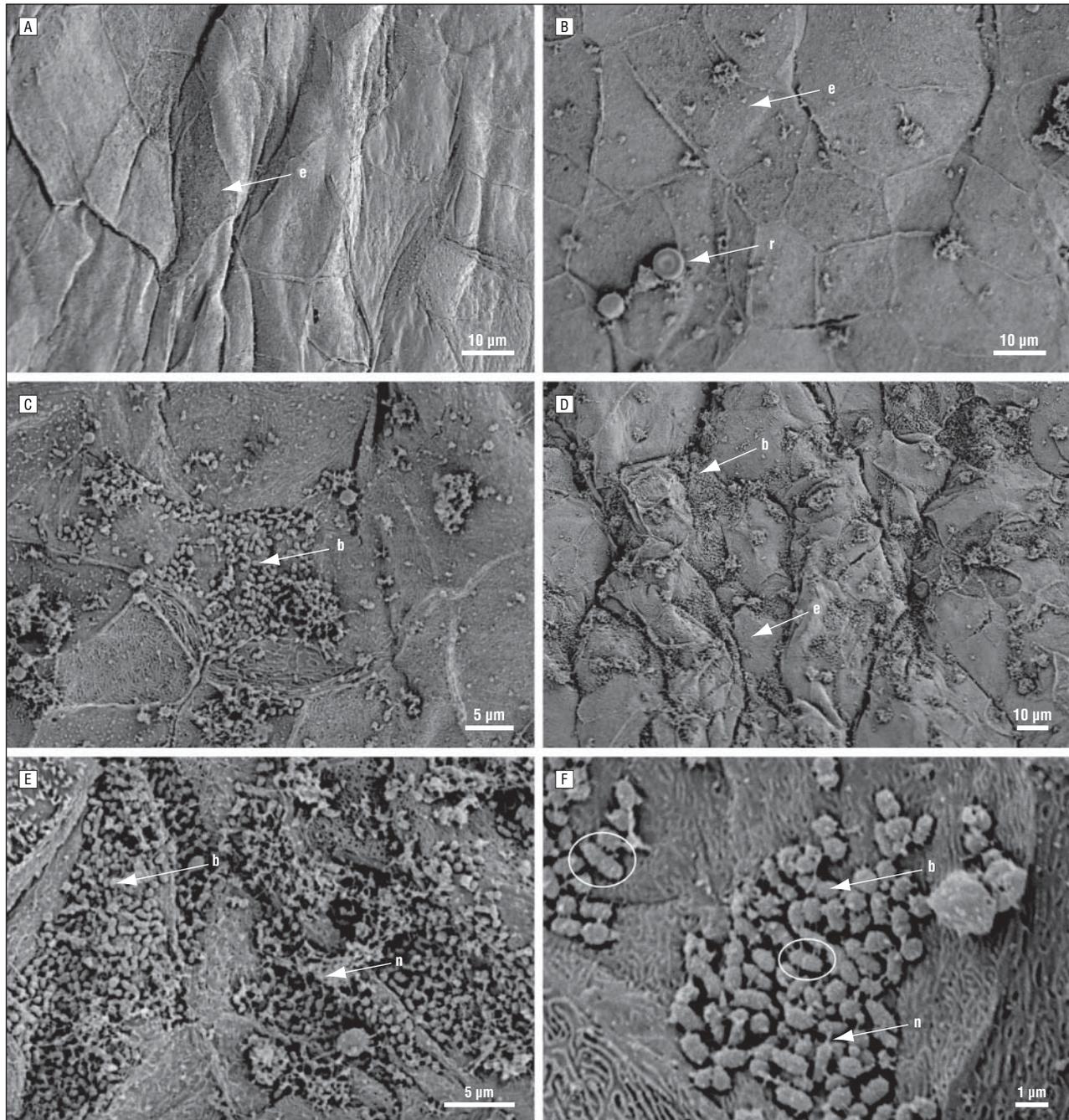
## RESULTS

### TISSUE SPECIMENS

Twenty-four tonsils were collected. They were randomly selected for processing and for surface analysis by SEM or by CLSM.

### SCANNING ELECTRON MICROSCOPY

Analysis of various tonsils by SEM showed normal-appearing mucosa composed of epithelial cells with some small villi, ridges, or debris (**Figure 1A** and **B**). Attached bacteria were present on the surface of the specimen and could be clearly distinguished from smaller anorganic materials or irregularities of the epithelium nearby (**Figure 1C** and **D**). The number of attached bac-



**Figure 1.** Scanning electron microscopy images of human tonsils. Epithelial cells (e) are associated with red blood cells (r). Attached bacterial cells (b) were organized in microcolony formations and were connected in a networklike organization (n). Dividing bacteria were observed (encircled cells). A, Normal-appearing mucosa is composed of epithelial cells with some small villi. B, Small isolated debris and red blood cell on the surface of normal-appearing mucosa. C, Attached bacteria organized in microcolony formations. D, Microcolony formations between epithelial cells and in small depressions or crypts with widespread colonization of the tonsil surface. E, Bacterial cells connected by extracellular materials resulting in a networklike organization. F, Dividing bacterial cells observed in the microcolonies.

teria was variable among specimens. The cells were not evenly distributed over the entire surface of the specimen but rather were clustered and formed microcolonies (Figure 1D). These microcolonies were mostly located in small depressions between epithelial cells. Higher magnifications confirmed that the microcolony formations were preferentially located at the junction of the epithelial cells in small holes or crypts (Figure 1E and F). Bacterial cells were connected by extracellular ma-

terial, which could represent a glycocalyx matrix. Bacteria seemed to be organized in a scaffolding network located in small crypts. Dividing bacterial cells were observed within the microcolonies (Figure 1F). Because of the presence of blood cells and clots, the surfaces of 10 specimens could not be evaluated for the presence of attached bacteria, especially in the crypts of the tonsil. Attached bacteria were seen on the surface of the tonsils in 5 (35.7%) of 14 remaining specimens.

## CONFOCAL LASER SCANNING MICROSCOPY

To visualize bacterial cells and the surrounding glycocalyx matrix (which is indicative of bacterial biofilm formation) present on the surface of tonsillar tissue, double staining was performed using propidium iodide and Con A FITC. Bacterial cells and nuclei of tonsillar cells stained red, whereas binding of Con A resulting in green staining indicated the presence of a bacterial glycocalyx. **Figure 2** shows single bacterial cells alone (Figure 2A) or encased in a glycocalyx matrix (Figure 2B). Biofilms were observed at the outside (Figure 2C) and in crypts (Figure 2D) of the tissue section. A Con A-staining matrix generally, but not exclusively, encased bacterial clusters (Figure 2E). Most of the bacteria were cocci shaped. Interconnected bacteria were encased in a scaffolding network composed of extracellular matrix, suggesting a 3-dimensional architecture of biofilm formations (Figure 2F). All 24 tonsils were suitable for CLSM analysis. Seventeen tonsils (70.8%) showed evidence of mucosal biofilms.

### COMMENT

The results from the present study demonstrate the presence of bacterial biofilms on tonsillar tissue obtained by resection from patients with chronic or recurrent tonsillitis using classic SEM analysis and a novel CLSM imaging approach to demonstrate mucosal biofilms in human tissue. This CLSM approach allowed the detection of both bacterial cells and the glycocalyx matrix of a biofilm in a single-tissue section.

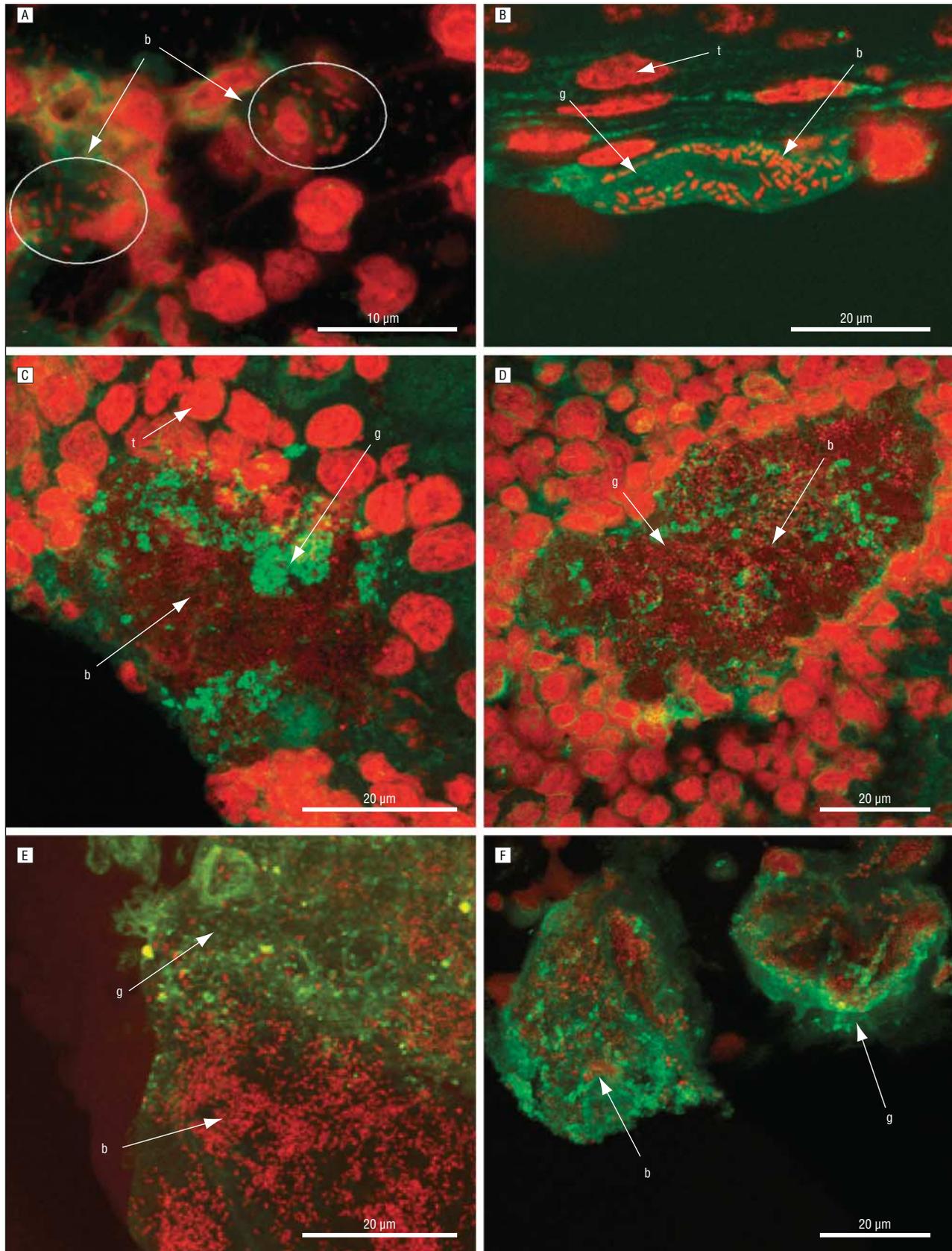
The demonstration of biofilms in human tissue sections is technically challenging and has so far relied mostly on the use of SEM and transmission electron microscopy (**Table**). Scanning electron microscopy imaging of the tonsil surface showed the presence of single and clustered bacteria attached to the surface and in the crypts (Figure 1C-F). In addition, these images clearly showed bacterial cell division (Figure 1F). Bacterial cells present in a biofilm are usually encased in a hydrated (typically 95%-99%) matrix, which is prone to dehydration artifacts. When using SEM or transmission electron microscopy, only the remnants of the original hydrated structure can be visualized.<sup>17,19</sup> In our study, SEM analysis was hampered by the presence of blood cells on the surface of the tissue, masking bacterial presence on these surfaces. Therefore, not all of the tissue specimens collected were suitable for SEM analysis.

In contrast to electron microscopy, preparation of tissue for CLSM does not require dehydration and better preserves the structure of a biofilm.<sup>19</sup> To our knowledge, CLSM has not been used to detect biofilms on human tonsils that show the presence of bacterial cells and the glycocalyx matrix of a biofilm in a single section. Ehrlich et al<sup>12</sup> used an animal model to monitor biofilm formation using a bacterial viability kit (Live/Dead BacLight; Molecular Probes, Eugene, Ore). However, no attempts in that study were made to stain glycocalyx. Post<sup>14</sup> investigated biofilms on tympanostomy tubes removed from 3 children by CLSM using green fluores-

cent staining for bacteria identification, without staining for glycocalyx. In a recent study,<sup>26</sup> biofilm formation by nontypeable *Haemophilus influenzae* was demonstrated in the middle ear of infected chinchilla using lectin staining to detect sialylation associated with biofilm formation. Bacterial cells in our study were easily identified based on their size and morphologic features, despite the fact that the nuclei of tonsillar cells also stained using propidium iodide.<sup>12</sup> We used Con A to detect sugar residues present in glycocalyx, using a technique previously described.<sup>17,22</sup> Concanavalin A binds to mannose residues that are not unique for the bacterial glycocalyx. Nevertheless, we observed marked colocalization of green Con A staining with clusters of bacterial cells (Figure 2). The staining of the matrix with Con A FITC was not homogeneously distributed. The presence of dark areas within the biofilm can be explained by (1) the existing water channels, (2) the heterogeneous production of the matrix and the types of exopolysaccharides within the biofilm, and (3) the absence of Con A binding to the matrix.<sup>18,28</sup> Together, our data show that CLSM analysis of propidium iodide- and Con A-stained tissue sections provides a novel approach to demonstrate the presence of mucosal biofilms in human tissue.

So far, the presence of mucosal biofilms in human tissue has been primarily reported using SEM or transmission electron microscopy. Several authors<sup>11,15,25,27</sup> recently described biofilms in chronic sinusitis using gram staining and transmission electron microscopy, techniques that require a dehydration process. Electron microscopy has been used to demonstrate biofilm formations on indwelling silicone rubber tracheoesophageal voice prostheses,<sup>29</sup> endotracheal tubes removed from intubated neonates,<sup>30</sup> and frontal recess stents in patients with chronic rhinosinusitis.<sup>31</sup> In these studies, the structure of the biofilm matrix could not be studied because of problems inherent in the technique. Because of the combination of techniques used, our study allowed the demonstration of both bacteria and glycocalyx. Confocal laser scanning microscopy with double staining was superior to SEM in our study for demonstrating the presence of human mucosal biofilms in tonsillitis.

Compared with the published findings about biofilms on tonsillar tissues,<sup>10</sup> we observed a similar biofilm prevalence of 70.8% among our patients with tonsillitis. In addition, our data suggest the dynamic process of biofilm formation and development through (1) the metabolic activity of cell division, (2) the multiplicity of biofilm formations observed, and (3) the association of individual attached cells and mature biofilms. Chole and Faddis<sup>10</sup> speculate that biofilms found in 73.3% (11/15) of their tonsillitis specimens could account for the recalcitrant nature of some cases of recurrent tonsillitis. The concept that tonsillitis might be a biofilm illness is supported by the observation that bacteria in biofilms are remarkably resistant to host defenses and antibiotics.<sup>2,5-8,24,32-34</sup> However, a limitation of the present study is the lack of a control group. Tissue from an appropriate control group is ethically problematic to obtain because it should be composed of tonsillar tissue from age-matched control subjects who never had infection of the upper airways. This was not feasible in our study.<sup>10</sup>



**Figure 2.** Confocal laser scanning microscopy images demonstrating bacterial biofilms on human tonsils. Bacterial cells (b) in biofilm, as well as surrounding nuclei of tonsillar cells (t), stained red (propidium iodide). Green fluorescent staining (concanavalin A) around bacteria indicates the presence of glycocalyx (g). A, Bacterial cells seen as single cells (encircled cells). B, Bacterial cells seen encased in a glycocalyx matrix. C, Biofilm formations shown on the surface of the tissue. D, Biofilm formations shown in crypts. E, Concanavalin A staining matrix mostly, but not exclusively, encasing bacterial clusters. F, Interconnected bacteria encased in a scaffolding network of glycocalyx forming a 3-dimensional structure.

**Table. Review of Mucosal Biofilms Reported In Situ on the Mucosa of Upper and Lower Airways in Human or Animal Models**

Source	Study Model	Nature of Chronic Infection	No. of Specimens	Investigation
Lam et al, <sup>13</sup> 1980	Human	Postmortem lung of patients with CF	2	SEM
Post, <sup>14</sup> 2001	Animal	Otitis media	17	SEM
Chole and Faddis, <sup>9</sup> 2002	Human	Cholesteatoma	24	Gram, TEM
	Animal	Cholesteatoma	22	Gram, TEM
Ehrlich et al, <sup>12</sup> 2002	Animal	Otitis media	45	SEM, CLSM
Chole and Faddis, <sup>10</sup> 2003	Human	Tonsils	19	Gram, TEM
Cryer et al, <sup>11</sup> 2004	Human	Sinusitis	16	SEM
Ramadan et al, <sup>15</sup> 2005	Human	Sinusitis	5	SEM
Perloff and Palmer, <sup>16</sup> 2005	Animal	Sinusitis	22	SEM
Sanclément et al, <sup>25</sup> 2005	Human	Sinusitis	30	SEM, TEM
Jurcisek et al, <sup>26</sup> 2005	Animal	Middle ear	18	SEM, CLSM
Ferguson and Stolz, <sup>27</sup> 2005	Human	Sinusitis	4	TEM

Abbreviations: CF, cystic fibrosis; CLSM, confocal laser scanning microscopy; Gram, gram staining; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Another difficulty is that hypertrophied tonsils contain pathogenic bacteria. In the study reported by Chole and Faddis,<sup>10</sup> 3 of 4 enlarged tonsils that were removed because of hypertrophy and obstruction had evidence of biofilms. Hence, the presence of biofilms does not prove causality in chronic tonsillitis, and further studies are needed to elucidate whether the presence of biofilm formation is associated with the pathogenesis of tonsillitis. If the presence of biofilms is involved in the mechanisms of chronic tonsillitis, this notion would have implications for the development of treatment modalities for patients with chronic or recurrent tonsillitis that are focused on the eradication of biofilms.<sup>3</sup>

## CONCLUSIONS

The results from our study show that CLSM combined with fluorescent staining of both bacteria and glycocalyx provides a novel approach for the demonstration of mucosal biofilms in human tissue. More investigation is needed to evaluate the role of biofilm formation in the pathogenesis of chronic or recurrent tonsillitis.

**Submitted for Publication:** January 29, 2006; final revision received September 16, 2006; accepted September 24, 2006.

**Correspondence:** Romain E. Kania, MD, PhD, Department of Otorhinolaryngology–Head and Neck Surgery, Lariboisière Hospital, Assistance Publique-Hôpitaux de Paris and Laboratoire de Neurobiologie des Réseaux Sensorimoteurs, 2 rue Ambroise Paré, 75010 Paris, France (romain.kania@lrh.ap-hop-paris.fr).

**Author Contributions:** Drs Kania, Tray Ba Huy, Hiemstra, Bloemberg, and Grote, Ms Lamers, and Mr Vonk had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Kania, Lamers, Vonk, Tran Ba Huy, Hiemstra, Bloemberg, and Grote. *Acquisition of data:* Kania and Bloemberg. *Analysis and interpretation of data:* Kania, Hiemstra, Bloemberg, and Grote. *Drafting of the manuscript:* Kania, Lamers, Vonk, Hiemstra, and Bloemberg. *Critical revision of the manu-*

*script for important intellectual content:* Kania, Tran Ba Huy, Hiemstra, Bloemberg, and Grote. *Obtained funding:* Kania, Tran Ba Huy, Hiemstra, and Bloemberg. *Administrative, technical, and material support:* Kania, Lamers, Vonk, and Bloemberg. *Study supervision:* Hiemstra, Bloemberg, and Grote.

**Financial Disclosure:** None reported.

**Funding/Support:** This study was supported by the 2004 Prix de Recherche Perspective en Otorhinolaryngologie from Sanofi Synthelabo Aventis (Dr Kania) and by the NRJ Foundation, Institut de France (Dr Tran Ba Huy).

**Acknowledgment:** We are grateful to the following attending otorhinolaryngologists and head and neck surgeons who assisted us in collecting specimens: R. Baatenburg de Jong, MD, PhD; J. Frijns, MD, PhD; K. Brenkman, MD, PhD; F. Otten, MD, PhD; J. Jansen, MD, PhD; N. Peek, MD; C. Rus, MD; R. Bonnet, MD; and M. Schmitt, MD.

## REFERENCES

- Costerton JW, Geesey GG, Cheng KJ. How bacteria stick. *Sci Am*. 1978;238:86-95.
- Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science*. 1999;284:1318-1322.
- Parsek MR, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol*. 2003;57:677-701.
- Potera C. Forging a link between biofilms and disease. *Science*. 1999;283:1837-1839.
- Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. *Trends Microbiol*. 2005;13:34-40.
- Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol*. 2004;2:95-108.
- Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. *Annu Rev Microbiol*. 2002;56:187-209.
- Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet*. 2001;358:135-138.
- Chole RA, Faddis BT. Evidence for microbial biofilms in cholesteatomas. *Arch Otolaryngol Head Neck Surg*. 2002;128:1129-1133.
- Chole RA, Faddis BT. Anatomical evidence of microbial biofilms in tonsillar tissues: a possible mechanism to explain chronicity. *Arch Otolaryngol Head Neck Surg*. 2003;129:634-636.
- Cryer J, Schipor I, Perloff JR, Palmer JN. Evidence of bacterial biofilms in human chronic sinusitis. *ORL J Otorhinolaryngol Relat Spec*. 2004;66:155-158.
- Ehrlich GD, Veeh R, Wang X, et al. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA*. 2002;287:1710-1715.
- Lam J, Chan R, Lam K, Costerton JW. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect Immun*. 1980;28:546-556.

14. Post JC. Direct evidence of bacterial biofilms in otitis media. *Laryngoscope*. 2001; 111:2083-2094.
15. Ramadan HH, Sanclement JA, Thomas JG. Chronic rhinosinusitis and biofilms. *Otolaryngol Head Neck Surg*. 2005;132:414-417.
16. Perloff JR, Palmer JN. Evidence of bacterial biofilms in a rabbit model of sinusitis. *Am J Rhinol*. 2005;19:1-6.
17. Akiyama H, Hamada T, Huh WK, et al. Confocal laser scanning microscopic observation of glycocalyx production by *Staphylococcus aureus* in skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus. *Br J Dermatol*. 2003; 148:526-532.
18. Lawrence JR, Neu TR. Confocal laser scanning microscopy for analysis of microbial biofilms. *Methods Enzymol*. 1999;310:131-144.
19. McLean RJC, Bates CL, Barnes MB, McGowin CL, Aron GM. Methods of studying biofilms. In: Ghannoum M, O'Toole GA, eds. *Microbial Biofilms*. Washington, DC: ASM Press; 2004:379-413.
20. Kennedy JF, Palva PMG, Corella MTS, Cavalcanti MSM, Coelho LCB. Lectins, versatile proteins of recognition: a review. *Carbohydr Polym*. 1995;26:219-230.
21. Strathmann M, Wingender J, Flemming HC. Application of fluorescently labelled lectins for the visualization and biochemical characterization of polysaccharides in biofilms of *Pseudomonas aeruginosa*. *J Microbiol Methods*. 2002;50:237-248.
22. Akiyama H, Huh WK, Yamasaki O, Oono T, Iwatsuki K. Confocal laser scanning microscopic observation of glycocalyx production by *Staphylococcus aureus* in mouse skin: does *S aureus* generally produce a biofilm on damaged skin? *Br J Dermatol*. 2002;147:879-885.
23. Akiyama H, Morizane S, Yamasaki O, Oono T, Iwatsuki K. Assessment of *Streptococcus pyogenes* microcolony formation in infected skin by confocal laser scanning microscopy. *J Dermatol Sci*. 2003;32:193-199.
24. Post JC, Stoodley P, Hall-Stoodley L, Ehrlich GD. The role of biofilms in otolaryngologic infections. *Curr Opin Otolaryngol Head Neck Surg*. 2004;12:185-190.
25. Sanclement JA, Webster P, Thomas J, Ramadan HH. Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis. *Laryngoscope*. 2005; 115:578-582.
26. Jurcisek J, Greiner L, Watanabe H, Zaleski A, Apicella MA, Bakaletz LO. Role of sialic acid and complex carbohydrate biosynthesis in biofilm formation by non-typeable *Haemophilus influenzae* in the chinchilla middle ear. *Infect Immun*. 2005; 73:3210-3218.
27. Ferguson BJ, Stolz DB. Demonstration of biofilm in human bacterial chronic rhinosinusitis. *Am J Rhinol*. 2005;19:452-457.
28. Neu TR, Lawrence JR. Lectin-binding analysis in biofilm systems. *Methods Enzymol*. 1999;310:145-152.
29. Leunisse C, van Weissenbruch R, Busscher HJ, van der Mei HC, Dijk F, Albers FW. Biofilm formation and design features of indwelling silicone rubber tracheoesophageal voice prostheses: an electron microscopical study. *J Biomed Mater Res*. 2001;58:556-563.
30. Zur KB, Mandell DL, Gordon RE, Holzman I, Rothschild MA. Electron microscopic analysis of biofilm on endotracheal tubes removed from intubated neonates. *Otolaryngol Head Neck Surg*. 2004;130:407-414.
31. Perloff JR, Palmer JN. Evidence of bacterial biofilms on frontal recess stents in patients with chronic rhinosinusitis. *Am J Rhinol*. 2004;18:377-380.
32. Costerton W, Veeh R, Shirtliff M, Pasmore M, Post C, Ehrlich G. The application of biofilm science to the study and control of chronic bacterial infections. *J Clin Invest*. 2003;112:1466-1477.
33. Hoyle BD, Costerton JW. Bacterial resistance to antibiotics: the role of biofilms. *Prog Drug Res*. 1991;37:91-105.
34. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*. 2002;15:167-193.