ORIGINAL ARTICLE

Evidence of Bacterial Biofilms in Nasal Polyposis

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Abstract

Introduction: The pathogeny of chronic rhinosinusitis with nasal polyposis (CRS/NP) has not been elucidated. Bacterial exotoxins have been implicated in many inflammatory chronic diseases, such as chronic otitis, chronic tonsillitis, cholesteatomas, and more recently CRS/NP. We propose that the bacteria in CRS/NP are not only present in a planktonic state, but also occur in microbial communities as biofilms.
Objective: To determine and characterize the presence of biofilms in CRS/NP.
Methods: We performed a prospective study in 12 patients undergoing endoscopic sinus surgery for nasal polyposis. Ten patients without CRS/NP who underwent septoplasty were included as a control group. Tissue samples were obtained from the inferior turbinate mucosae. The bacteria were isolated and typified and the material was examined in vitro using a spectrophotometer, and in vivo using optical microscopy and confocal scanning laser microscopy.
Results: Moderate to high in vitro biofilm-forming capacity was detected in 9 out of 12 patients with CRS/NP (mean [SD] optical density values of between 0.284 [0.017] and 3.337 [0.029]). The microorganisms isolated were *Staphylococcus* (5 patients), *Streptococcus viridans, Pseudomonas aeruginosa, Enterococcus faecalis* and *Streptococcus viridans/Corynebacterium*. Biofilms were demonstrated in vivo in 2 patients and no biofilm structures were evident in any of the controls.
Conclusion: This study demonstrates the presence of bacterial biofilms in patients with CRS/NP. This chronic inflammatory factor might contribute to nasal mucosa damage, increased inflammatory cells in tissue, and the subsequent hyperplasic process.

Key words: Biofilms. Nasal polyposis. Chronic rhinosinusitis. Optical microscopy. Confocal scanning laser microscopy.

Resumen

Introducción: Todavía no se ha explicado la patogenia de la rinosinusitis crónica con poliposis nasal (RSC/PN). Se ha sugerido la implicación de las exotoxinas bacterianas en muchas enfermedades inflamatorias crónicas, como la otitis crónica, la tonsilitis crónica, los colesteatomas y, más recientemente, la RSC/PN. En este artículo se propone que las bacterias en la RSC/PP no solo se encuentran presentes en estado planctónico, sino que también se observan en comunidades microbianas como biopelículas.
Objetivo: Determinar y caracterizar la presencia de biopelículas en la RSC/PN.
Métodos: Se realizó un estudio prospectivo en 12 pacientes sometidos a cirugía endoscópica sinusal para poliposis nasal. Diez pacientes sin RSC/PN que se sometieron a septoplastia se incluyeron como grupo de control. Se obtuvieron muestras de tejido de la mucosa del cornete inferior. Las bacterias se aislaron y tipificaron, y se estudió el material in vitro utilizando un espectrofotómetro, e in vivo mediante microscopía óptica y microscopía confocal de barrido láser.
Resultados: Se detectó una capacidad *in vitro* de formación de biopelícula moderada o alta en 9 de los 12 pacientes con RSC/PN (valores de densidad óptica medios [DE] de entre 0,284 [0,017] y 3,337 [0,029]). Los microorganismos aislados fueron *Staphylococcus* (5 pacientes), *Streptococcus viridans, Pseudomonas aeruginosa, Enterococcus faecalis* y *Streptococcus viridans/Corynebacterium*. Dos pacientes presentaron biopelículas in vivo y no se hallaron indicios de estructuras de biopelícula en ninguno de los controles.
Conclusión: En este estudio se demuestra la presencia de biopelículas bacterianas en pacientes con RSC/PN. Este factor inflamatorio crónico puede contribuir a causar daños en la mucosa nasal, un aumento de células inflamatorias en el tejido y el consiguiente proceso hiperplásico.

Palabras clave: Biopelículas, poliposis nasal, rinosinusitis crónica, microscopía óptica, microscopía confocal de barrido láser.

Introduction

The association between bacterial colonization and different forms of chronic rhinosinusitis with and without nasal polyps remains unclear. The most recent hypothesis suggests the involvement of superantigen (SA)-induced inflammation, particularly involving enterotoxins from *Staphylococcus aureus*. According to this theory, enterotoxins might activate a type 2 T helper cell response, leading to an inflammatory process. The most important cytokine involved is interleukin 5, responsible for the recruitment of eosinophils, which are the main cells involved in the production of nasal polyps [1-2].

Bacteria exist in 2 main forms: as free-floating planktonic replicating cells and in biofilms, which are defined as organized communities of collaborating bacteria that are attached to an inert or living surface contained in a self-produced polymeric matrix primarily composed of exopolysaccharides, nucleic acids, and proteins [3-6]. The structural nature of biofilms and the characteristics of sessile cells produce resistance against antimicrobial agents, resulting in an environment that affords protection against adverse conditions and the host´s defenses [7-9].

The planktonic form of bacteria has greatly helped to improve our understanding of acute infections. Chronic infections, in turn, are more closely related to the presence of biofilms, with current research indicating an important role for bacterial biofilms in recurrent or chronic infections, including those which are not responsive to culture-appropriate antibiotic therapy. In particular, biofilms are involved in chronic otitis, chronic tonsillitis, cholesteatomas, and other inflammatory and infectious disorders [5,6]. The link between biofilms and chronic infectious disease is currently a subject of investigation [7].

Furthermore, bacterial exotoxins have been found to be involved in the pathogenesis of many inflammatory chronic diseases, such as atopic dermatitis, asthma, and more recently, chronic rhinosinusitis with nasal polyposis (CRS/NP). A possible role of *S. aureus* in polyposis has been suggested by numerous studies that have demonstrated the presence of immunoglobulin (Ig) E antibodies against enterotoxins and inflammatory changes in the nasal polyp [10,11]. This bacterium, and possibly others, produces toxins that act as superantigens and conventional allergens, possibly providing the initial trigger for the inflammation of the lateral wall of the nose [11-13]. Biofilms could be the reservoir of bacteria responsible for chronic and persistent inflammation.

We propose that bacteria are not only present in a planktonic state, but also occur in microbial communities with sessile cells, leading to the formation of biofilms. The aim of this study was to determine and characterize the presence of these biofilms in nasal polyposis. A secondary aim was to establish the presence of staphylococcal biofilms in nasal polyps in support of the superantigen theory.

Methods

Study Design and Population

We performed a prospective observational study of nasal polyps from patients with CRS/NP who underwent endoscopic sinus surgery at the Otolaryngology Department of Sanatorio Allende, in Córdoba, Argentina. The group consisted of 5 women and 7 men (mean age, 43 years; range, 24-81 years) with a diagnosis of CRS/NP confirmed by endoscopy and a sinus computed tomography (CT) scan. The patients' relevant clinical history and physical examination findings were obtained following confirmation of diagnosis. Information on asthma, allergy, aspirin sensitivity, previous surgery, and antimicrobial therapy before surgery was recorded. CT scans were scored by means of the Lund-Mackay staging system. All the material collected during endoscopic sinus surgery was sent for anatomopathologic examination. Ten patients scheduled for septoplasty for nasal obstruction, without a history or physical evidence of recurrent sinus infections or nasal discharge, served as a control group. Tissue samples were obtained from the inferior turbinate mucosa. The ethics and research committee of Sanatorio Allende and the Faculty of Chemical Sciences of the Universidad Nacional de Córdoba approved the study, and patients gave their written consent before donating tissue for the study.

Bacterial Isolation and Biofilm Quantification

The bacteriological study was performed by scraping the surface of the polyps with a scalpel and examining the samples following Gram staining. The samples were seeded in blood agar and thioglycollate (anaerobic) medium prior to incubation for 72 hours. Identification of the bacteria was performed using classical biochemical tests and antimicrobial susceptibility was tested using the Kirby-Bauer disk diffusion method.

In vitro biofilm-forming capacity was assessed in the bacteria isolated using a previously described method by Tool & Kotler [14]. An overnight culture corresponding to an optical density (OD) of 0.8 at 600 nm was analyzed with a spectrophotometer (UV-1601 PC Visible Spectrophotometer; Shimadzu, Kyoto, Japan). Dilutions of the bacterial suspension (1/100) were distributed in 96-well plates and incubated at 37°C without agitation for 24 hours. After drying, staining for adherent biofilms was performed using crystal violet 1%. A quantitative assessment of biofilm formation was made by extracting the crystal violet with 200 µL/well of bleaching solution (ethanol/glacial acetone, 70:30). The intensity of the coloration was determined by measuring the absorbance at 595 nm using a microplate reader (Model 680; BioRad, Hercules, California, USA) [15].

The mean OD590nm value was determined using 4 replicates and interpreted according to the following scale: positive (≥0.24), weak (≥0.12 and <0.24), and negative: (<0.12) [16].

Biofilm Study

The criteria for the in vivo classification of bacterial biofilms were the presence of characteristic bacterial morphology and micro and macrocolonies for examination by optical microscopy and the presence of a surrounding polysaccharide blush and tower formation by confocal scanning laser microscopy (CSLM) [3,4].

The presence of biofilms in nasal polyp samples obtained

during surgery was studied by Gram staining. The material was cut with a microtome and observed under an optical microscope (Zeiss Axiovert; Carl Zeiss, Jena, Germany). Semiquantification of the bacterial biofilms was performed according to the following arbitrary scale: 0 micro or macrocolonies (-); <1 microcolony in 50 fields (+); <1 macrocolony in 50 fields (++); 1-9 micro or macrocolonies in 50 fields (+++); and ≥10 micro or macrocolonies in 50 fields (++++).

The biofilms were observed by CSLM as described below [6]. Prior to imaging, they were rinsed with sterile 50 mM potassium phosphate buffer (pH 7.2; no autofluorescence detected) for 10 minutes and then stained with propidium iodide and fluorescein isothiocyanate (FITC). The propidium iodide was excited at 520 nm, with monitoring of emission at 620 nm; the corresponding figures for FITC were 495 nm and 525 nm, respectively. Intact biofilms were examined nondestructively using a Fluoview FV1000 Espectral Olympus CSLM (Olympus Latin America, Miami, Florida, USA) equipped with a UPlanSApo 100X/1.40 oil UIS2 Olympus oil immersion lens (NA 1.40; Olympus, Melville, NY). Optical sections of 0.87 µm were collected from the full thickness of the biofilms. For each sample, images from 3 randomly selected positions were obtained and analyzed using an Olympus Fluoview (FV1000; Olympus, Tokyo, Japan). For image analysis, 3 investigators (N.A.V., I.A., and M.G.P.) evaluated the images independently in a blinded retrospective manner.

Controls

Biofilm- and nonbiofilm-producing bacterial strains of each of the species tested were used as references. Four wells with 200 µL of TSB were added as negative controls and to obtain a background value, which was then subtracted from values obtained from the wells containing cells.

Results

Twelve patients with CRS/NP who underwent functional endoscopic sinus surgery were included in the study; 75% of these (n=9) had asthma and 83.3% (n=10) had a diagnosis of allergic rhinitis. It is well known that the risk of recurrence of nasal polyposis is associated with many factors. In our group, 41.6% of the patients (n=5) had ASA triad. The Lund-Mackay CT scan scores for the group ranged between 13 and 24, with an average of 21 points, reflecting extensive paranasal sinus involvement. The score was higher than 20 points for 75% of the group (n=9). Finally, 58.3% of the patients (n=7) had a history of sinus surgery, which is another predictor of relapse. Some patients had been treated with antimicrobial therapy (directed against the planktonic bacteria) before surgery. Details of the patients' characteristics are shown in Table 1.

The pathologic analysis revealed inflammatory polyps of the nasal mucosae, with lymphocytic and eosinophilic cellular infiltration in all cases.

The 12 patients and 10 controls were analyzed for the presence of biofilms in vivo and in vitro. Bacterial strains were isolated in 9 of the 12 patients with CRS/NP. The microorganisms detected were *S. aureus* (in 3 patients), *Streptococcus viridans* and coagulase-negative staphylococci (in 2 patients), and *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Streptococcus viridans* associated with Corynebacterium (all in 1 patient each) (Table 2). Planktonic bacteria were found in the controls in isolated forms, with no evidence of biofilm structures in any of the samples.

In vitro biofilm-forming capacity was detected in all the bacterial isolates from the patients with CRS/NP. Staphylococcal biofilms were found in the nasal polyps of 5 patients. Quantification of the isolated strains by spectrophotometry was positive with high in vitro biofilm-forming capacity and absorption values that ranged from 0.284 (SD, 0.017) to 3.337 (0.029) of OD590nm in all cases (Table 2).

Bacteria were identified by Gram staining for optical microscopy and by fluorescent staining (with propidium iodide and FITC) for CSLM. Optical microscopy revealed the presence of in vivo biofilms in 2 patients, with 1 or 2 macrocolonies per 50 fields (+++) (Table 2). Some structures were present on the surface of the polyp in one of the patients, whereas in the others they were located in the inner part of the

Table 1. Clinical Summary

Patient No.	Sex	Age, y	Asthma	Allergic Rhinitis	Aspirin Sensitivity	Lund and Mackay Score	History of Surgery	Previous Antibiotic Therapy
1	F	25	Yes	Yes	No	23	No	Yes
2	F	50	Yes	Yes	No	20	Yes	No
3	M	43	Yes	Yes	Yes	24	Yes	No
4	M	24	No	Yes	No	13	No	No
5	M	81	Yes	Yes	Yes	24	Yes	No
6	M	35	Yes	Yes	No	14	No	Yes
7	F	49	Yes	Yes	Yes	24	Yes	No
8	M	39	No	No	No	18	Yes	No
9	F	32	Yes	Yes	Yes	20	Yes	No
10	F	53	Yes	Yes	No	24	Yes	Yes
11	M	28	No	Yes	No	22	No	Yes
12	M	59	Yes	No	Yes	23	No	No

Abbreviations: F, female; M, male.

Table 2. Bacterial Biofilm Results

Patient No.	Isolated Bacteria	Biofilm Quantification		
		UV-Quantification, OD.59nm (SD)	Scale	Semiquantitative Assessment by Optical Microscopy
1	No development	–	–	–
2	*Streptococcus viridians*	3.337 (0.029)	Positive	–
3	*Staphylococcus aureus*	1.237 (0.014)	Positive	–
4	*Staphylococcus aureus*	0.332 (0.003)	Positive	–
5	*Pseudomonas aeruginosa*	0.956 (0.062)	Positive	–
6	No development	–	–	+++
7	*Streptococcus viridans/ Corynebacterium*	0.312 (0.019)	Positive	+++
8	Coagulase-negative staphylococci	0.316 (0.019)	Positive	–
9	*Staphylococcus aureus*	0.428 (0.012)	Positive	–
10	*Enterococcus faecalis*	0.428 (0.017)	Positive	–
11	No development	–	–	–

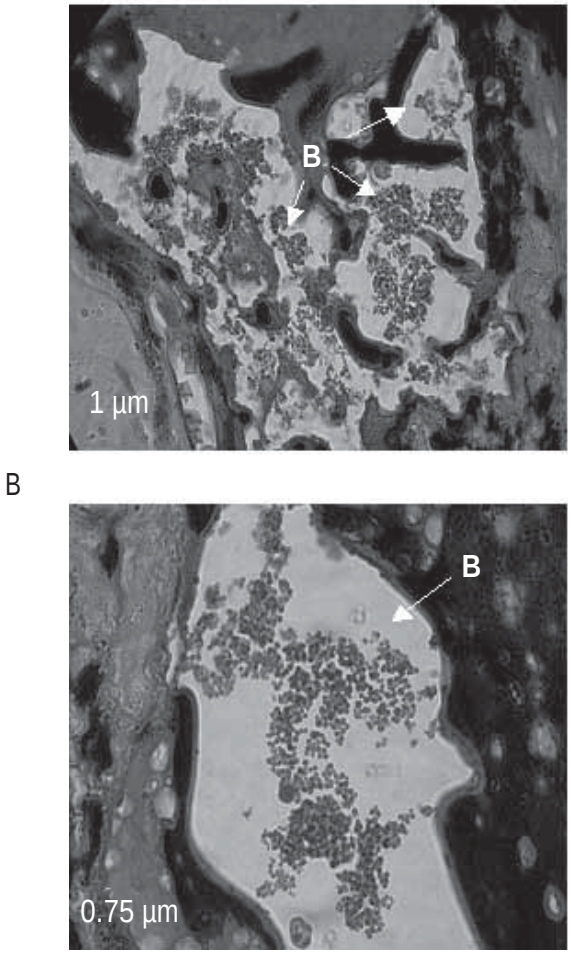

Figure 1. Biofilms in nasal polyps studied under an optical microscope following Gram staining. Bacterial biofilms within invaginations of nasal polyps. The colony appears as a densely packed mass of bacteria. (Original magnification: A, ×100; B, ×200).

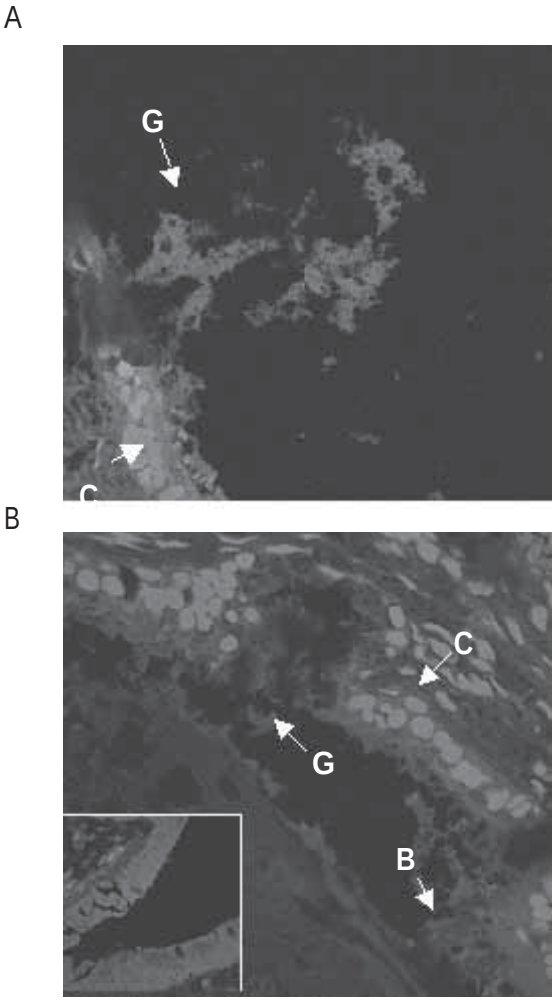

Figure 2. Biofilm structure observed by confocal scanning laser microscopy. Note the stained bacteria and the surrounding diffuse glycocalyx. A, nasal polyp surface; B, polyp invagination and negative control (inset). B indicates bacteria; G, glycocalyx; and C, host cells.

invaginations (Figure 1). Specific demonstration of biofilms by CSLM was based on the fluorescence of bacterial DNA and the glycopolysaccharide of the matrix (Figure 2). No biofilm structures were evident in any of the controls.

Discussion

It is now known that 99% of all bacteria exist in biofilms, with only 1% living in a planktonic state. Recent publications have reported that at least 65% of all human bacterial infections involve biofilms [8-17]. In relation to this, Chole et al [3,4] described the presence of biofilms in tonsillitis and chronic otitis. In addition, Sanclement et al [17], on studying surgical specimens from patients with chronic rhinosinusitis by scanning electron microscopy, demonstrated the presence of a 3-dimensional structure, glycocalix, and water channels. However, they were not able to detect the bacteria within the biofilms using this method. Using electron microscopy, Palmer et al [5] studied the nasal mucosae of 16 patients with chronic sinusitis and different types of infection. The presence of biofilms was detected in 4 of the patients, in all cases in association with bacterial infection but not with nasal polyps.

In the present study, we demonstrated the presence of biofilms in vivo in 2 patients with CRS/NP. We also isolated 2 bacteria in another patient, a finding which could have important implications for the study of mixed biofilms. Although the bacteria isolated showed a high capacity to form biofilms in vitro, these structures were not visible by optical microscopy or CSLM in all cases. This might have been because the samples were very small and represented only a minute fraction of the total surface of the polyps. Biofilms can have a patchy distribution on the mucosa, and there may also have been limitations with the technical procedure. Furthermore, bacteria within biofilms are not easily cultured because they are protected by the matrix and nutritional requirements and culture times vary between species. Moreover, there might not have been enough bacterial inoculum from the collected tissue to give a positive culture. It should also be noted that some patients had received antibiotic therapy prior to surgery. Other authors have shown that bacterial biofilms are not present in sinus mucosa samples from control individuals, in agreement with our results [17,18]. A recent publication showed biofilms in diffuse nasal polyposis and in chronic rhinosinusitis, with evidence of biofilm presence detected in 7 patients with nasal polyps [19].

The bacteria in these biofilms, while protected from host defenses and antibiotics, actively metabolize and produce endotoxins and other virulence factors. This may, however, perpetuate an inflammatory host response, even in the absence of culturable planktonic bacteria [4]. The production of local endotoxins in nasal polyposis may also lead to chronic inflammation [3]. We detected the presence of staphylococcal biofilms in nasal polyps in 5 patients, providing further evidence in support of the superantigen theory.

CRS/NP is a multifactorial disease, but its associated chronic persistent inflammation is undoubtedly a major component of the disease [20]. This study demonstrates the presence of another chronic inflammatory factor, bacterial biofilms, which might contribute to nasal mucosa damage, increased numbers of inflammatory cells in the tissue, and subsequent hyperplasia.

Further work is required to determine the full significance of bacterial biofilms in human nasal polyposis. However, the corroboration of the presence of biofilms in 2 patients in vivo, and the demonstration of high in vitro biofilm-forming capacity in all of the bacteria isolated, suggest a nexus between CRS/NP and chronic inflammation or infection. Evaluation of samples with CSLM, the gold standard method, is important. However, the possibility of viewing biofilms with an optical technique may also be helpful in ordinary practice and contribute to improving the overall management of these patients. Further research is required to clarify the impact of these biofilms on the pathology and symptoms of patients who develop nasal polyposis.

Acknowledgments

Natalia Angel Villegas is a research fellow of CONICET and María Gabriela Paraje is a member of the Research Career of CONICET. The authors wish to thank C. Mas, M.C. Sampedro, and P. Icely for their excellent technical assistance. This work was supported by a grant from the Secretaría de Ciencia y Tecnología (Secyt 2008-2009).

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Manuscript received September 29, 2009; accepted for publication, November 20, 2009.

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