Environmental Scanning Electron Microscopy for Biofilm Detection in Tonsils

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Introduction and objective: To describe an environmental scanning electron microscopic method for the study of biofilms in clinical samples. A comparison with standard scanning electron microscopy is performed.
Patients and method: Nine patients with a past history of recurrent tonsillitis underwent tonsillectomy. Samples from each patient were obtained for both conventional and environmental scanning electron microscopy. The tonsils removed from 2 patients with sleep apnoea syndrome were used as controls.
Results: Eight of 9 tonsils had biofilms on their surface. Scanning electron microscopy showed accumulations of bacteria covered by fibrillar structures resulting from the sample dehydration process. Environmental scanning electron microscopy provided a view of bacteria embedded in a homogeneous, amorphous substance that was preserved during the examination.
Conclusions: Environmental scanning electron microscopy permits the imaging of wet systems at different degrees of dehydration. It therefore allows researchers to observe biofilms in their natural hydrated state.

Key words: Environmental scanning electron microscopy. Biofilm. Tonsillitis.

Microscopia electrónica de barrido ambiental para la detección de biopelículas en las amígdalas
Introducción y objetivo: Describir un método ambiental con microscopio electrónico de barrido para el estudio de biopelículas en muestras clínicas. Se realiza una comparación con la microscopia electrónica de barrido convencional.
Pacientes y método: Se intervino de amigdalectomía a 9 pacientes con antecedentes de amigdalitis de repetición. Se obtuvieron muestras de cada uno para microscopia electrónica de barrido tanto convencional como ambiental. Como controles se empleó las amígdalas extirpadas de 2 pacientes intervenidos por síndrome de apnea del sueño.
Resultados: De las 9 amígdalas, 8 mostraron biopelículas en su superficie. La microscopia electrónica de barrido convencional mostró acumulaciones de bacterias cubiertas de estructuras fibrilares, originadas en el procedimiento de deshidratación de las muestras. La técnica ambiental proporcionó una imagen de las bacterias sumergidas en una sustancia homogénea y amorfa, que se pudo conservar durante el examen microscópico.
Conclusiones: La microscopia electrónica de barrido en su modalidad ambiental permite la observación de muestras húmedas en diferentes grados de deshidratación. Por lo tanto, permite al investigador la observación de las biopelículas en su estado natural de hidratación.

Palabras clave: Microscopio electrónico de barrido ambiental. Biopelícula. Amigdalitis.

INTRODUCTION

Repeated tonsillitis is one of the most common diseases in children and young adults. Frequently, treatment with appropriate antibiotics fails, even though microbiological culture shows that the causal bacterium is sensitive to these drugs.[1] Other infections of the organism show a similar resistance to conventional treatments. In recent years, a new hypothesis has been proposed to explain the resistance of acute and chronic bacterial infections to the usual antibiotic

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treatments: a special pattern of bacterial growth called biofilms. Biofilms have been defined as "a structured community of bacterial cells embedded in a polymer matrix manufactured by them and adhered to an inert or living surface."[2] Using Gram staining and transmission electron microscopy, Chole et al[3] have discovered biofilms in tonsils which had been removed from patients with repeated tonsillitis.

Various imaging techniques have been employed in the investigation of biofilms. Although it is considered that confocal laser scanning microscopy (CLSM) is the reference technique, because it provides both morphological and functional information with excellent spatial resolution,[4] it is very costly and is available at very few research centres. This is why other, more affordable techniques such as scanning electron microscopy are very often employed. However, this technique requires dehydration of the sample prior to the examination, which leads to major distortions and artefacts in the actual images in the case of wet biological simples.

The purpose of this work is to describe the use of environmental scanning electron microscopy (ESEM) for the study of biofilms in patient samples, since it allows the conservation of biological characteristics while at the same time offering excellent image resolution. A comparison is made with conventional scanning electron microscopy. To this end, fragments of tonsils extracted in common surgeries have been used.

PATIENTS AND METHOD

Nine tonsils were obtained from as many adult patients undergoing tonsillectomy for repeated tonsillitis. The average age was 29 years (19-43). Control samples were obtained from tonsils taken from patients with sleep apnoea syndrome who had not suffered tonsillitis for at least the previous 10 years (aged 46 and 51 years). All patients gave their consent for the study. The project was reviewed and approved by the hospital's ethics committee.

After surgical excision, one of the tonsils was sectioned through a crypt to obtain 2 halves, and the tissue corresponding to the inner walls of the crypt was separated. One of the pieces was processed for conventional scanning electron microscopy, by fixation in a solution of 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer for 48 h and subsequent dehydration in ethanol at consecutive concentrations of 10%, 30%, 70%, 90%, and 100%. The critical state was obtained with an Emitec 850 system. Samples were coated with a Bio-Rad SC-650 system and examined through a Phillips XL-30 microscope with digital scanning.

The other fragment of crypt was submerged in saline solution and was taken fresh to the environmental microscope, a Philips XL-30 model with tungsten filament and equipped with a Peltier cooling device. The sample was placed inside the chamber of the environmental microscope, the temperature was set to 2ºC and a pressure of 4 Torr was slowly generated in the chamber, in order to control the drying process and observe the sample. Photographs at were obtained 500 and 1000 times magnifications.

The microphotographs obtained with each technique were processed with the Adobe Photoshop 7.0 program (Adobe Systems Inc., San Jose, California, USA) in order to improve the contrast.

RESULTS

In conventional scanning electron microscopy, biofilms are regarded as dense accumulations of bacteria. In environmental scanning electron microscopy, biofilms are regarded as accumulations of bacteria immersed in an amorphous substance. By comparison, the normal mucosa was seen as a smooth surface with isolated and sporadic

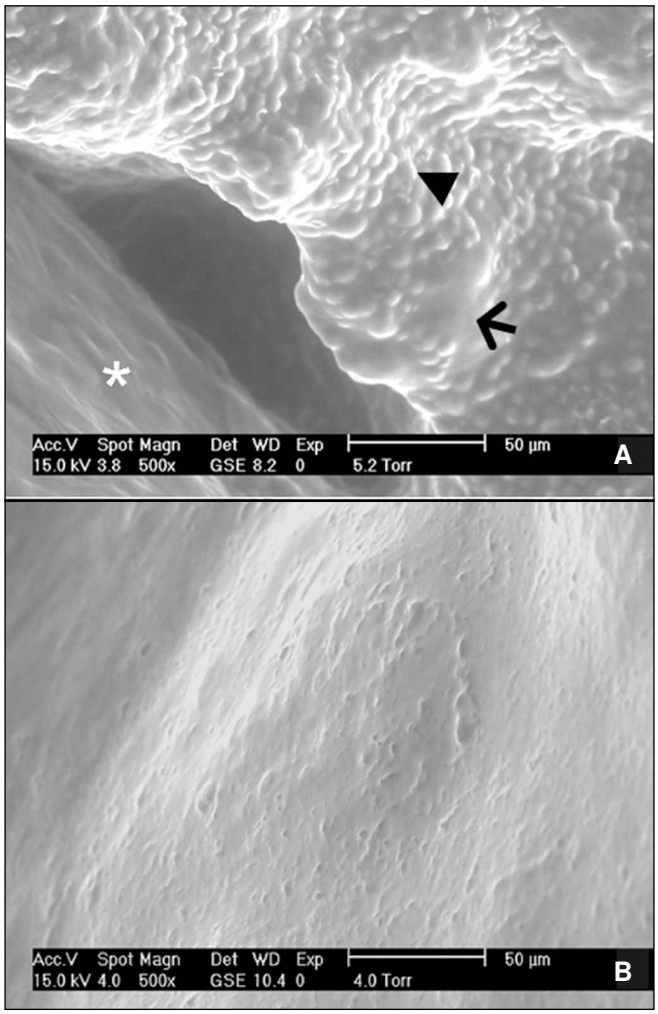

Figure 1. Environmental scanning electron microscopy images. A: biofilm inside a tonsil crypt with a hydrated and intact glycocalyx in which bacterial cells are submerged (arrowhead). An area of normal mucosa can be seen at the bottom left of the image (*) (×500). B: normal mucosa in a sample taken from a control patient (×500).

bacteria (Figures 1A and B). The amorphous matrix may vary in thickness depending on temperature and pressure inside the chamber. After examination of the first sample, the optimal conditions for viewing biofilms were defined as 2ºC for temperature and 4-5 Torr for pressure.

Accumulations of bacteria compatible with biofilms were found in 8 of the 9 cases studied. Conventional microscopy showed biofilms as accumulations of bacteria with spherical and rod shapes coated with fibre structures which the procedure caused through dehydration of the glycocalyx of mucopolysaccharides (Figure 2B).

Environmental microscopy showed numerous spherical cellular structures submerged in an amorphous substance, compatible with bacterial cells embedded in a highly hydrated matrix (Figure 1A).

Biofilms were not identified with any technique in the control samples (Figure 1B).

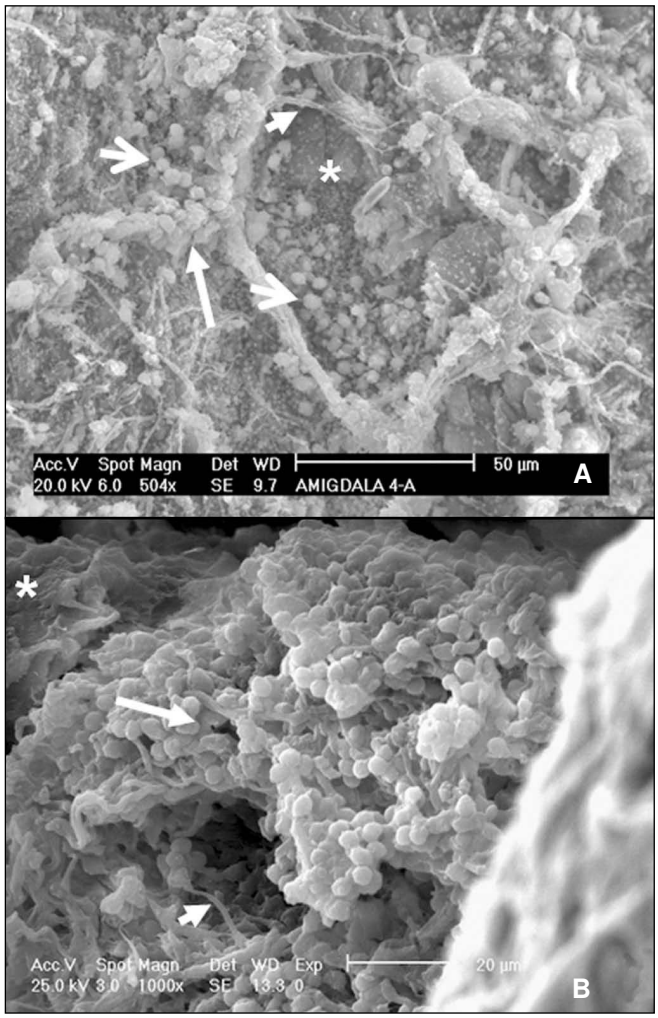

Figure 2. Images from the conventional scanning electron microscope showing groups of bacterial cells and remnants of glycocalyx after the dehydration of tonsil fragments.
A: small areas of the mucous layer of the crypt, covered with small rounded structures that could correspond to dehydration artefacts (*); bacterial accumulations can be seen (open arrows) as well as fibre residues of glycocalyx (short arrow) (×504).
B: tower-shaped structure composed of multiple layers of bacteria (long arrow) with scattered fibre remnants of glycocalyx.

DISCUSSION

The concept of biofilm emerged in the seventies when Characklis[5] described the presence of bacterial films in industrial water pipes which were very resistant to cleaning methods. Bacterial biofilms are structured groups of bacteria surrounded by a polymer matrix produced by them and adhered to an inert or biological surface.[2] Once attached, the bacteria secrete polysaccharides providing a matrix with high water content, gradually forming large colonies. Bacteria living in these colonies are called sessile bacteria. These structures can release isolated bacterial cells (planktonic bacteria) or fragments of biofilm which act as septic emboli and may cause further acute episodes.

The vast majority of bacteria in the wild are believed to grow as a biofilm, because this form of growth provides a very effective means of defence against external aggressions. The matrix or glycocalyx surrounding the bacterial cells slows down or even prevents the penetration of antibiotic drugs, complement factors, and macrophages. Sessile bacteria undergo physiological changes and alterations in gene expression that make them less susceptible to antibiotics, and a significant percentage are in a slowed state of growth,[6] with reduced oxygen consumption, and there is a higher rate of plasmid exchange, where resistance to antibiotics is encoded,[7] between the bacteria making up the biofilm.

Biofilms may play an important role in many chronic or recurrent infections in the area of otolaryngology and in connection with implanted devices: tonsillitis, colesteatoma,[8] chronic sinusitis,[9] and infections associated with endotracheal prostheses and ventilation tubes.[10] It has been shown that several bacteria involved in otolaryngological infections can grow as a biofilm, such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*.[10,11] The Centers for Disease Control and Prevention in the United States estimate that 65% of all bacterial infections in humans are due to biofilms.[12] The concept of biofilm as a cause of recurrent infections could explain some puzzling phenomena such as in vivo resistance to antibiotics which are effective in vitro, the absence of positive cultures in patients with clear signs of bacterial infection and the need to remove the infectious focus ultimately through surgery.[13] This may be the case of patients with repeated tonsillitis who do not respond to antibiotic treatments and eventually need to undergo tonsillectomy.

In the study, biofilms were found in all but 1 patient with repeated tonsillitis. The negative finding could be explained by the small size of the samples observed under microscopy.

Scanning electron microscopy has represented a breakthrough in the past 40 years. The sweeping of the sample surface with an electron beam and the detection of

secondary or reflected electrons provides excellent resolutions of 10 nm or less. However, it requires the existence of a large vacuum and a metal coating and does not allow the examination of samples which produce water vapour when placed in a vacuum chamber, as this interferes with the electron beam. Therefore, biological samples require a long dehydration process which is very destructive and increases the risk of introducing artefacts and destroying the more delicate structures. The metal coating can also hide fine surface details in biological samples.[14] Some authors have found that the external polysaccharide layer and much of the bacterial flora may disappear during the critical state.[15]

Environmental scanning electron microscopy is a substantial modification of the conventional technique that allows visualization of virtually any sample without dehydration or conductive dipping, as it allows the introduction of a gaseous atmosphere in the sample chamber (therefore also called "wet mode"). There are openings throughout the column that limit the pressure and help maintain vacuum gradients, so that there is an optimal vacuum in the electron beam while preserving a low vacuum in the sample chamber. Water vapour is the gas most commonly used in this technique and it acts as an amplifier of the secondary electrons signal released from the surface of the sample after the first scan by the electron beam. The positive ions resulting from this amplification help to compensate the negative charge generated, and thus there is no need for a conductive metal coating of the samples. A special, positively charged gas detector collects the signal amplified by secondary electrons. The atmosphere inside the sample chamber is stabilized by a Peltier cooling device that reduces the temperature to 3-5ºC, so that the pressure of water vapour is limited to a few Torr.[14,16]

This technique in wet mode provides spatial resolutions of 10 nm or less. Wet samples, such as biological tissues in their original state can be examined. The fact that the processing of the sample is minimal (transport in a container with saline solution) reduces the time spent on the study and the economic costs, while also reducing the possibility of introducing artefacts. Since the environment of the sample can be altered dynamically, the hydration and dehydration processes of the sample can be followed as they take place. Thus, hydration provides excellent images of the surface of the biofilm, while gradual dehydration allows a clearer view of the bacterial cells as the amorphous matrix loses water and becomes finer.

However, despite the numerous applications of environmental electron microscopy for biological samples, a series of difficulties have been reported with regard to the handling of wet samples. The water condensed in the sample may obscure the vision of very fine structural details, such as microvilli. This can be resolved with a slight fixation and dehydration of the sample to eliminate some of the water and increase the resistance of cells to the conditions of environmental electron microscopy.[14] Differences in electrical charge in the sample may make the topography confusing. Moreover, a fresh sample cannot be observed for more than 30 minutes and only one examination can be performed. Conventional scanning electron microscopy, in comparison, allows multiple observations. If the examination has to be delayed, the sample must be frozen until the study can be done, in which case the sample must be thawed slowly. This may introduce artefacts into the image. Frozen biofilm samples have shown areas of breakage and formation of holes and channels, probably due to a slight dehydration and sublimation of ice.[17] Another drawback of this technique is that quantitative measurements cannot be made. The lowest possible amplification is 200 times, so the sample cannot be observed in its entirety.[14] Finally, it is difficult to obtain an optimal resolution, because the presence of an aqueous film on the surface of the sample limits topographic analysis, although for some authors these disadvantages are largely outweighed by the possibility of conducting further experiments in the sample chamber.[18] In our opinion, the possibility of obtaining more realistic images of biofilms compensate for these drawbacks.

In conclusion, environmental scanning electron microscopy offers researchers a reliable method to study in detail the structure of biofilms, their presence in diseased tissues obtained in the operating theatre, the relations between the polysaccharide matrix and bacterial cells and the changes in both components of the biofilm after the implementation of local and general treatment. An improvement in the technology can be expected, in order to derive the maximum benefit from the microscopic study of biological tissue.

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