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International Journal of Pediatric Otorhinolaryngology
45 (1998) 15–20

INTERNATIONAL JOURNAL OF
Pediatric
Oto
Rhino
Laryngology

# Initial events in the pathogenesis of acute tonsillitis caused by *Streptococcus pyogenes*[1]

Markus Lilja [a], Simo Räisänen [b], Lars-Eric Stenfors [a,*]

[a] *Departments of Otolaryngology, University of Tromsø, Box 5, Breivika, N-9038 Tromsø, Norway*
[b] *Clinical Microbiology, Central Hospital, Kokkola, Finland*

Received 25 March 1998; accepted 20 May 1998

**Abstract**

Bacterial and epithelial cell samples were obtained, within 24 h of onset of pharyngeal symptoms, from the palatine tonsils of nine patients (four female and five male; age range 10–40 years, median age 23) with acute tonsillitis, culture-positive for *Streptococcus pyogenes*. The specimens were examined using fluorescein isothiocyanate- (FITC) and gold-labelled antiserum to *S. pyogenes* and fluorescence, scanning electron and transmission electron microscopy. *S. pyogenes* could be identified both in the mucous layer covering the tonsils and attached to the surface epithelial cells. Long chains of coccus-shaped bacteria could be seen encroaching on the epithelial cell borders. *S. pyogenes* can apparently penetrate the mucous barrier, attach to the epithelial cells, spread from cell to cell and possibly penetrate into the outermost layer of the epithelial cells. These events in turn provoke cytokine production and/or complement activation, which induce inflammatory reaction in the tonsillar tissue. 

*Keywords:* Tonsils; $\beta$-haemolytic streptococci; Bacterial adherence; Bacterial penetration; Inflammation; Immunofluorescence; Immunohistochemistry

## 1. Introduction

It is well established that both viral and bacterial microorganisms can cause acute tonsillitis (AT). Viruses such as Epstein-Barr-, adeno- (types 3, 4, 7, 14, 21), influenza-, parainfluenza-, coxsackie A-, herpes simplex- (types 1 and 2), rhino- and coronaviruses have been reported. The most common bacterial pathogens in this context are *Streptococcus pyogenes*, *Neisseria gonorrhoideae*, *Corynebacterium diphtheriae* and *Borrelia Vincenti* [1]. *S. pyogenes*, a Group A $\beta$-haemolytic streptococcus (GABHS), is said to cause roughly 20–40% of all AT cases [2,3].

* Corresponding author. Tel.: +47 776 27390; fax: +47 776 27369; e-mail: onhles@rito.no

[1] This paper was presented in part at the XVI World Congress of Otorhinolaryngology, Head and Neck Surgery, Sydney, Australia, March 2–7, 1997.

0165-5876/98/$ - see front matter 
*PII* S0165-5876(98)00071-8

Microorganisms entering the oral and/or nasal cavities can be trapped and neutralized by the mucous blanket and removed by the clearance system. But some microorganisms, among them *S. pyogenes*, can even penetrate the mucous film and attach to the epithelial lining [4,5]. The biomolecular stages in the interaction between the microorganisms and host at the level of the mucosal membranes are far from being completely understood.

The purpose of the present study was to elucidate more closely the bacterial/cellular events on the tonsillar surfaces during the early stage of an AT infection caused by *S. pyogenes*. To be able to do this it is absolutely necessary to localize the pathogens on the tonsillar surfaces. In this study we used fluorescence (FM), scanning electron (SEM) and transmission electron microscopy (TEM) for the immunfluorescence and immunocytochemical assays.

## 2. Material and methods

The clinical material comprised nine patients (four female, five male; age range 10–40, median age 23 years) suffering from classical AT symptoms including dysphagia, elevated body temperature, and general feeling of being unwell. Their palatine tonsils showed signs of acute inflammation: an inflamed surface, pus plugs in the tonsillar crypts and a yellowish exsudate partly covering the tonsillar surfaces. A rapid identification test for *S. pyogenes* in swabbed material obtained from the palatine tonsils proved positive (Streptest, Pharmacia, Sweden) and subsequent aerobic culturing on blood-agar and chocolate-agar plates (incubation in an atmosphere of 5% $CO_2$ for 24–48 h at 37°C) could confirm the initial bacteriological diagnosis. Swabbed material was obtained from both tonsils by rotating a cotton wool tipped swab on the tonsillar surfaces within 24 h of onset of the pharyngeal symptoms. No antibiotics had been administered prior to sampling. All material was loosened from the swab by rinsing it in 8 ml physiological saline. The samples were homogenized by extruding the cell suspension twice through a 20 G needle in order to disrupt clumps. The cell suspension was then pressed through a filter (pore size 5 $\mu$m; Sartorius, Ministart, NML), which allowed unattached bacteria to pass but trapped epithelial cells with and without attached bacteria. Both cell fractions were used in the subsequent analysis. The cell mixtures were given a light centrifugation for 10 min at 1500 rpm to harvest the cells at the bottom of the tubes. The supernatants were carefully discharged using a Pasteur pipette. Thereafter, the precipitates were adjusted to 0.5 ml with physiological saline. The samples were then treated as follows:

### *2.1. Identification of S. pyogenes using FITC-labelled antiserum (Immunofluorescence assay)*

One drop of the cell suspension was placed on a clean glass slide and allowed to dry. One drop of fluorescein isothiocyanate (FITC)-labelled rabbit anti-human *S. pyogenes* serum was added (FA Streptococcus Group A FITC-conjugated reagent for direct serogrouping of Streptococcus Group A, order number 2318-56-6*, Difco Labs, Detroit, Michigan). The antiserum was diluted to 1:50 in phosphate-buffered saline. As negative controls, we used $\beta$-haemolytic streptococci Groups B, C, F and G from our laboratory and *Streptococcus sanguis* I, *S. sanguis* II and *S. mitis* biovar I SK 142 kindly provided by Dr M. Kilian, Aarhus, Denmark. The specimens were evaluated in a Leitz fluorescence microscope.

### *2.2. Scanning electron microscopic studies (SEM)*

Coverglasses (10 mm in diameter) were washed in acetic acid and subsequently in alcohol. Poly-L-lysine (Sigma, St Louis, MO) was dissolved in water (0.1%), applied to one side of the glass and dried overnight. A drop of cell suspension was placed on the coverglass, dried in air, and placed in increasing concentrations of alcohol before being critical point dried using $CO_2$. The coverglasses were mounted on aluminium stubs, covered with a 200 nm layer of gold and observed in a JEOL 5300 scanning electron microscope.

### 2.3. Transmission electron microscopic studies (TEM) with gold-labelled antiserum to S. pyogenes (Immunohistochemical assay)

To 0.5 ml cellular mixture was added 0.5 ml 4% glutaraldehyde buffered in 0.2 M phosphate fixative. After fixation, the mixture was centrifugated at 16000 rpm for 1–2 min. The supernatant was pipetted away and the cells diluted in 100 μl 10% gelatin. The sample was given a brief centrifugation (15 s) and kept on ice for 1 h. The cell pellet was excised, immersed in 2.3 M sucrose containing 20% poly(vinyl)pyrrolidine (PVP-sucrose) for 1 h, placed on frozen aluminium specimen pins by immersion in liquid nitrogen and sectioned using a Reichert Ultracut S ultramicrotome with an FCS cryochamber (Leica, Deerfield, IL). Thin sections (approximately 30–60 nm) were cut with glass or diamond knives, retrieved from the knife surface with a drop of methyl cellulose mixed with sucrose. The specimens were then mounted on carbon-coated grids, which were placed in 1% fish skin gelatin (FSG) for 15 min. After two washes in PBS, the grids were placed in 0.12% glycine for 5 min. After further washing in PBS (twice) the grids were placed in rabbit antiserum (diluted in FSG 1:50) to *S. pyogenes* (Difco Labs, Detroit, MI) for 30 min. After renewed washing in PBS (five times) the grids were placed in protein A-gold diluted in 1% FSG for 20 min (gold particle 10 nm). After a final wash in PBS (five times), the grids were contrasted with methylcellulose/3% uranylacetate (9:1) for 10 min and then examined in a JEOL 1010 transmission electron microscope at 80 kV (method described in detail elsewhere, [6,7]).

As negative controls, we used similarly processed samples obtained from three healthy individuals, culture-negative for *S. pyogenes* but with massive growth of Group K $\alpha$-haemolytic streptococci (*S. salivarius)* and from two individuals with growth of Group C $\beta$-haemolytic streptococci (*S. equisimilis*). In addition, plain cultures of *Neisseria sicca*, *S. mitis* and *S. salivarius* from our own laboratory were used.

## 3. Results

### 3.1. Fluorescence microscopic studies

In all samples obtained from the AT patients, coccus-shaped bacteria, expressing positive affinity to FITC-labelled antiserum to *S. pyogenes*, were identified. Usually the bacteria appeared in pairs or short chains. In samples containing epithelial cells, the bacteria showing positive affinity for FITC-labelled antiserum formed chains consisting of five to eight cocci. The bacteria stained yellow, whereas the epithelial cells stained red (Fig. 1).

### 3.2. SEM studies

Epithelial cells with attached bacteria were regularly found. The bacteria usually formed colonies consisting mainly of cocci. In addition, the colonies harboured variously sized rods, which were intermingled in the colonies. Long coccal chains of up to 80–100 cocci/diplococci were regularly found. These cocci did not 'respect' the epithelial cell borders but could encroach upon seven to 10 different epithelial cells. The ends of such coccal chains regularly disappeared into bacterial colonies formed by variously sized cocci and rods (Fig. 2A, B).

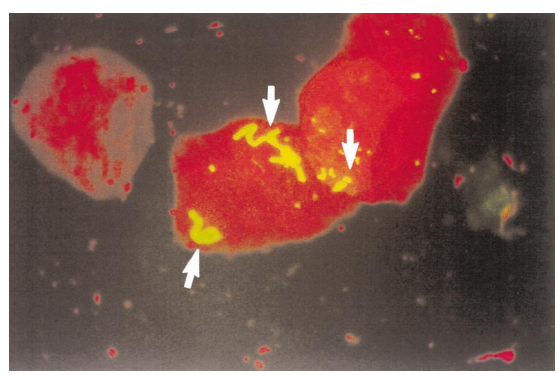

Fig. 1. Chain of coccus-shaped bacteria (arrows) displaying positive reactivity to FITC-labelled antiserum to *S. pyogenes* × 3500.

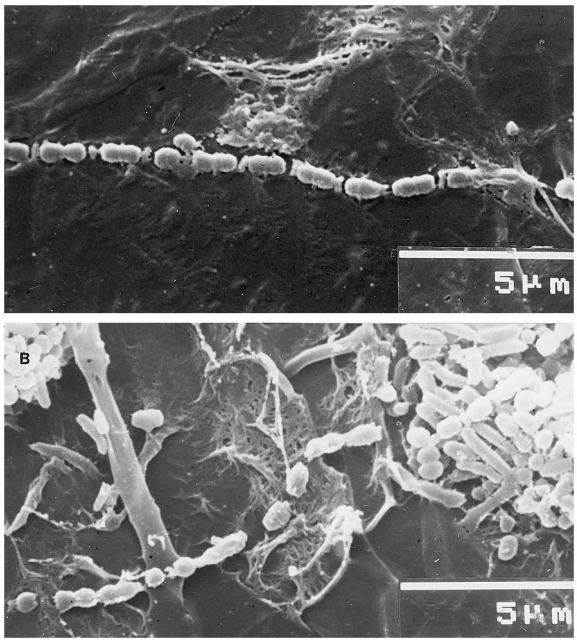


Fig. 2. SEM pictures of tonsillar surface epithelial cells showing a long bacterial chain formed by cocci (A) encroaching on several epithelial cells and (B) ending up in a bacterial colony of variously sized cocci and rods. Bar 5 $\mu$m.

### 3.3. TEM studies

In all samples, coccus-shaped bacteria with affinity for immunogold-labelled antiserum to *S. pyogenes* could be identified both in samples with plain bacteria and in samples containing epithelial cells with attached bacteria (Fig. 3A). As positive bacteria, we chose those bacteria coated with more than seven gold particles. Each gold particle measured to 10 nm. In the bacterial mixture, positively labelled bacteria were intermingled with variously sized cocci and rods without gold-labelling. Many bacteria attached to the epithelial cells proved positive by immunogold-labelling. These bacteria were frequently diplococci, or formed short chains. Close contact was noted between the fimbriae of the bacteria and projections on the epithelial cell surface. No bacteria with positive affinity to gold-labelled antiserum to *S. pyogenes* were detected among the controls (Fig. 3B).

## 4. Discussion

Most studies concerning the bacteriology of AT infections caused by *S. pyogenes* have been based on standard bacterial culturing and/or rapid detection of bacterial antigens by use of precipitation reactions. By using refined methods, and taking advantage of immunofluorescence and immunocytochemical techniques, there are better prospects of elucidating the intial stages of AT etiopathogenesis. Recently, we showed that *S. pyogenes* microorganisms have a definite affinity for the epithelium covering lymphatic tissue [8]. Moreover, we showed that *S. pyogenes* colonies were remarkably increased in number in relation to other aerobic colonies on the tonsillar surfaces during an AT infection. It seems likely that the increase in *S. pyogenes* bacteria during infection was mainly due to the attachment of the bacteria

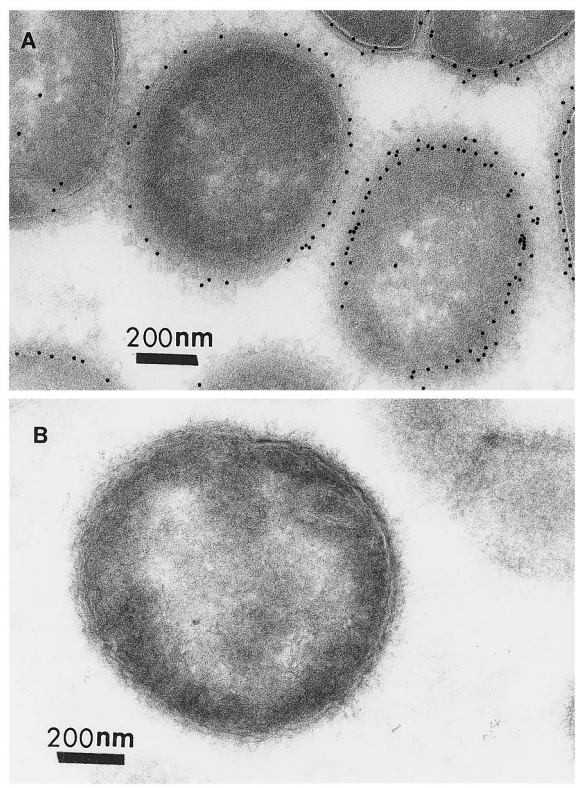


Fig. 3. TEM pictures of coccus-shaped bacteria exhibiting (A) positive (*S. pyogenes*) and (B) negative (*Neisseria sicca*) reactivity to gold-labelled antiserum to *S. pyogenes*. Bar 200 nm.

to the surface epithelium, as has been suggested recently [4,8,9]. Attachment of pathogenic bacteria to tonsillar epithelium could only be recognized during an on-going AT infection, but not among healthy carriers of *S. pyogenes* [8]. The first essential stage in the etiopathogenesis of AT must therefore be the penetration of the mucous film covering the palatine tonsils. Both $\alpha$- and $\beta$-haemolytic streptococci possess the ability to penetrate mucous effusion material [10].

The next important stage in the etiopathogenesis of AT is the subsequent attachment of the pathogenic microorganisms to the epithelial cells. In this context, the M-protein forming an essential part of the fimbriae covering the surface of *S. pyogenes* bacteria plays a crucial role [11,12]. M protein is the most abundant protein on the surface of virulent streptococci. Lipoteichoic acid complexed with this bacterial cell surface protein is suggested to act as the adhesive molecule, with the counterpart being fibronectin on the epithelial cell [13].

When attached to the epithelial cells, the bacteria can form colonies on the epithelial cell surface, but they also have the ability to penetrate into the cell, as has been shown in both in vitro and in vivo studies [14–16]. It is self-evident that antibiotics, with property to penetrate into the cells are advantageous in the treatment of these types of diseases [17]. A rapid desquamation of such infected cells must be regarded as being of the utmost importance in host defence. Any factor hindering desquamation of these infected cells will increase the susceptibility to *S. pyogenes* infection. In our study we recognized only a few intracellular pathogens. Thus our findings were in accord with a previous report [18] which showed that bacteria do not to any great degree penetrate the tonsillar epithelium during acute pharyngotonsillitis.

Local spreading of *S. pyogenes* infection on the tonsillar surface is ultimately an important stage in the etiopathogenesis of AT. An important way of spreading appeared from the present SEM study. These bacteria divide in one plane and therefore often form diplococci and chains. We noted that long chains of coccus-formed bacteria encroached on several epithelial cells (Fig. 2A). Under healthy conditions, bacterial colonies on tonsillar surfaces appeared to 'respect' the cell borders [19], whereas during an AT infection this did not seem to be the rule. That the pathogenic *S. pyogenes* bacteria under healthy conditions are kept in check by non-pathogenic $\alpha$-streptococci might well be the case, as was recently suggested [20]. The SEM studies in this paper showed that the coccal chains ended up in a bacterial colony consisting of variously sized bacteria (Fig. 2B).

Immunoglobulin production by the tonsillar tissues definitely plays an important role in the combat of bacterial infections [21]. Whether the induction of an AT infection caused by *S. pyogenes* is due to local suppression of the acquired immune system, or to an inadequate innate immune system of the palatine tonsils (phagocytosis, lysozyme, complement activation, bacterial opsonization) requires further investigation. The present study did show, however, that attachment of *S. pyogenes* microorganisms to the surface epithelium of the palatine tonsils can definitely provoke an inflammatory reaction of the underlying tissues, apparently via cytokine release and/or complement activation [22].

## Acknowledgements

We thank Helga-Marie Bye, Tom-Ivar Eilertsen, Randi Olsen and Reidar Myklebust at the Department of Electron Microscopy, University of Tromsø, Norway, for technical assistance.

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