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Staphylococcus aureus controls interleukin-5 release in upper airway inflammation

C. Bachert[a,b,*], G. Holtappels[a], M. Merabishvili[c], T. Meyer[d], A. Murr[d], N. Zhang[a], K. Van Crombruggen[a], E. Gevaert[a], U. Völker[d], B.M. Bröker[e], M. Vaneechoutte[c], F. Schmidt[d,**]

[a] Upper Airways Research Laboratory, Ghent University, Ghent, Belgium
[b] Division of ENT Diseases, CLINTEC, Karolinska Institute, Stockholm, Sweden
[c] Laboratory Bacteriology Research, Faculty of Medicine & Health Sciences, Ghent University, Ghent, Belgium
[d] Interfaculty Institute for Genetics and Functional Genomics, Department Functional Genomics, ZIK-FunGene, University Medicine Greifswald, Greifswald, Germany
[e] Department of Immunology, University Medicine Greifswald, Greifswald, Germany

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ABSTRACT

Staphylococcus aureus is a frequent colonizer of the upper airways in chronic rhinosinusitis with nasal polyps, but also resides intramucosally; it has been shown that secreted staphylococcal proteins such as enterotoxins and serine proteases induce the release of cytokines such as IL-5. We have analyzed nasal polyp tissue freshly obtained during routine surgery, which did or did not contain cultivatable *S. aureus*, to study spontaneous IL-5 production by nasal polyp tissue over 24 and 72 h in tissue culture. In *S. aureus*-positive samples we interfered by killing the bacteria using antibiotics or *S. aureus* specific intravenous staphylococcal phages (ISP), active or heat-inactivated. Phage-neutralizing antibodies were used to demonstrate the specificity of the phage-mediated effects. We monitored *S. aureus* colony forming units, and identified *S. aureus* proteins by mass spectrometry. We demonstrate that cultivatable *S. aureus* may be found in type-2 inflamed nasal polyps; the pathogen is replicating within 24 h and secretes proteins, including enterotoxins and serine proteases. The presence of *S. aureus* was associated with a significantly higher release of IL-5. Killing of *S. aureus* by antibiotics or specific ISP significantly reduced the IL-5 release. The suppressive activity of the bacteriophage on IL-5 be abolished by heat inactivation or anti-phage antibodies.

Biological significance: In this study, we used high resolution mass spectrometry to identify *S. aureus* proteins directly in infected nasal polyp tissue and nasal polyp tissue incubated over 24 and 72 h in culture. We discovered bacterial proteins including enterotoxins and serine proteases like proteins. These experiments indicate a direct role of *S. aureus* in the regulation of IL-5 production in nasal polyps and may suggest the involvement of bacterial proteins detected in the tissues.

1. Introduction

Staphylococcus aureus has been associated with chronic inflammatory airway diseases, as the bacteria may colonize the airways and release proteins such as staphylococcal enterotoxins (SEs) and related molecules which might severely impact the mouse and human immune system [1,2]. SE-IgE, specific IgE antibodies to staphylococcal enterotoxins (SEs), as a marker of immune contact with *S. aureus*, have been associated with asthma throughout Europe [3], and specifically severe asthma [4,5], as well as chronic rhinosinusitis with nasal polyps (CRSwNP; [2,6]). The abundancy of SE-IgE was related to more severe disease, as manifested by the presence of comorbid asthma and recurrence of disease after surgery [6,7]. Recently, Chen et al. [7a] demonstrated that SEs could function as an allergen and as a superallergen in nasal polyp tissue. The release of high concentrations of cytokines, including type 2 cytokines such as IL-5, has been observed upon exposure of mucosal tissue to SEs [8] and serine proteases (spl's) [18].

Abbreviations: IL, interleukin; *S. aureus*, *Staphylococcus aureus*; CRSwNP, chronic rhinosinusitis with nasal polyps (nasal polyps); MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; ISP, Intravenous Staphylococcal Phage; ECP, eosinophil-cationic protein; APS, anti-ISP phage serum; CFU, colony-forming units; Spl, serine protease like protein; SE, staphylococcal enterotoxin; TSST-1, toxic-shock-syndrome toxin-1; PNA-FISH, peptide nucleic acid–fluorescence in situ hybridization

* Correspondence to: C. Bachert, Upper Airways Research Laboratory, Ghent University, De Pintelaan 185, 9000 Gent, Belgium.
** Correspondence to: F. Schmidt, Interfaculty Institute for Genetics and Functional Genomics, Department Functional Genomics, ZIK-FunGene, University Medicine Greifswald, Friedrich-Ludwig-Jahnstr. 15a, D-17487 Greifswald, Germany.
E-mail addresses: claus.bachert@ugent.be (C. Bachert), frank.schmidt@uni-greifswald.de (F. Schmidt).

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S. aureus has been shown to permanently colonize the nose in about a third of European adults, but can be found in up to 85% of patients with chronic rhinosinusitis with nasal polyps [9]. However, no difference in the ability of production of classical superantigens has been found between colonizing *S. aureus* strains obtained from CRSwNP patients vs. healthy controls [10]. In contrast to healthy mucosal tissue, however, *S. aureus* is frequently found intramucosally and intracellularly in CRSwNP tissues [11]; furthermore, macrophages in severely inflamed polyp tissues are at least in part alternatively activated and unable to effectively phagocytize and kill *S. aureus*, as shown by Krysko et al. [12], and may allow the pathogen to survive and to secrete proteins intramucosally. Although we have shown recently that secreted proteins from *S. aureus* can be detected in nasal polyp tissue [12a], and that both SEs and spl's have the potential to induce the release of IL-5 upon exposure to human nasal mucosal tissue [8,18], it has not been demonstrated yet that *S. aureus* can be cultured from nasal polyp tissue and is spontaneously producing and releasing proteins "ex-vivo" into the mucosal tissue of CRSwNP patients, associated with the release of type-2 inflammatory cytokines.

We therefore aimed to use ex vivo human tissue, freshly obtained from CRSwNP patients during surgery for their disease, to study the presence of *S. aureus*, its secretome, and effects of the released proteins on the mucosal inflammation, measured here as spontaneous release of Interleukin-5 (IL-5). To demonstrate the direct link between the activity of the germ and cytokine release, we intended to eradicate tissue *S. aureus* using both antibiotics and germ-specific bacteriophages; and to evaluate the release of secreted staphylococcal proteins into the mucosal tissues by proteomics.

2. Material and methods

2.1. Patients and sample collection

Study subjects were selected on the basis of a documented medical history of chronic rhinosinusitis with nasal polyps (CRSwNP), a pathological nasal endoscopy confirming bilateral nasal polyps and a pathological CT-Scan of the sinuses, according to the current European position paper on sinusitis and nasal polyps [13]. Tissue samples from the ethmoidal sinuses were collected from patients during endoscopic sinus surgery procedures, which were indicated for clinical reasons, independent of this study. Surgery was performed at the Department of Otorhinolaryngology at Ghent University Hospital, Belgium. The local Ethics Committee approved the study (B670201112019), and written informed consent was obtained from all patients prior to surgery. Clinical data of the patients are summarized in Table 1.

2.2. Tissue culture for identification of bacteria

Several nasal polyps from each patient with a diameter of at least 5 mm were washed vigorously in 0.9% NaCl, cut open with a sterile scalpel to obtain small tissue pieces from the center of the polyp, which were cultured overnight at 37 °C in Tryptic Soy Broth (TSB). This was followed by plating 10 µL of the TSB-overnight culture on Columbia agar with 5% sheep blood as well as on Mannitol Salt Agar (MSA), a selective growth medium for *S. aureus* (all media from Becton Dickinson, Erembodegem, Belgium). After further incubation for 24 h at 37 °C, bacterial colonies were identified by MALDI-MS (Bruker Daltonics, Germany).

For quantifying *S. aureus* in the tissue culture experiments, decinormal serial dilutions from the ex vivo nasal polyp supernatant samples were prepared and 100 µL of each dilution was inoculated onto MSA plates. Colonies were counted after incubation for 24 h at 37 °C. All culture dilutions were done in triplicate. The detection threshold for *S. aureus* and *Staphylococcus epidermidis* in the overnight cultures was 100 CFU/mL.

After overnight culture several colonies were picked, using a 1 µL disposable loop, and spotted evenly over the wells of the MALDI target plate. The preparations were covered with 1 µL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and dried for 2 min at RT. A bacterial test standard (BTS 255343, Bruker Daltonics, Germany) was used as positive control for MALDI spectra, and an empty well covered with matrix served as a negative control. Mass spectra were generated and analyzed with a Microflex Biotyper™ spectrometer (Bruker Daltonics) and Bruker Daltonics' flexControl version 3.0 software, using the manufacturer's standard settings [13a].

2.3. Bacteriophages

The LPS-free *S. aureus* bacteriophage ISP was received from Eliava IBMV (Tbilisi, Georgia) in 2002 and is currently maintained in the phage collection of the Laboratory Bacteriology Research Ghent University, Belgium. For the phage propagation we used the clinical *S. aureus* strain '13 S44 S9' isolated from a burn wound at the Burn Wound Centre (Queen Astrid Military Hospital, Brussels, Belgium) in 2006. The bacterial strain and the phage were cultured on Select Alternative Protein Source Luria Bertani media (Becton Dickinson, Erembodegem, Belgium). The agar overlay method with modifications as described earlier [14] was used to obtain a high titer (11 log plaque-forming units (PFU)/mL) phage lysate. Briefly, 1 mL of phage suspension containing 4 log PFU of ISP was mixed with 3.0 mL of molten (45 °C) Alternative Protein Source Luria Bertani top agar (0.7%) and 0.1 mL of a host bacterial suspension (end concentration of 8 log CFU/mL). This mixture was plated onto Petri dishes, filled with a bottom layer of 1.5% Alternative Protein Source Luria Bertani agar and incubated at 37 °C for 16–18 h. The top agar layer was scraped off and centrifuged for 20 min at 6000 × *g*. The supernatant was filtered through a 0.45 µm membrane filter (Sartorius Stedim Biotech, Göttingen, Germany). Phage lysate was subsequently purified from endotoxins using a commercially available kit (Endotrap Blue, Hyglos, Germany) according to the instructions of manufacturer.

The obtained phage lysate was ultracentrifuged at 25,000 × *g* for 1 h at 4 °C and the pellet was resuspended in ten times less volume of a 0.9% NaCl solution. Phage particles were enumerated by the agar overlay method [14]. Briefly, decinormal serial dilutions (from log(0) to log(−10)) of the bacteriophage suspension were prepared. One milliliter of each dilution was mixed with 3.0 mL of molten (45 °C) 0.7% Alternative Protein Source Luria Bertani LB top agar and 0.1 mL of a host bacterial suspension (end concentration of 8 log CFU/mL) and plated in triplicate onto 90 mm diameter Petri dishes (Plastiques Gosselin, Menen, Belgium), filled with a bottom layer of 1.5% Alternative Protein Source Luria Bertani agar and incubated for 18–24 h at 37 °C. To estimate the original bacteriophage concentration, plates with 100–1000 plaques were counted. Each titration was performed in triplicate and the means were calculated.

Heat inactivation of ISP was performed at 90 °C for 15 min. Anti-ISP

Table 1
Patient's clinical data.

Patient clinical data	*S. aureus* negative CRSwNP	*S. aureus* positive CRSwNP
Number of subjects	8	9
Age (y), median (range)	47 (31–77)	47 (28–81)
Gender (male/female)	4/4	5/4
Atopy	6	6
Asthma	6	5
IgE (kU/l)	350.9 (IQR: 251.4–432.1)	428.5 (IQR: 171.4–571.9)
SE-IgE positive	3	3
IL-5 (pg/mL)	270.9 (IQR: 216.2–956.6)	151.6 (IQR: 61.3–326.0)
ECP (µg/l)	13,365 (IQR: 5918–20,075)	10,846 (IQR: 3461–32,230)