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Intracellular residency is frequently associated with recurrent *Staphylococcus aureus* rhinosinusitis*

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SUMMARY

Aim: *The prevalence of intracellular Staphylococcus aureus organisms in the nasal mucosa of patients with recurrent infectious rhinosinusitis episodes was studied.*

Method: *Twenty-seven consecutive adult patients who failed medical management of chronic rhinosinusitis (CRS) of multiple origins, associated or not with nasal polyposis, were consecutively enrolled for endonasal sinus surgery (including partial middle turbinectomy, middle antrostomy, ethmoidectomy, sphenoidotomy) and followed for a 12-month post-operative period.*

Results: *Seventeen of these patients showed the presence of intracellular S. aureus as detected by confocal laser scan immunofluorescence microscopy in epithelial cells of surgical intranasal biopsy specimens. Nine of the patients with and two without intracellular bacteria yielded S. aureus in endoscopically guided cultures of middle meatus secretions, despite the recent administration of prophylactic antibiotics. Eleven of the 17 patients with intracellular S. aureus relapsed for rhinosinusitis within the 12-month follow-up period. Molecular typing of sequential S. aureus isolates demonstrated the persistence of unique patient-specific S. aureus clonotypes in nine of the patients with intracellular bacteria during the 12-month follow-up.*

Conclusion: *The presence of intracellular S. aureus in epithelial cells of the nasal mucosa is a significant risk factor for recurrent episodes of rhinosinusitis due to persistent bacterial clonotypes, which appear refractory to antimicrobial and surgical therapy.*

Keywords: Chronic rhinosinusitis, endoscopic sinus surgery, Staphylococcus aureus, recurrent infections, intracellular reservoir

INTRODUCTION

The etiology of chronic rhinosinusitis (CRS), a major common health care problem, is multifactorial, involving specific host immune characteristics as well as a variety of microbial pathogens [(1)]. The pathophysiology of the disease entity remains unclear although numerous potential causes are being explored.

Staphylococcus aureus, a major pyogenic pathogen [(2)] responsible for community- and hospital-acquired infections [(3-8)], is commonly found in CRS but its involvement in the development of nasal polyposis is still debated.

Several studies [(3,9)] have reported the asymptomatic, transient or permanent, carriage of *S. aureus* in the anterior nares of approximately 30-50% of the population, yet no link has been established between this observation and the incidence of staphylococcal rhinosinusitis. Persistent carriers [(10,11)] have higher *S. aureus* loads and a higher risk of acquiring *S. aureus* infection in general [(5,12,13)]. Neither prolonged courses of antibiotics nor nasal topical treatments as described by Müller et al. [(14)], leads to high rates of permanent eradication of *S. aureus* carriage.

While *S. aureus* was traditionally considered as an obligate extracellular pathogen colonizing many different types of host tissues and secreting various enzymes and toxins for tissue

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destruction, more recent in vitro studies indicate that *S. aureus* may also exhibit a facultative intracellular lifestyle [15,16-20]. *S. aureus* has been shown to survive not only within neutrophils thus contributing to infection [21], but also in epithelial cells, fibroblasts, endothelial cells [16-19] and cultured enterocytic or osteoblastic cells [15,20]. One proposed mechanism for *S. aureus* intracellular persistence is the formation of small colony variants, which protect them from host immune reactions and extend their survival within non-professional phagocytes [22].

We recently described the presence of intracellular *S. aureus* in middle meatus mucosal biopsies of a few CRS patients [23]. This clinical study evaluates the frequency of intracellular residency and its impact on recurrent CRS episodes by *S. aureus* in a larger cohort of consecutively enrolled patients.

PATIENTS AND METHODS

Patients

Twenty-seven outpatients attending the Rhinology-Olfactology unit of the ENT department (tertiary referral centre), whose clinical status required nasal surgery for persistent chronic rhinosinusitis (CRS), were prospectively enrolled from December 2003 to April 2004. These 27 patients (9 women/18 men), whose median age was 37 years (range 17-65 years), were included consecutively on the day of surgery and followed for a post-operative 12-month period. The study protocol was approved by the Ethical Committee of the Geneva University Hospitals and required informed consent of each patient. Inclusion criteria were chronic rhinosinusitis insufficiently relieved by more than 6 months of medical treatments, and eligible for surgical management. Exclusion criteria were HIV disease, primary ciliary dyskinesia, age under 16, and lack of patient's consent.

CRS symptoms were defined according to the ARIA classification [24]. Clinical examinations were undertaken with a 4 mm 0° rigid nasal endoscope. All patients underwent a preoperative CT-scan on which mucosal disease was evaluated using the Lund-MacKay scale to confirm CRS diagnosis [25]. Relapse episodes were identified by chronic nasal obstruction and/or chronic rhinorrhea and/or olfactory disorder and/or chronic headache related to sinonasal disease, associated with one or more of the following clinical signs: inflammatory or oedematous mucosa, polyps, tinted nasal secretions, crusting, cacosmia.

Before endoscopic sinus surgery, each patient received a standard prophylactic regimen consisting of oral prednisone (1mg/kg/day) for 5 days and 1g per day of co-trimoxazole for 10 days.

Endoscopic sinus surgery (including partial middle turbinectomy, middle meatotomy, ethmoidectomy, sphenoidotomy, eventually associated with access septoplasty) was performed on the patients under general anesthesia. Tissue specimens obtained during the surgical procedure were evaluated by routine anatomic, pathological, and histological procedures, and were screened for the presence of intracellular *S. aureus* as described below.

A standard protocol of post-operative care was applied to each patient, including nasal packing for 48 hours followed by nasal suctioning and removal of crusts, administration of topical steroids (momethasone or fluticasone), nasal douching with a saline solution, pain killers if needed and anti-inflammatory medication. Only symptomatic, post-operative patients yielding *S. aureus*-positive cultures in the middle meatus were treated with standard regimens of oral anti-staphylococcal antibiotics, in accordance with the in vitro sensitivity data (none of the *S. aureus* isolates displayed resistance to methicillin).

Immunofluorescent detection of intracellular S. aureus

Middle meatus biopsy specimens were embedded in OCT 4583 (Miles Scientific, Naperville, Illinois, USA) and frozen in precooled liquid isopentane. The presence of intracellular *S. aureus* in epithelial cells of surgical biopsy specimens was assessed by confocal laser scan immunofluorescence microscopy as described previously [23].

Bacterial identification and molecular typing

Microbiological cultures of middle meatus secretions were performed on swabs collected (i) immediately pre-operatively, (ii) during the study period when required for patients with relapsing CRS, and (iii) at 12-months after endonasal surgery. To avoid contamination by normal flora present in the anterior nares, the vestibule was disinfected with a chlorhexidine solution before the direct endoscopically guided sampling of patients' middle meatus secretions. Microbiological cultures

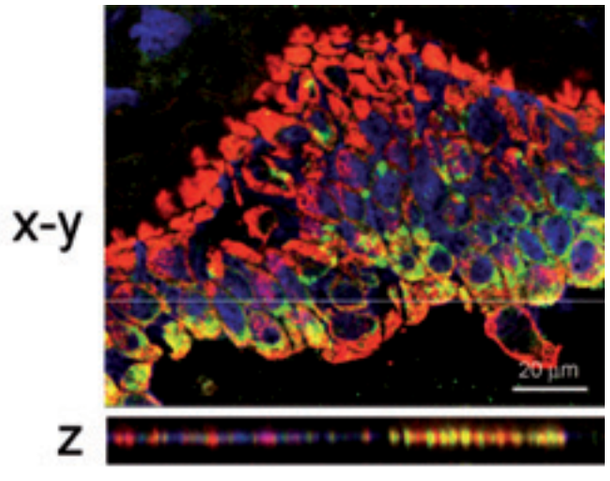

Figure 1. Intracellular localization of *S. aureus* in epithelial cells by confocal microscopy.

S. aureus were labeled with specific antibody (green), cell nuclei with TOTO-III (blue) and epithelial cells were visualized with anti-keratin (red). In top image, projection was constructed from confocal Z stacks (0.5 µm thick). Bottom image corresponds to a vertical view in the z plane obtained by combining the series of x-y scan taken along the z-axis. Line drawn in the top image indicates where the z-sections are generated.

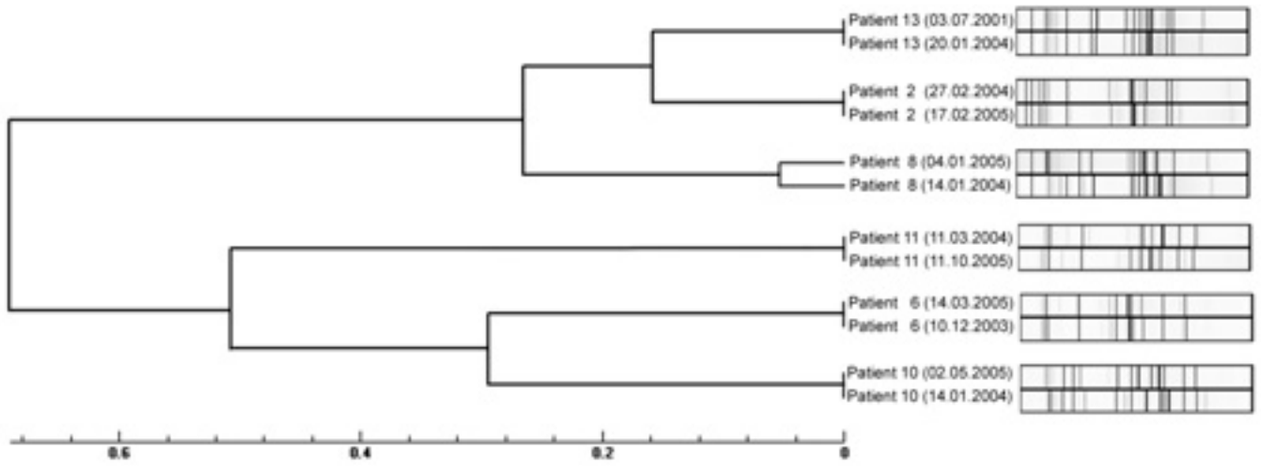

Figure 2. Demonstration by VNTR of clonal relationships between consecutive clinical isolates of 6 *S.aureus*-infected CRS patients.
Cluster-tree obtained for pairs of strains collected from de same patients having CRS. Each of the 6 patients, sampled at time intervals ranging from 1-3 years, carried specific isolates of the same strain (distance=0).

were performed on chocolate agar and Columbia colistin nalidixic agar (Becton Dickinson), and *S. aureus* was identified by using standard bacteriological criteria.
Molecular typing of sequential *S. aureus* isolates was performed by a recently described genotyping method of Variable-Number of Tandem Repeats (VNTR) [26].

Outcome evaluation
The following data were analyzed based on the files of the patients: age, sex, previous relevant medical history, including number of antibiotic courses for CRS, duration of symptoms, bacteriological sampling data in the middle meatus; status and clinical symptoms at time of surgery and at the 1-year control visit, microbiological results of middle meatus at t=0 (day of surgery) and t=1 year, immunofluorescence data on the biopsies taken during surgery, and molecular typing of sequential *S. aureus* isolates.

RESULTS

Table 1 shows the characteristics and outcomes of the 27 patients during the one-year post-operative follow-up. Patients were separated into two subgroups according to the presence or absence of intracellular *S. aureus*, detected in intranasal biopsy specimens by confocal laser scan immunofluorescence microscopy (Figure1).

Seventeen of the 27 patients had a positive score for intracellular *S. aureus* in their middle meatus at the time of nasal surgery (Table 1). Nine of those 17 patients also yielded *S. aureus*-positive cultures at the time of surgery, despite the recent administration of pre-operative, prophylactic antibiotics, according to the guidelines of our institution. The positive *S. aureus* cultures in nasal secretions of those 9 patients were very helpful for evaluating the clonal links of sequential, pre-operative and post-operative isolates of eventual CRS relapsing episodes, thus assessing or not the persistence of unique bacterial clonotypes during the one-year follow-up period. At one year after nasal surgery, 8 of those 9 patients still yielded *S. aureus*-positive cultures in their middle meatus, and 7 of them had a clinical score of CRS relapse. The persistent *S. aureus*-positive cultures in the 8 patients with intracellular *S. aureus* and in their middle meatus were found to be clonally related by VNTR after 12 months compared to those recovered at the time of nasal surgery (examples in Figure 2). Only 1 of the 9 patients with intracellular *S. aureus* improved after nasal surgery in showing no CRS relapse and no *S. aureus*-positive cultures during the one-year follow up.

Among the 10 patients having a negative score for intracellular *S. aureus*, two yielded *S. aureus*-positive cultures on their middle meatus, and only 1 of them had CRS relapse at one year, though without *S. aureus*-positive culture. All other 9 patients remained asymptomatic during the one-year follow-up, although some of them yielded positive cultures with commensal organisms (*coagulase negative Staphylococcus, Streptococcus*, others).
The median symptomatic period for the 17 patients with intracellular *S. aureus* was estimated to 72 months, and 8 of 12 evaluated patients had received systemic antibiotics for their CRS episodes before nasal surgery. The median symptomatic period of the 10 patients with no intracellular *S. aureus* was estimated to 18 months and 3 of 8 evaluated patients received antimicrobial therapy for rhinosinusitis prior to enrollment. Only 5 of those 10 patients accepted a control swab at the 1-year follow-up visit, which were all negative for *S. aureus.*

DISCUSSION

This study provides further evidence that the presence of intracellular *S. aureus* in epithelial cells of the middle meatus mucosa may represent a seeding reservoir for recurrent

Table 1. Patient characteristics and presence of *S. Aureus*.

Nr	Sex/age (years)	Symptom duration (months)	Surgery type	Previous AB courses for sinusitis	Intracellular SA	SA culture middle meatus		Relapse
					T = 0	T= 0	T= 1	
2	m / 59	120	ep2	2	+	+	+	yes
5	f / 46	120	semp2	0	+	+	-	0
6	m / 32	120	sem2	0	+	+	+	yes
8	m / 34	120	emp2	3	+	+	+	yes
10	m / 49	36	semt2	0	++	+	+	0
11	m / 55	120	emp2	NA	+	+	+	yes
12	f / 56	60	st	NA	++	+	+	yes
13	m / 32	72	emp2	NA	+	+	+	yes
14	f / 28	24	emp2	9	++	+	+	yes
15	m / 42	120	semt2	NA	+	-	-	yes
1	f / 58	120	semt2	10	++	-	-	yes
3	m / 22	12	empt2	4	++	-	+	yes
4	m / 47	24	semp2	1	++	-	-	yes
7	f / 36	120	sem2	12	+	-	-	0
9	m / 65	36	empt2	1	+	-	-	0
16	m / 28	72	sempt2	NA	++	-	ND	0
17	m / 27	3	st	0	++	-	ND	0
//////////								
21	f / 23	3	semt2	0	-	-	-	0
22	m / 43	12	semt2	NA	-	-	-	0
23	m / 27	24	semt2	4	-	-	-	0
24	m / 37	24	semp2	NA	-	-	-	0
27	m / 22	12	smt1	0	-	-	-	0
18	m / 40	6	emp2	0	-	-	ND	0
19	f / 17	48	ems2	10	-	-	ND	0
20	m / 26	NA	sem2	0	-	+	ND	0
25	f / 29	60	emt2	0	-	-	ND	0
26	m / 41	120	empt2	1	-	+	ND	yes

- Surgery type: “s” = septoplasty, “e” = ethmoidectomy, “m” = middle antrostomy, “p” = sphenoidotomy, “t” = partial middle turbinectomy, “1” = unilateral, “2” = bilateral
- Previous antibiotic (AB) courses (number of courses for sinusitis): “NA” = not available
- Intracellular SA: “++” = abundant, “+” = rare but seen, “-” = not seen
- SA culture from middle meatus (swab): “+” = present, “-” = absent, “ND” = not done; T = 0 (day of first swab and biopsies), T = 1 (1 year post-op)

episodes of rhinosinusitis [23]. This interpretation was further supported by molecular identification of persistent bacterial clonotypes in the nasal epithelial mucosa of CRS patients, which persisted for extended time periods despite nasal surgery and intensive antimicrobial therapy. Detection of intracellular *S. aureus* by confocal laser scan immunofluorescence microscopy requires a technically demanding protocol, which may potentially lead to false negative results due to difficulties in localizing the highly focalized bacterial reservoirs [23]. Even if the total number of patients evaluated for intracellular *S. aureus* residency was too low to estimate the general prevalence of this pathology within the overall population of CRS patients, their frequency among enrolled patients was quite impressive and would warrant further evaluation.

To facilitate presumptive identification of patients with putative *S. aureus* intracellular reservoirs, it is mandatory to perform nasal swab cultures under endoscopic guidance in order to avoid microbial contamination by resident vestibular microbial organisms. The demonstration of persistent *S. aureus* clonotypes in patients relapsing for CRS requires the storage of at least two consecutive frozen isolates from each patient, to allow their molecular typing by either pulsed-field gel electrophoresis [23] or using the recently developed VNTR assay [26].

Management of recurrent CRS episodes in patients yielding intracellular *S. aureus* reservoirs is difficult and problematic. The intracellular location combined with the lack of efficient bactericidal mechanisms in non-professional phagocytes are assumed to protect intracellular bacteria from professional

phagocytes and from antimicrobial agents whose action is mainly extracellular [27]. This study further established the short-term benefit of systemic antimicrobial therapy, which clearly alleviated symptoms of *S. aureus*-infected patients, but did not prevent relapsing of CRS episodes in those patients. To target intracellular bacteria, antimicrobial agents should be able to penetrate host cells containing *S. aureus* and exert optimal bactericidal activity, especially against slow growing bacteria [27]. The most effective anti-staphylococcal agent against intracellular infections is clearly rifampin, but its use in monotherapy is compromized by rapid emergence of high-level resistance [28-30]. To overcome this problem, a combined regimen of rifampin with a fluoroquinolone might be suggested, though the impact of this combined antimicrobial regimen for eradicating intracellular *S. aureus* reservoirs has yet to be established and warrants further studies. In the same line, the development of more elaborated in vitro assays that could monitor the impact of antimicrobial therapy against intracellular *S. aureus* would be most useful. In addition to systemic antibiotherapy, improved strategies of local therapy are currently tested. Preliminary data suggest that bid nasal lavage with 0.5% sodium hypochlorite in saline solution seems to improve the clinical status (manuscript in preparation). However the beneficial effects of such regimen does not seem to last over one month.

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