Evidence of an Intracellular Reservoir in the Nasal Mucosa of Patients with Recurrent *Staphylococcus aureus* Rhinosinusitis

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Severe infections due to *Staphylococcus aureus* require prolonged therapy for cure, and relapse may occur even years after the first episode. Persistence of *S. aureus* may be explained, in part, by nasal carriage of *S. aureus*, which occurs in a large percentage of healthy humans and represents a major source of systemic infection. However, the persistence of internalized *S. aureus* within mucosal cells has not been evaluated in humans. Here, we provide the first in vivo evidence of intracellular reservoirs of *S. aureus* in humans, which were assessed in endonasal mucosa specimens from patients suffering from recurrent *S. aureus* rhinosinusitis due to unique, patient-specific bacterial clonotypes. Heavily infected foci of intracellular bacteria located in nasal epithelium, glandular, and myofibroblastic cells were revealed by inverted confocal laser scan fluorescence and electron microscopic examination of posttherapy intranasal biopsy specimens from symptom-free patients undergoing surgery on the sinuses. Intracellular residence may provide a sanctuary for pathogenic bacteria by protecting them from host defense mechanisms and antibiotic treatment during acute, recurrent *S. aureus* rhinosinusitis.

*Staphylococcus aureus* is a major pathogen that has a high potential for colonizing anterior nares or other body sites of both infected patients and healthy carriers [1–3]. Colonized patients are a predominant source of *S. aureus* in hospitals, and a substantial proportion of hospitalized patients develop severe *S. aureus* infections from their own endogenous nasal reservoirs [2–5]. Conflicting observations exist on the molecular mechanisms of *S. aureus* nasal colonization, which have been studied mostly in chronic carriers. Binding properties of *S. aureus* cell-surface components (e.g., cell-wall components such as teichoic acids) [2, 6] or cell wall–associated proteins, known as “microbial surface components recognizing adhesive matrix molecules” [7, 8], with either carbohydrate-rich surface components of mucosal epithelial cells [2] or nasal mucus secretions [9], were extensively studied. Although it provides suitable explanation for initial binding and short-term carriage, extracellular binding of colonizing bacteria can hardly explain the long-term carriage of clonally identical strains of *S. aureus*, such as those found in a significant percentage of healthy carriers [4, 10–13]. Indeed, the presence of effective local defense mechanisms, such as the retrograde mucociliary transport, or locally secreted antimicrobial peptides [14, 15] should, in principle, lead to rapid elimination or replacement of colonizing organisms by exogenous strains [12, 16] and, thus, prevent long-term nasal carriage of identical strains.

Another mechanism that may potentially contribute to long-term endonasal persistence of *S. aureus* in some patients is the possibility of intracellular survival in a range of cell types frequently referred to as “nonprofessional phagocytes” [17, 18]. Notwithstanding its status as an extracellular pathogen [1], *S. aureus* reveals a considerable potential for in vitro invasion of epi-
Figure 1. Clinical course for 3 patients during a 3-year period (left panel). The occurrence of symptomatic episodes, either treated with systemic antibiotics or untreated, is indicated by green or red triangles, respectively. Black arrows indicate surgical biopsy specimens, and boxes represent *Staphylococcus aureus*-positive cultures. The nos. in boxes refer to strain isolates tested by pulsed-field gel electrophoresis (PFGE) (right panel).

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The epithelial and endothelial cells, osteoblasts, fibroblasts, and human embryonic kidney cell lines [18–28]. Several studies have demonstrated that fibronectin-binding proteins promote *S. aureus* internalization by binding to circulating or cellular fibronectin, which, in turn, acts as a molecular bridge to the host cell fibronectin receptor integrin αβ, [17–21, 23, 24, 26–29]. Integrin-mediated invasion of *S. aureus* into nonprofessional phagocytes is promoted by activation of Src family protein-tyrosine kinases [25, 28]. These in vitro data suggest that intracellular reservoirs might provide a sanctuary for *S. aureus* by protecting them from extracellular host defense mechanisms and from the most commonly used antibiotics, such as β-lactams, which do not penetrate into cells [17, 18]. However, evidence of such intracellular reservoirs and their in vivo contribution to persistence of *S. aureus* in humans is still lacking. The objective of the present study is to provide morphological evidence of intracellular foci of *S. aureus*, which may act as potential reservoirs for the multiple recurrent episodes of rhinosinusitis caused by unique, patient-specific clonotypes of this pathogen.

**PATIENTS, MATERIALS, AND METHODS**

**Patients.** This study was performed in compliance with the ethical guidelines established by the University Hospitals of Geneva. From January 2000 to December 2001, 750 outpatients were treated for persistent rhinosinusitis in the rhinology unit of our institution by endoscopic examination of the nasal cavities. The presence of polyps, crust, and/or purulent secretions in the middle meatus was observed in 135 patients (18%), of whom 39 also yielded *S. aureus*-positive cultures in the middle meatus, conducted by endoscopy. Three of these patients required more-extensive investigations because they experienced multiple rhinosinusitis episodes during a 3-year period (range, 12–18 episodes) and regularly relapsed with acute symptoms of nasal obstruction, anterior and posterior rhinorhea, and headache within 2–3 months after standard systemic antimicrobial therapy and daily topical administration of nasal steroids (400 μg of mometasone-17furoas) [30]. During each acute episode, patients’ nasal mucosa, which was evaluated by endoscopic examination of the middle meatus, displayed an erythematous and edematous epithelium and the presence of polyps with purulent secretions (patients 1 and 3). The diagnosis of rhinosinusitis was established by direct coronal computed tomographic scanning, which showed inflammatory mucosa thickening ≥5 mm in ≥2 paranasal sinuses [31]. Direct endoscopically guided sampling of patients’ middle meatus revealed the recurrent presence (during consecutive rhinosinusitis episodes) of *S. aureus* but not of other upper respiratory pathogens, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. Patients yielding *S. aureus*-positive cultures in the middle meatus were treated by standard regimens of oral antistaphylococcal antibiotics, in accordance with the in vitro sensitivity data (none of the *S. aureus* isolates displayed resistance to methicillin), combined with topical administration of nasal steroids (400 μg of mometasone-17furoas), as recommended elsewhere [30]. At 2 weeks after completion of treatment, the overall therapeutic response of each patient was assessed for relief of symptoms, reduction in size of polyps, and disappearance of crusts and/or purulent discharge, as evaluated by endoscopic examination of the nasal cavities.

Endoscopic nasal surgery—including partial middle turbiectomy, middle meatotomy, and anterior ethmoidectomy—was performed with 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 microbial pathogens.

**Bacteriological identification and molecular typing.** Swabs of nasal secretions or biopsy specimens (disrupted in a sterile tissue grinder in 500 μL of 0.9% NaCl) were plated on blood, chocolate, and Columbia colistin nalidixic agar (Becton Dickinson), and then *S. aureus* was identified according to standard bacteriological criteria. Molecular typing of sequential *S. aureus* isolates was performed by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA digested with *Sma*I (Bio-Rad), by use of a CHEF MAPPER system (Bio-Rad) [32]. DNA banding patterns of the different gels were analyzed by use of the software GelCompar (version 4.1; Applied Math) and interpreted according to criteria published elsewhere [32].

**Indirect immunofluorescence, confocal laser scanning microscopy, and light microscopy.** Nasal biopsy specimens were embedded in OCT 4583 (Miles Scientific) and frozen in precooled liquid isopentane. Three-micrometer cryostat sections were fixed in acetone for 5 min at −20°C and stained with anti-*S. aureus* antibodies (mouse IgM; Chemicon International) combined with antibodies directed against either human keratin (wide-spectrum screen rabbit polyclonal antibody [Dako]) or α-smooth muscle actin mouse IgG2a [33] or human CD45 (mouse IgG1 [BD Biosciences PharMingen]). Control experiments assessed the specificity of anti-*S. aureus* antibodies directed against staphylococcal peptidoglycan, which, under the conditions used for immunofluorescence, did not cross-react with *Staphylococcus epidermidis* or other gram-positive species. Subsequently, samples were incubated with Alexa 488–labeled anti-mouse IgM antibodies (Molecular Probes) and with secondary tetramethylrhodamine isothiocyanate–conjugated antibodies recognizing either rabbit (Jackson ImmunoResearch Labs) or the different mouse isotypes (Southern Biotechnology Associates); the secondary antibodies were diluted in PBS solution containing 1:100 human serum and 1:1000 nuclear dye (TOTO-III; Molecular Probes) and were mounted in polyvinyl alcohol mounting medium. An inverted confocal laser scan fluorescence microscope (Carl Zeiss) was used to collect 3-D image sets. Stacks of confocal images were processed by use of IMARIS software (version 3.2; Bitplane AG) for 3-D view of the infected cells. Simultaneous detection, by confocal imaging, of immunostained *S. aureus* and cytoplasmic components (e.g., cytokeratin) on 0.5-μm-thick cross-sectional images was performed to assess the intracytoplasmic localization of bacteria.

For light microscopic examination, pieces of tissue were fixed in 10% neutral buffered formal and embedded in paraffin. Four-micrometer serial sections were stained with either hematoxylin-eosin or blue alanine Masson’s trichrome or were immunostained with anti-*S. aureus* antibodies. Immunoperoxidase staining was...
Figure 3. Presence of *Staphylococcus aureus* in epithelial cells of nasal epithelium, evaluated by transmission electron microscopy (TEM) (arrows; B), immunohistochemical methods (brown; C), and immunofluorescence (*S. aureus* in green, keratin in red, and nuclei in blue; D). A. Schematic representation of the multistep protocol used to optimize the detection of *S. aureus* by TEM. The highly infected areas (star corresponding to the dashed circle in C) were selected by microscopy (step 1), and fragments were punched from the paraffin-embedded block (step 2) and processed for TEM. Insets in B and C represent a zoomed portion of each image. ci, cilia from the cell apical surface; ep, epithelial cell; N, nucleus; pm, plasma membrane. Bars in C and D, 20 μm. Original magnification in B, ×2200; original magnification in inset, ×23,000.

performed as described elsewhere [34]. Sections were examined by use of an Axiophot photomicroscope (Carl Zeiss), and images were acquired by use of an Axiocam camera (Carl Zeiss).

**Transmission electron microscopy (TEM).** Highly infected areas were selected by microscopic examination of paraffin-embedded tissues stained, by immunohistochemical methods, with anti-*S. aureus* antibodies, as described above, and punch fragments of the paraffin-embedded block were then deparaffined in xylol and ethanol, followed by fixation in 1% osmium tetroxide for 1 h and subsequent dehydration and embedding in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined by use of a CM10 electron microscope (Philips).

**RESULTS**

The time course of multiple symptomatic rhinosinusitis episodes (range during a 3-year period, 12–18) for each of the 3 patients is shown in parallel with *S. aureus*–positive cultures of middle meatus samples and courses of systemic antimicrobial therapy combined with daily topical administration of nasal steroids (figure 1). PFGE data were used to assess the presence of single, patient-specific clonotypes of *S. aureus* during multiple rhinosinusitis episodes (figure 1).

Histological analysis of posttherapy intranasal biopsy specimens from patients with *S. aureus* rhinosinusitis undergoing surgery on the sinuses showed the presence of diffuse fibrotic areas surrounding mucous-producing glands (figure 2B) that were characterized by expression of α-smooth muscle actin (data not shown), an actin isoform typical of myofibroblastic cells, which are abundant and persistent in fibrotic inflammatory tissue [35]. Although the presence of *S. aureus* was easily detected by microbiological cultures of biopsy specimens, its detection by use of a cell wall–specific monoclonal antibody was far more difficult, because of highly irregular spatial distribution. Intracellular location of *S. aureus* was assessed by confocal immunofluorescence microscopic examination of nasal specimens that combined immunodetection of *S. aureus* with specific markers of different cell types. *S. aureus* frequently colocalized with cytokeratin (intracytoplasmic epithelial–specific intermediate filament protein) [36] in mucous glands (figure 2C) and nasal epithelial cells (figure 3D). By use of Z-scan, *S. aureus* clusters were also observed in myofibroblastic cells at the same level as was α-smooth muscle actin, which appeared to form a constricting ring around bacteria (figure 2D).
positive professional phagocytic cells distributed throughout the connective tissue, in epithelium, or in fibrotic areas (figure 2E). Confocal microscopic analyses provided further evidence that approximately one-third of the S. aureus-infected cells, regardless of the cell type, carried >10 bacteria.

Intracellular S. aureus were also seen by TEM examination of highly infected foci (selected by microscopic examination of tissue sections stained, by immunochemical methods, with anti-S. aureus) (figure 3C) that had been punched from paraffin-embedded tissue and reprocessed for TEM (figure 3A). This protocol was required for accurate localization of S. aureus-containing areas that were dispersed within nasal mucosa specimens. TEM examination (figure 3B) confirmed the presence of numerous intracytoplasmic S. aureus in nasal epithelial cells.

**DISCUSSION**

The mechanisms of persistence and recurrence of S. aureus in humans are still partly speculative. The lack of in vivo morphological evidence of intracellular S. aureus reservoirs, which may contribute to clinically important S. aureus–persistent diseases, might be explained, in part, by technical difficulties in localizing the highly focalized S. aureus intracellular reservoirs [17]. Despite its in vitro potential for invading a range of nonprofessional phagocytes [18–28], S. aureus has no dedicated molecular system of penetration, in contrast to enteroinvasive bacteria [37].

The fate of internalized S. aureus after endocytosis in cell culture models may be determined by the interplay between host cell defense mechanisms and the intrinsic virulence of internalized S. aureus strains. A frequently described scenario is that of initial intraphagosomal survival, followed by S. aureus escape into the cytoplasm, followed by intracellular replication and eventually leading to host cell apoptosis [18, 38–44]. However, in vitro studies showing rapid (within 24–48 h) triggering of apoptosis by intracellular S. aureus are of uncertain relevance for explaining long-term persistence of bacteria in vivo. In this context, an important determinant shown to significantly improve the long-term persistence of bacteria in vivo. In this context, an important determinant shown to significantly improve the long-term persistence of bacteria in vivo.

**References**

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