Antibiotic-induced persistence of cytotoxic *Staphylococcus aureus* in non-phagocytic cells

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**Objectives:** After infection of non-phagocytic cells, some *Staphylococcus aureus* strains are able to survive and kill their host cells. The purpose of this study was to determine the action of various antibiotics on the survival of host cells and/or intracellular *S. aureus*.

**Methods:** Murine keratinocyte (PAM212) and fibroblast (mKSA) cell lines were infected with cytotoxic *S. aureus* and cultured in the presence of various antibiotics at graded concentrations. The viability of host cells was measured 24 h after infection. To determine the bacterial viability within host cells, cellular lysates were prepared and colony forming units were quantified using a spiral plater. Host cells infected with fluorescein isothiocyanate (FITC)-labelled *S. aureus* were analysed by flow cytometry and microscopy to determine the subcellular localization *S. aureus*.

**Results:** Oxacillin, vancomycin, gentamicin, ciprofloxacin and trimethoprim/sulfamethoxazole did not rescue host cells from cell death induced by intracellular *S. aureus*. In contrast, linezolid, rifampicin, azithromycin, clindamycin, erythromycin and quinupristin/dalfopristin suppressed the cytotoxic action of *S. aureus*. After withdrawal of antibiotics, intracellular *S. aureus* regained cytotoxic activity and killed their host cells. Only rifampicin was able to eliminate intracellular *S. aureus* completely within 72 h. In contrast, clindamycin, azithromycin and linezolid induced a state of intracellular persistence of viable *S. aureus*.

**Conclusions:** Antibiotics commonly used for the management of *S. aureus* infections appear to create a niche for invasive intracellular *S. aureus*, which may play an important role for persistence and recurrence of infection. Because of its unique ability to eliminate intracellular *S. aureus*, rifampicin appears to be valuable for the treatment of invasive *S. aureus* infections.

**Keywords:** anti-infective agents, staphylococcal infections, microbial susceptibility tests, cell death

**Introduction**

*Staphylococcus aureus* is a highly virulent pathogen posing an increasing challenge both in community-acquired and nosocomial infections.1 *S. aureus* is a frequent cause of severe diseases, ranging from wound and soft tissue infections to endocarditis and septic shock. Although *S. aureus* represents the prototype of an extracellular pathogen, recent investigations revealed its ability to infect and survive in various types of host cells, both professional phagocytes and non-phagocytic cells.2–7 Moreover, long-term intracellular persistence of small colony variants of *S. aureus* has been described in association with chronic osteomyelitis, cystic fibrosis and skin infections.8–11 The facultative intracellular persistence of staphylococci may play an important role in the pathogenesis, because this localization protects them from both humoral and cell-mediated immune responses. The intracellular habitat of *S. aureus* calls for antibiotics with intracellular activity toward *S. aureus*. However, antibiotics are routinely tested only for their in *vitro* activity on extracellular bacteria. Obviously, the intracellular activity of antibiotics can significantly differ from that exerted extracellularly. In general it seems impossible to deduce the intracellular antibacterial activity of antibiotics from standard susceptibility tests.12,13 Up to the present time, the intracellular activity of antibiotics on *S. aureus* has been examined with polymorphonuclear leucocytes and macrophages.14 These cell types represent an important part of the host defence and clear bacteria from the organism. However, little attention has been devoted to the fate of non-professional phagocytic host cells after invasion by staphylococci during antimicrobial therapy.

We have previously shown that for the majority of clinical *S. aureus* isolates, uptake by non-phagocytic host cells results in
elimination of bacteria by cell-autonomous defence mechanisms. However, approximately one-third of clinical S. aureus isolates not only survived in, but also killed their eukaryotic host cells, which was accompanied by increased in vivo virulence. The survival of non-phagocytic host cells such as endothelial, epithelial or parenchymal cells may have important implications because of their metabolic, structural and barrier functions. Therefore, a successful anti-staphylococcal therapy should include the elimination of intracellular bacteria and the rescue of host cells from staphylococci-induced cell death. The aim of this study was therefore to determine which antibiotics exert bactericidal activity on intracellular cytotoxic S. aureus strains in non-phagocytic host cells and whether antibiotics can prevent S. aureus-induced cell death.

Materials and methods

Antibiotics

Oxacillin, clindamycin, vancomycin, erythromycin, trimethoprim/sulfamethoxazole, rifampicin and gentamicin were obtained from Sigma (Taufkirchen, Germany). Linezolid, quinupristin/dalfopristin, ciprofloxacin and azithromycin were purchased from Pharmacia (Peapack, NJ, USA), Aventis Pharma (Frankfurt am Main, Germany), Bayer (Leverkusen, Germany) and Pfizer (Karlsruhe, Germany), respectively.

Antibodies

Lysosomes were stained with anti-LAMP-1 monoclonal antibody (BD Pharmingen, San Diego, CA, USA) followed by cyanin-3 (Cy3)-coupled anti-rat IgG secondary antibody (Dianova, Hamburg, Germany).

Bacteria

S. aureus ATCC 29213 reference strain was used throughout this study. S. aureus grown overnight at 37°C in LB broth were diluted with fresh broth and cultured until mid-logarithmic phase of growth (OD600 = 0.3). Bacteria were harvested, washed with PBS and plated on Mueller-Hinton agar. Colony counting instructions.

Fluorescein isothiocyanate (FITC)-staining of S. aureus

Bacterial pellets of $1 \times 10^{10}$ cfu were resuspended in carbonate buffer (pH 9.0) containing 100 mg/L FITC isomer I (Sigma) for 1 h at room temperature. Staphylococci were extensively washed with PBS, adjusted with 10% glycerol to $10^{7}$ cfu/mL and kept at $-70$°C until use. No significant reduced viability or fluorescence intensity during the freeze-thaw procedure was observed.

Eukaryotic cell culture

Mouse keratinocyte PAM212 and fibroblast mKSA cell lines were cultured in RPMI 1640 (Biochrom, Berlin, Germany) or DMEM (Biochrom), respectively. Both media were supplemented with 10% heat-inactivated fetal calf serum (Biochrom), 100 U/mL penicillin G and 100 mg/L streptomycin sulphate (Biochrom); 18 h before infection, $5 \times 10^{5}$ cells were seeded in six-well plates (total volume = 3 mL) (Nunc, Wiesbaden, Germany). Viability was monitored using Trypan Blue (Biochrom) exclusion.

cfu determination

Infected cells were lysed in PBS containing 0.05% Triton X-100. Lysates were sonicated for 5 min at 4°C. This procedure was found to be most effective for resolving the bacterial clumps. Then, lysates were diluted with PBS and plated on Mueller–Hinton agar using a spiral plater (EDDY-Jet, IUL Instruments, Königswinter, Germany). Colony counting and cfu determination were carried out according to the instructions of the manufacturer.

Invasion assay

Before infection, cells were washed with growth medium without antibiotics and kept for 1 h at 37°C. Then $10^{10}$–$10^{8}$ FITC-stained staphylococci were added per well. After 1 h of incubation, cells were detached from the culture plate and washed with growth medium. Extracellular staphylococci were killed by incubation of cells with 100 mg/L lysozyme (Sigma) for 7 min at 37°C. This treatment was found to be effective at eradicating all extracellular S. aureus. Cells were washed with growth medium complemented with antibiotics and reseeded in a new six-well plate (total volume = 3 mL). After 24 h of incubation, cells were harvested and analysed by flow cytometry and microscopy. Host cell viability was measured using Trypan Blue exclusion. For fluorescence microscopy, cells were grown on cover slips. After the invasion procedure, cells were rinsed with PBS and fixed for 15 min with 4% paraformaldehyde. Cells were permeabilized with 0.1% saponin before staining with antibodies specific for the lysosome specific marker LAMP-1. Specimens were mounted on microscopy slides in 10% glycerol supplemented with 100 mg/mL DABCO (diazabicyclo[2.2.2]octane, Sigma). Images were acquired using an Axioscop 2 microscope (Zeiss, Oberkochen, Germany) equipped with an AxioCam charge-coupled device camera and analysed using Axiovision software (Zeiss).

Flow cytometry

The supernatants of the infected cultures were removed to preserve detached cells. Subsequently the adherent cells were harvested by standard trypsin treatment and combined with cells from the supernatants. Cell samples were washed once with PBS and submitted to analysis by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany). Data were acquired in linear mode for the forward and side scatter and logarithmic mode for green fluorescence. No gates were applied. For each sample, $10^{6}$ cells were measured. Data were presented as the distribution of green fluorescence in the cell population. Non-infected cells served as negative control to set the cut-off marker for the discrimination of FITC-negative and -positive cells.

Statistical analysis

Experimental data were assessed with a two-tailed unpaired Student’s $t$-test for comparison between means. $P$ values of <0.01 were considered to be statistically significant.

Results

Antibiotic-induced protection of S. aureus-infected host cells

Mouse PAM212 keratinocytes or mKSA fibroblasts were infected at a multiplicity of infection (MOI) of 200 with S. aureus strain ATCC 29213 that has been shown to belong to the cytotoxic fraction of S. aureus strains. In the absence of antibiotics, S. aureus ATCC 29213 kills >76% of host cells within 24 h (Figure 1). When various
Antibiotics were added directly after infection, only clindamycin, rifampicin, azithromycin, erythromycin, linezolid and quinupristin/dalfopristin rescued host cells from *S. aureus*-induced cell death. In contrast, oxacillin, vancomycin, gentamicin, ciprofloxacin and trimethoprim/sulfamethoxazole did not prevent the death of infected host cells even when used at concentrations corresponding to the highest serum level achievable in vivo. There was no difference between the effect of antibiotics on intracellular *S. aureus* in keratinocytes or fibroblasts. Antibiotics that rescued keratinocytes from *S. aureus*-induced cell death also rescued fibroblasts. No *S. aureus* could be detected in any culture supernatants, indicating that each of the antibiotics effectively suppressed growth of extracellular *S. aureus* released from dead host cells.

To determine the efficacy of antibiotics that protected *S. aureus*-infected host cells, antibiotics were added at graded concentrations to *S. aureus*-infected host cells. Each of the antibiotics rescued both fibroblasts (Figure 2) and keratinocytes (not shown) in a dose-dependent manner. It should be noted that clindamycin protected host cells at a concentration even below 1 × MIC, whereas quinupristin/dalfopristin achieved complete protection only at >10 × MIC. In relation to their respective MICs, a hierarchy becomes apparent where clindamycin shows the greatest efficacy followed by linezolid, azithromycin, rifampicin, erythromycin and quinupristin/dalfopristin (Figure 2). Indeed, the concentrations of quinupristin/dalfopristin and erythromycin required to be effective were near or even beyond the previously reported respective maximal serum concentrations.\(^19,20\) Thus, these results demonstrate that only clindamycin, rifampicin, azithromycin and linezolid exert antibacterial activity on intracellular *S. aureus* at concentrations relevant to the clinical situation.

Reversibility of protective effects

In host cells treated with rifampicin, FITC-labelled *S. aureus* were readily detectable by flow cytometry 24 h after infection (Figure 3), suggesting that the *S. aureus* were still viable. The killing of intracellular *S. aureus* occurs in mature lysosomes and is associated with reduced pH. As FITC is not fluorescent in the acidic pH range found in the lysosomal compartment, it is unlikely that fluorescence measured in flow cytometry came from dead bacteria or bacterial fragments. The reduction in fluorescence 24 h after infection was the result of growth of *S. aureus*, as dividing bacteria quickly lose FITC-staining.\(^15\) Alternatively, a minor fraction of *S. aureus* might not escape the lysosomal compartment and be killed. In order to test whether the remaining *S. aureus* present in host cells after a 24 h period of exposure to antibiotics were still able to exert cytotoxic activity, infected murine fibroblasts were first incubated with either rifampicin, linezolid, or clindamycin for 24 h and then extensively washed to remove the respective antibiotic. As shown in Figure 4, upon removal of the antibiotic, persisting *S. aureus* became cytotoxic again and killed their host cells within 24 h, indicating that, in principle, the antibiotic-mediated inhibition of cytotoxicity of *S. aureus* is a reversible effect. Compared with clindamycin, azithromycin and rifampicin, the reversibility of linezolid on *S. aureus*-related cytotoxic activity appeared less pronounced.

Antibiotic-induced persistence of intracellular *S. aureus*

When *S. aureus* infected host cells were analysed for viable bacteria over a long period of time, only high dose rifampicin proved effective in reducing the cfu of *S. aureus* (Figure 5). In the presence of 7 mg/L rifampicin, the number of cfu of *S. aureus* declined rapidly to become undetectable within 3 days. In contrast, even when used at maximal achievable in vivo concentrations, linezolid, clindamycin or azithromycin reduced the viability of *S. aureus* with significantly slower kinetics, leaving a considerable number of viable *S. aureus* after 6 days of treatment.

Subcellular localization of *S. aureus* in rifampicin-treated host cells

Host cells kill ingested non-cytotoxic *S. aureus* by means of phagolysosomal fusion and subsequent degradation by oxygen radicals,
NO and proteases. Cytotoxic *S. aureus* can avoid such killing by escaping from the endolysosomal compartment.\textsuperscript{15} We thus addressed the question of whether inhibition of protein synthesis by rifampicin might redirect cytotoxic *S. aureus* to the phagolysosomal degradation pathway. As shown in Figure 6, *S. aureus* did not co-localize with the lysosomal marker LAMP-1 in cells treated with rifampicin, indicating that cytotoxic *S. aureus* retain their phagosomal escape mechanisms even in the presence of rifampicin.

**Discussion**

The aim of this study was to determine the intracellular anti-staphylococcal activities of antibiotics in non-phagocytic host cells. It is well established that *S. aureus* invade non-phagocytic cells including keratinocytes, fibroblasts and endothelial cells.\textsuperscript{2–7} As previously shown, a significant fraction of clinical *S. aureus* isolates not only invades but also survives within and kills their non-phagocytic host cells, which seems to have implications for the pathogenicity and virulence of *S. aureus in vivo*.\textsuperscript{15}

Our experimental models demonstrate that not all antibiotics known to be active against *S. aureus in vitro* protect infected host cells from *S. aureus*-mediated cell death. A lack of protection with oxacillin or vancomycin is not surprising because of their inability to cross the plasma membrane. However, even among the antibiotics known to readily enter eukaryotic cells, significant differences were observed with respect to protection of host cells. Compared with their respective MICs, clindamycin appeared to be the most effective antibiotic, whereas ciprofloxacin completely failed to protect host cells. The possible reasons for that discrepancy are numerous: (i) the degree of plasma membrane permeability may determine concen-
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Surprisingly, only one single antibiotic, rifampicin, effectively eradicated *S. aureus* in non-phagocytic host cells. This is in stark contrast to previous studies which showed that other antibiotics like clindamycin, erythromycin, quinupristin/dalfopristin and quinolones accumulate in eukaryotic cells and enhance intracellular killing of *S. aureus* in neutrophils and macrophages. However, one major limitation of the *S. aureus*/neutrophil model is the very efficient killing mechanism of the neutrophils against this bacterium. The neutrophil model is more sensitive in detecting possible synergies between phagocytic oxygen-dependent killing mechanisms and intracellular antimicrobial activity of antibiotics. In phagocytes, *S. aureus* is contained in the phagolysosomal compartment and may be co-ingested and co-localize with antibiotics. It has been also shown that uptake into pinocytic vesicles and their fusion with the phagosomes can transport antibiotics even to bacteria that already persisted intracellularly. Such activity was not reported for non-phagocytic cells. Additionally, *S. aureus* can evade the phagosome in non-phagocytic cells. The distinct subcellular localization of *S. aureus* might impair, at least in part, the accessibility for antibiotics. A high intracellular concentration of a given antibiotic does not guarantee effective intracellular antimicrobial activity. What counts is the concentration of free active antibiotic where the bacteria are. Thus, the concentrations of clindamycin, linezolid, erythromycin, azithromycin and quinupristin/dalfopristin acting on *S. aureus* in combination can explain the differences between the antibiotics with regard to their intracellular activity.

Figure 5. Clindamycin, azithromycin and linezolid induce intracellular persistence of cytotoxic *S. aureus*. mKSA cells were infected with *S. aureus* and incubated with rifampicin (7 mg/L, circles), clindamycin (10 mg/L, triangles), linezolid (18 mg/L, squares) or azithromycin (7 mg/L, diamonds). The concentrations of antibiotics correspond to the respective maximum serum levels.

Figure 6. Subcellular localization of *S. aureus* in the presence of rifampicin. PAM212 cells were infected with FITC-labelled *S. aureus* and treated with 1 mg/L rifampicin for 24 h; 24 h after infection, cells were fixed and stained with anti-LAMP-1 antibody followed by Cy3-labelled secondary antibody. Images were acquired using FITC filter (lower left), rhodamine (upper right), superimposition of fluorescence images (upper left) or bright light image (lower right).
in the cytoplasm may be low in relation to the total amount of drug present in the cell.

In the past, numerous attempts have been made to correlate the in vivo efficiency of antibiotics with their intracellular action on S. aureus including accumulation, subcellular distribution and antibacterial activity. The in vitro killing activity, however, has been difficult to correlate with the therapeutic success. For example, whereas the efficacy of rifampicin is consistent in both the in vitro and in vivo systems that have been studied, ciprofloxacin, which kills S. aureus sequestered within neutrophils in vitro, was ineffective in an abscess animal model. In contrast, clindamycin was found to be effective in the treatment of experimental S. aureus infections, but did not enhance killing of bacteria by neutrophils in vitro. Because of these apparent discrepancies, it remains controversial whether antibiotics that kill intracellular staphylococci may have an advantage in vivo over antibacterial drugs that do not.

Strikingly, the many reports on the in vitro efficacy of antibiotics in S. aureus infections are most consistent with respect to their intracellular activity in non-phagocytic cells shown in this study. We would like to emphasize that these considerations are only relevant to cytotoxic S. aureus strains. Non-cytotoxic S. aureus isolates are efficiently eliminated by eukaryotic host cells in the presence as well as in the absence of antibiotics.

Finally, our observation that clindamycin, azithromycin and linezolid do not kill intracellular S. aureus but instead induce long-term persistence provides a plausible explanation why these antibiotics may not be ideal for the management of an infection caused by an invasive, cytotoxic S. aureus strain. These antibiotics do efficiently clear extracellular S. aureus from the circulation or tissues, and yet at the same time may produce a reservoir of viable intracellular S. aureus that escape cell-autonomous defence mechanisms, innate and specific immune responses, and, last but not least, diagnostic detection. Persistent S. aureus do not remain contained by their host cells. After removal of antibiotics, S. aureus may reactivate their cytotoxic potential, escape their host cells, and eventually cause recurrence of infection.

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