Intracellular Activity of Antibiotics against *Staphylococcus aureus* in a Mouse Peritonitis Model

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Antibiotic treatment of *Staphylococcus aureus* infections is often problematic due to the slow response to therapy and the high frequency of infection recurrence. The intracellular persistence of staphylococci has been recognized and could offer a good explanation for these treatment difficulties. Knowledge of the interplay between intracellular antibiotic activity and the overall outcome of infection is therefore important. Several intracellular in vitro models have been developed, but few experimental animal models have been published. The mouse peritonitis/sepsis model was used as the basic in vivo model exploring a quantitative ex vivo extracellular and intracellular differentiation assay. The intracellular presence of *S. aureus* was documented by electron microscopy. Five antibiotics, dicloxacillin, cefuroxime, gentamicin, azithromycin, and rifampin (rifampicin), were tested in the new in vivo model; and the model was able to distinguish between their extracellular and intracellular effects. The intracellular effects of the five antibiotics could be ranked as follows as the mean change in the log10 number of CFU/ml (Δlog10 CFU/ml) between treated and untreated mice after 4 h of treatment: dicloxacillin (3.70 Δlog10 CFU/ml) > cefuroxime (3.56 Δlog10 CFU/ml) > rifampin (1.86 Δlog10 CFU/ml) > gentamicin (0.61 Δlog10 CFU/ml) > azithromycin (0.21 Δlog10 CFU/ml). We could also show that the important factors during testing of intracellular activity in vivo are the size, number, and frequency of doses; the time of exposure; and the timing between the start of infection and treatment. A poor correlation between the intracellular accumulation of the antibiotics and the actual intracellular effect was found. This stresses the importance of performing experimental studies, like those with the new in vivo model described here, to measure actual intracellular activity instead of making predictions based on cellular pharmacokinetic and MICs.

*Staphylococcus aureus* is a major human pathogen that causes both community- and hospital-acquired infections (35). It causes a diverse array of infections ranging from relatively minor skin and wound infections to more serious and life-threatening diseases such as pneumonia (20, 46), endocarditis (48), osteomyelitis (17, 29), arthritis (1), and meningitis (40). Some of these types of *S. aureus* infections, e.g., endocarditis, are associated with high rates of mortality (25 to 50%), despite antimicrobial treatment (48, 49, 57). Furthermore, *S. aureus* infections are often persistent and are associated with treatment difficulties, such as a slow response to antibiotic treatment and recurrences, that lead to an extended duration of antimicrobial therapy (11, 13, 31). The antimicrobial treatment of *S. aureus* infections has also become more difficult due to the emergence of multidrug-resistant strains (3, 4).

Several factors may help explain the capacity of staphylococci to avoid the actions of antibiotics. Biofilm formation might be the main reason for a deficient antibiotic effect when foreign bodies are involved in the staphylococci infections (12, 15, 53). Otherwise, the intracellular presence of the bacteria could offer a good explanation for the slow response to antibiotics, since bacteria located intracellularly might be protected from the effects of antibiotics (55).

*S. aureus* has classically been classified as an extracellular pathogen (21). Conversely, several reports have established that *S. aureus* internalizes and survives within professional and even nonprofessional mammalian phagocytes (7, 19, 24, 25, 26, 27). The attitude is therefore changing toward classifying *S. aureus* as a facultative/opportunistic intracellular pathogen (13, 36, 41, 42, 55).

Having an intracellular target for antimicrobial therapy is more complex than having an extracellular target, because intracellular antimicrobial activity further depends on the penetration into and accumulation in the cell, cellular metabolism, the subcellular disposition, and the bioavailability of the drug. The bacterial responsiveness to antibiotics can also change intracellularly (54, 55). Antimicrobial activity is therefore often impaired intracellularly (6, 56).

To date, this knowledge of the intracellular presence of *S. aureus* has not influenced the choice of antibiotic to be used for the treatment for *S. aureus* infections. Penicillinase-stable penicillins, for instance, are considered the mainstay of treatment for methicillin-susceptible *S. aureus* infections (5, 23, 35), even though penicillins are usually considered not to penetrate cells (8, 30, 50).

Recurrent *S. aureus* infections may also, at least partly, be explained by the intracellular presence of the bacteria. Gresham et al. demonstrated that polymorphonuclear neutrophils with intracellular *S. aureus* isolated from the peritoneums of infected mice could cause a new infection by intraperitoneal injection of these cells into healthy mice.
activities of antimicrobials against S. aureus. Only a few in vivo models have been developed for the study of intracellular antibiotic action. In this study, we report the development of an in vivo model that can be used to study the intracellular activities of antibiotics against S. aureus. The model was tested in mice, and the results are compared to data from the literature, which is why these dosages were estimated on the basis of calculation of the surface area.

The MIC for DCX was estimated by using an oxacillin Etest. The MICs for DCX and oxacillin are identical, according to the Clinical and Laboratory Standards Institute (14).

Here we present an in vivo model that can be used to study the intracellular activities of antimicrobials against S. aureus.

(Materials and Methods) Bacterial strains and growth conditions. A clinical strain of S. aureus from a patient with bacteremia, strain E19977 (Statens Serum Institut, Copenhagen, Denmark), was used throughout the study. The strain is penicillin resistant and methicillin susceptible. The organism was grown and quantified on 5% blood agar plates. Inocula were prepared by measurement of the optical density at 546 nm. The accurate bacterial count (CFU/ml) was quantified by the use of 10-fold dilutions obtained by spotting each dilution (20 μl) in duplicate on agar plates. Inocula were prepared by measurement of the optical density at 546 nm. The accurate bacterial count (CFU/ml) was quantified by the use of 10-fold dilutions obtained by spotting each dilution (20 μl) in duplicate on agar plates.

The detection limit was 25 CFU/ml. Saline with 0.1% (vol/vol) Triton X-100 was used in the dilutions to prevent the effect of bacterial clumping. Dilution and spotting were performed in one step, and dilutions containing both bacteria and Triton X-100 were never performed with the analyzer; and the WBCs were discriminated into granulocytes (WBC) count was determined with an automatic hematology analyzer (Medonic CA620 VET; Boule Medical AB, Stockholm, Sweden). WBC differentials were performed with the analyzer; and the WBCs were discriminated into granulocytes, monocytes, and lymphocytes.

Light microscopy. The sample preparations were fixed with a flame, stained with methylene blue (1%; Sigma Diagnostics, Hillerod, Denmark), and studied with light microscopy at ×1,000 magnification.

Electron microscopy. The suspension of cells and bacteria was fixed by adding glutaraldehyde in 0.1 M caccodylate buffer (pH 7.2) containing 0.01 M CaCl₂ to a final concentration of 3% (vol/vol). After 60 min of fixation at 4°C, the samples were centrifuged at 300 × g for 15 min. To increase the cell concentration, three-fourths of the supernatant was removed. Procedures for further preparation of the samples were as described previously (2).

Separation of intracellular and extracellular S. aureus in the peritoneal wash. Samples from two mice were pooled (1:1) to ensure a minimum sample volume of 2.0 ml. The total amount of bacteria in the pooled sample was quantified before any other procedure was performed. Then, 1.5 ml of the pooled samples was transferred to micro-test tubes (Eppendorf AG, Hamburg, Germany) and

### Materials and Methods

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extracellular bacteria. A cell-free bacterial suspension was run in parallel as a suspension was incubated at room temperature for 7 min to kill the remaining bacteria. The killing was recorded for 24 h (Fig. 1). GEN, DCX, CXM, and RIF all showed bactericidal effects (>2-log_{10} unit decreases). GEN was the most effective drug at these concentrations, with a 4-log_{10} decrease achieved within the first 4 h. RIF had a slower but more persistent bactericidal effect than DCX and CXM (a 2-log_{10} unit decrease within the first 12 h), which could be due to the longer half-life applied for this drug in the in vivo model (Table 1). AZM showed a bacteriostatic effect for the first 8 h, but in line with the antibiotic washout, growth occurred in parallel with the control growth. For all five antibiotics tested, regrowth of the bacteria occurred (Fig. 1).

RESULTS

MIC determination and time-kill curves in vitro. The MICs for DCX, CXM, AZM, GEN, and RIF against S. aureus E19977 are shown in Table 1. The strain was susceptible to all five antibiotics, according to the guidelines of the Clinical and Laboratory Standards Institute (14). The in vitro time-kill profiles of the five antibiotics were tested by use of the in vitro kinetic model. Peak (maximum) concentrations (C_{max}) corresponding to the free drug (fC_{max}) (Table 1) were applied, and the killing was recorded for 24 h (Fig. 1). GEN, DCX, CXM,
The changes in the numbers of CFU/ml after 4 h of treatment are shown in Fig. 3A to I. The bacterial counts in the peritoneums of untreated mice increased 1.94 log10 units in total, 3.65 log10 units intracellularly, and 1.88 log10 units extracellularly. All antibiotics except AZM produced significant decreases in colony counts in total, extracellularly, and intracellularly compared to the colony counts in the untreated control group by the Dunnett’s multiple-comparison test.

The effects of the five antibiotics were compared by Tukey’s multiple-comparison test. The effects of the antibiotics on the total colony counts, the extracellular colony counts, and the intracellular colony counts were compared. The levels of significance between groups are marked by asterisks: *, 0.05 > P > 0.01; ***, 0.01 > P > 0.001; ***, P < 0.001; and NS, nonsignificant.

The effects of the five antibiotics fell into two significantly different groups when the total effects were compared, as follows:

- DCX = CXM = RIF > GEN = AZM
  - NS
  - NS
  - ***
  - NS

DCX, CXM, and RIF showed the best total effect, and their effects did not differ significantly from each other. GEN and AZM showed the poorest effect.

The five antibiotics fell into four significantly different groups when the extracellular effects were compared, as follows:

- RIF > DCX = CXM > GEN > AZM
  - ***
  - NS
  - ***
  - ***

RIF showed the best effect extracellularly, while DCX, CXM, and GEN showed intermediate effects. AZM showed the poorest effect.

Finally, when the intracellular effects were compared, the antibiotics fell into three groups, as follows:

- DCX = CXM > RIF > GEN = AZM
  - NS
  - ***
  - NS
  - **

DCX and CXM showed the best intracellular effects, while RIF showed an intermediate effect and GEN and AZM showed the poorest intracellular effects.

The effect of GEN in vivo was very poor compared to the effect of GEN seen in vitro (Fig. 1). The effect of RIF intracellularly differed remarkably from its effect extracellularly (Fig. 3G to I).

**Timing of treatment onset and bacterial challenge.** The influence of different timing intervals between inoculation and treatment onset was explored. Mice were treated 1, 2, or 3 h after inoculation with a single dose of DCX (60 mg/kg of body weight) or RIF (10 mg/kg) (four mice per group). All mice were sampled after 4 h of treatment; but the duration of infection was 5, 6, or 7 h, respectively. Vehicle-treated control groups were included; and they were also sampled 5, 6, or 7 h after inoculation.

The change in log10 CFU/ml (Δlog10 CFU/ml) between the treated and the untreated mice after 4 h of treatment according to the time of treatment after inoculation is shown in Fig. 4. The values of Δlog10 CFU/ml between the treated and the untreated mice were calculated by using untreated mice with the same times of infection (i.e., 5, 6, or 7 h). The effects of the two drugs in all three fractions (total, intracellular, and extracellular) were influenced by the time of treatment; i.e., the effect decreased with an increasing time between inoculation and treatment onset. For DCX the difference was most noticeable for the intracellular count, and for RIF the difference was most noticeable for the extracellular count.

**Dose-response relationship extra- and intracellularly.** Mice were treated with five different doses of DCX (200, 60, 30, 20, or 10 mg/kg; 4 to 16 mice per group) 2 h after inoculation. The mice were sampled after 4 h of treatment. Vehicle-treated control groups were included. The Δlog10 CFU/ml correlated to the dose given (log10 mg/
kg) is shown in Fig. 5. Significant dose-response correlations were recorded both for the total count and when the counts were separated into the extra- and intracellular compartments. The untreated control group formed a part of the dose-response curve by including the colony count for the control group as a very small concentration (0.3 mg/kg). The static dose for each compartment was calculated by interpolation in GraphPad Prism software. The static doses were 47.2 mg/kg for the intracellular compartment (Fig. 5B), 18.2 mg/kg for the extracellular compartment (Fig. 5C), and 59.8 mg/kg for the effect in total (Fig. 5A).

**Effect of one dose versus effect of three doses on extra- and intracellular S. aureus at 24 h.** Mice were treated with one or three doses of DCX (200 mg/kg) or RIF (60 mg/kg) and received the first dose 2 h after inoculation (four mice per group). The three-dose regimen was administered every 8 h. The mice were sampled 19 h after treatment onset. Untreated control groups were included, but only for 6 h of infection. At this point, untreated mice met the clinical signs of irreversible sickness and were euthanized.

Time-kill curves displaying the changes in the colony counts (CFU/ml) in the peritoneums of the mice over time after treatment with both RIF and DCX in relation to the number of doses given are shown in Fig. 6. RIF did not show a dose-dependent effect on the total, extracellular, or intracellular colony counts. On the contrary, the infection outcome was highly affected by the number of doses given in the mice treated with DCX.

In total, a decrease in the colony counts of approximately 2 log_{10} units during the 19 h of treatment was estimated for the mice receiving one and three doses of RIF and mice receiving three doses of DCX. For the mice receiving only one dose of
DCX, however, regrowth appeared at between 4 and 19 h of treatment. Therefore, compared to the bacterial level before treatment, a decrease of less than 1 log10 unit after 19 h of treatment was estimated for these mice.

In the extracellular compartment, a reduction of approximately 2.5 log10 units appeared within the first 4 h of treatment for mice treated with RIF, and no regrowth appeared for the following 15 h, irrespective of the number of doses. For mice receiving three doses of DCX, a reduction of 2 log10 units appeared during the 19 h of treatment. For the mice receiving only one dose, a reduction of less than 1 log10 unit occurred.

None of the dosing regimens were able to reduce the colony counts below the bacterial level intracellularly at the start of treatment. The result obtained with the three-dose regimen with DCX, however, was static compared to the starting bacterial level, while the one-dose regimen with DCX resulted in an increase of the colony count of 1 log10 unit. Treatment with RIF resulted in a decrease of 1 log10 unit as opposed to the starting bacterial level, irrespective of the number of doses given.

No change in MIC between that for the original bacteria injected and that for the bacteria retrieved from the mice after 19 h of treatment with RIF was observed.

**DISCUSSION**

An in vivo model was developed to study the relationship between the intracellular presence of *S. aureus* and antimicrobial effects of five antibiotics.

As exemplified by the test with the five different antibiotics (DCX, CXM, AZM, GEN, and RIF), the model allowed a distinction between antistaphylococcal effects extracellularly and intracellularly (Fig. 3).

When we compared the present results with the results of in vitro studies with various cell lines, we found that they were similar; Barcia-Macay et al. studied the intra- and extracellular antistaphylococcal effects of 16 different antibiotics in vitro (6) and reported an impaired intracellular antibacterial effect compared to the extracellular effect after 24 h of exposure to drugs at concentrations corresponding to the MIC, 10× MIC, and Cmax (human doses). Similar to our findings for AZM, they reported decreases in counts of less than 1 log10 unit both intracellularly and extracellularly, irrespective of the antibiotic concentration. For oxacillin, they showed, as we did for DCX in vivo, a good intracellular effect, but only at high concentrations. Finally, when they tested RIF and GEN, they showed an intracellular effect markedly lower than the extracellular effect (6).

When data from the new in vivo model are processed, some methodological pitfalls should be considered. (i) When the extracellular and intracellular counts for one sample were added, the sum was always less than the total count for the same sample, indicating a loss of bacteria during the ex vivo separation assay. During centrifugation of the cell suspension in order to isolate the cells and the intracellular bacteria from the extracellular bacteria, sedimentation of the extracellular bacteria from the supernatant also occurred. This resulted in an underestimation of the extracellular count and could at least partly explain the loss of bacteria during the separation assay. Cell lysis might also occur during the separation assay, which could confuse the separation of intra- and extracellular bacteria, and it could also contribute to the loss of bacteria during the lysostaphin washout step. Cell lysis, however, was very dependent on the sample processing procedure and was restrained by careful sample handling during the whole separation assay. Thus, only the results for samples that have undergone the exact same isolation procedure should be compared. Furthermore, it is important that the counts for each fraction are evaluated separately, whether it is the total, extracellular, or intracellular bacterial counts.

(ii) The separation assay induced false-positive intracellular
bacteria that consisted of up to 1.6% of the extracellular bacteria. This bias, however, would influence the conclusions only in situations with a high extracellular bacterial load and a low intracellular bacterial load.

(iii) After 2 h of infection, the variation in the intracellular bacterial counts (IQ range, $10^{2.4}$ to $10^{4.1}$ CFU/ml; $n = 16$) was larger than the variation in the total and the extracellular counts (Fig. 3). Furthermore, the intracellular count increased more dramatically (3.65 log$_{10}$ units) in the control group than the total and the extracellular counts did (1.94 and 1.88 log$_{10}$ units, respectively). This indicates that the number of intracellular bacteria is not static; i.e., it encompasses both potential intracellular bacterial growth (Fig. 2B and D) and extracellular bacteria that are internalized because of phagocytosis. Such a dynamic nature of infection must, however, be expected throughout the entire infectious process, since phagocytosis and, to some extent, cell lysis continue. Furthermore, the migration of new cells to the infection site also continues. Owing to this increased initial variation, standardization of the intracellular inoculum before antibiotic treatment is difficult.

In general, as shown by us and others, it is difficult to predict the intracellular effects of antibiotics. The finding of a poor intracellular effect for AZM was surprising, since AZM is known to accumulate to a great extent inside cells and to thus have a potentially good intracellular effect (6, 9, 54). On the contrary, the β-lactams showed a good intracellular effect, even though they do not accumulate in cells (9, 45, 55). This indicates that intracellular accumulation alone is not an indicator of intracellular activity, as was previously assumed. According to Van Bambeke et al. (55), the intracellular activities of antibiotics depend on a wide range of other factors besides intracellular presence, which explains the impaired intracellular activities of antibiotics that are often recorded. The impaired effect could be caused by (i) different subcellular locations of the antibiotic and the bacteria or (ii) increased MICs intracellularly due to the impaired expression of the antibacterial activity of the antibiotic (e.g., drug metabolism, changed local pH, or protein binding) or altered bacterial responsiveness (e.g., a changed bacterial metabolism or growth rate). All these parameters make prediction of the actual intracellular effect
difficult, which increases the relevance of experimental models for measurement of intracellular activity.

The results obtained for RIF and DCX in this study evidently show how the antibiotic exposure time can change the intracellular activity profile of an antibiotic; the results presented in Fig. 6 display very different activity-time profiles for the two antibiotics. The interpretation of the effect obtained for the animals that received a single dose depends on whether the colony counts were obtained after 4 or 21 h. With the short-term results alone, we would conclude that DCX is superior to RIF intracellularly. However, with the longer-term results, we would conclude that RIF is superior to DCX intracellularly after the administration of a single dose. Since the onset of the extracellular killing activity of RIF is very rapid, RIF must reach the infection site quickly. Intracellular accumulation of RIF is expected, but the time required for RIF to reach intracellular equilibrium and its subcellular location could be slow (the equilibrium time is unknown), which would explain the slow intracellular response (6, 9). The results for DCX show another pattern (Fig. 6) and indicate a lack of accumulation of DCX, since there was a reasonable short-term effect but no long-term effect after the administration of a single dose. Furthermore, the rapid onset of the intracellular effect of DCX could reflect rapid penetration and the rapid achievement of subcellular equilibrium.

Knowledge of the dose-response relationship is also crucial when antibiotic activity extra- and intracellularly is explored, as shown by the results of the dose-response study with DCX (Fig. 5). The dose-response curves clearly display the need for the use of higher doses to obtain an effect in the intracellular compartment compared to the doses required to achieve an effect in the extracellular compartment.

Finally, our results revealed that the time between inoculation and treatment onset in vivo were also critical to the final outcome of the infection (Fig. 4). Changes in the subcellular placement of intracellular S. aureus during different infection stages have been reported by several study groups (7, 24, 52). These subcellular changes would theoretically affect the factors mentioned by Van Bambreke et al. (55) and would result in a change in the antimicrobial effect over time. Therefore, the timing of treatment onset
should also be considered when intracellular activity studies are planned and when results are compared.

Both the in vivo and the in vitro models used to assess intracellular antibacterial effect have some advantages, disadvantages, and limitations. The in vivo model has the advantage of allowing the study of concepts in a whole-body system; for example, it includes a fully functional immune system, whole-body drug kinetics are occurring, and the nature of the intracellular infection is dynamic. However, the infection course in vivo is more difficult to standardize, as mentioned above, and it can be difficult to differentiate between an actual antibiotic effect and other in vivo influences. Furthermore, one can only speculate how the response in the mouse model resembles the response that would take place in the actual clinical situation, since immune defenses are highly species specific. The advantage of using cell lines instead of an in vivo model is that the intracellular effect can be explored in different cell types of different host origins, including those of human origin. A cell line model also allows the exploration of isolated mechanisms and effects. However, in vitro models cannot easily simulate drug kinetics as they exist in animals and cannot evaluate the interplay between a fully functional immune system and antibiotic treatment. So far the in vivo animal model and in vitro cell line models complement each other. Continued research may show that the in vitro models alone may sufficiently predict the intracellular effect.

In conclusion, a new in vivo model was developed to explore the extra- and intracellular activities of antimicrobials against \textit{S. aureus}. The new model complements existing in vitro models well by providing the opportunity to perform more complex studies in a whole-organism system. Studies of particular interest that could be performed with the new model include dose-response studies, drug development and screening assays, pharmacokinetic and pharmacodynamic studies, combination treatments, staphylococcal virulence studies, relapse studies, and others. Studies with this model could be complemented by in vitro studies with cell line models to emphasize the conclusions. When studies with the new in vivo model are planned, the study design should be considered carefully, since exposure time, dose selection and frequency, and the time between inoculation and treatment onset are highly critical to the final conclusions.

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