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 extra- 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 extra- and intracellular effects. The intracellular effects of the five antibiotics could be ranked as follows as the mean change in the $\log_{10}$ number of CFU/ml ($\Delta\log_{10}$ CFU/ml) between treated and untreated mice after 4 h of treatment: dicloxacillin (3.70 $\Delta\log_{10}$ CFU/ml) > cefuroxime (3.56 $\Delta\log_{10}$ CFU/ml) > rifampin (1.86 $\Delta\log_{10}$ CFU/ml) > gentamicin (0.61 $\Delta\log_{10}$ CFU/ml) > azithromycin (0.21 $\Delta\log_{10}$ 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

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TABLE 1. MICs, antibiotic parameters in humans, and experimental dosages used for single-dose in vivo and in vitro studies of activities of antibiotics against *S. aureus* E19977

Antibiotic	MIC (mg/liter)	Clinical parameters				Experimental dosage		Reference(s)
		Human dosage and route[a]	Human C_{max}[b] (mg/liter) (total drug)	Protein binding in human serum (%)	Half-life in humans (h)	In vivo (mg/kg)	In vitro[c] (mg/liter)	
DCX	0.5[d]	1,000 mg p.o.	30	95	1.0	200[e]	1.5	37, 38, 43, 47
CXM	2.0	1,000 mg i.m.	26–40	30	1.5	200[e]	20.0	37, 38, 43, 47
AZM	0.5	1,000 mg p.o.[f]	1[f]	50	10.0[g]	1	0.5	16
GEN	0.38	240 mg i.m.	10	10	2.0	10	9.0	16, 39
RIF	<0.016	600 mg p.o.	10	80	3.0	10	2.0	16, 28

[a] Conventional dosages and route of administration to humans (p.o., per oral administration; i.m., intramuscular administration).

[b] Commonly observed C_{max} in serum after administration of the drug at the dosages and by the route stated for humans.

[c] The initial concentration added to the in vitro kinetic model corresponding to the free peak concentration of the antibiotic in humans after administration of a single dose.

[d] 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).

[e] Murine dosages expected to yield simulated C_{max}s in mice that are the same as those in humans. For these drugs, however, it was not possible to find appropriate data from the literature, which is why these dosages were estimated on the basis of calculation of the surface area (22).

[f] The most conventional dosage used is 500 mg given orally, but due to the accumulation of the drug, a dosage of 1,000 mg and the corresponding C_{max} were used to obtain more actual conditions for the drug.

[g] Half-life corresponding to the initial half-life of azithromycin.

(24). They also demonstrated that intracellular survival was linked to the global regulator *sar*, which regulates multiple virulence factors in *S. aureus*. These two observations could together indicate that intracellular survival is a part of the pathogenesis of *S. aureus*.

Appropriate models for the testing of the intracellular activities of antimicrobials against *S. aureus* are needed. Several in vitro models that use various cells and cell lines are available for the study of intracellular *S. aureus* (6, 10, 18, 24, 44, 51), but only a few in vivo models have been developed.

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

(Part of this study was presented at the 16th European Congress of Clinical Microbiology and Infectious Diseases, Nice, France.)

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. The detection limit was 25 CFU/ml. Saline with 0.1% (vol/vol) Triton X-100 (T-8787; Sigma-Aldrich Inc., St. Louis, MO) was used in the dilutions to prevent the clumping of the bacteria during whirl mixing, as increased whirl mixing compounds the effect of bacterial clumping. Dilution and spotting were performed in one procedure, and dilutions containing both bacteria and Triton X-100 were never stored for more than 1 min before they were spotted on agar plates. Bacterial cell lysis did not occur due to the short time of exposure to Triton X-100. Mucin (M-2378; Sigma-Aldrich Inc.) was used as an adjuvant for the inoculation of mice. The mucin stock solution was prepared in saline, sterilized, and adjusted to physiological pH before use.

Antimicrobial agents. When possible, antibiotics were procured as the commercial product registered in Denmark for parenteral use; dicloxacillin (DCX) as Diclocil (Bristol-Myers Squibb Company, New York, NY) cefuroxime (CXM) as Zinacef (GlaxoSmithKline, Brentford, Middlesex, United Kingdom), azithromycin (AZM) as Zitromax (Pfizer Inc., New York, NY), and gentamicin (GEN) as Garamycin (Schering-Plough Corporation, Kenilworth, NJ). Rifampin (RIF; rifampicin) was procured from Sigma-Aldrich Inc. RIF stock solutions were made in

dimethyl sulfoxide (D-8418; Sigma-Aldrich Inc.). The final concentration of dimethyl sulfoxide in the solutions used for the animal studies never exceeded 10%.

In vitro studies. The MICs were determined by the Etest, according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

A previously described (34) in vitro kinetic model was used for the in vitro kinetic studies. The model simulates antibiotic elimination in a bacterial culture without a change to the inoculum.

Bacteria in exponential phase of growth were applied to the model at a final density of $10^{6.3}$ CFU/ml. Clinical pharmacokinetic conditions were simulated (Table 1). Growth controls were included in each test run. Samples for quantitative cultures were withdrawn after 0, 2, 4, 6, 8, 12, and 24 h.

Mouse peritonitis/sepsis model. The mouse peritonitis/sepsis model has been described previously (22, 32). In brief, outbred female NMRI mice (HsdWin: NMRI; weight, 25 to 30 g; Harlan Netherlands, Horst, The Netherlands) were used throughout the study. The mice had free access to chow and water. The mice were inoculated by intraperitoneal (i.p.) injection of 0.5 ml bacterial suspension containing $10^{7.7}$ CFU/ml and 5% (wt/vol) mucin. To achieve a sterile immune response, 0.5 ml of a 2.0 mg/ml zymosan A suspension (Z-4250; Sigma-Aldrich Inc.) was injected i.p., as described previously (33).

Mice were euthanized before sample collection. A peritoneal wash was performed by injecting 2.0 ml sterile saline i.p. The resulting fluid, which contained both murine cells and bacteria, was collected with a pipette after the peritoneum was opened. Antibiotic treatment (0.25 ml) was administered subcutaneously. Humane end points consisted of clinical signs of irreversible sickness. If the mouse had these signs, the mouse was immediately euthanized. All animal experiments were approved by the Danish Animal Experimentation Inspectorate (license no. 2004/561-835).

WBC count and differentiation in peritoneal wash. The white blood cell (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, midsize cells, and lymphocytes.

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

Electron microscopy. The suspension of cells and bacteria was fixed by adding glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.01 M CaCl$_2$ 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
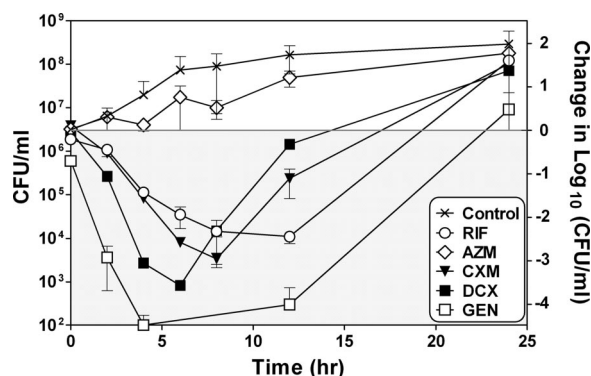
FIG. 1. Results of time-kill curve studies performed by use of the in vitro kinetic model by applying both concentrations corresponding to the fC_{max} measured in humans after administration of a single dose of the antibiotic and simulated human serum elimination half-lives for the five antibiotics studied. The graph shows the change in CFU/ml in the culture flask over a 24-h period (mean and range, $n = 2$) for RIF, AZM, CXM, DCX, and GEN. The left ordinate displays the actual numbers of CFU/ml, and the right ordinate displays the changes in the numbers of $\log_{10}$ CFU/ml compared to the amount of bacteria present before the antibiotic was applied. The gray area displays decreased colony counts compared to the starting bacterial count, and the white area displays increased colony counts compared to the starting bacterial count.
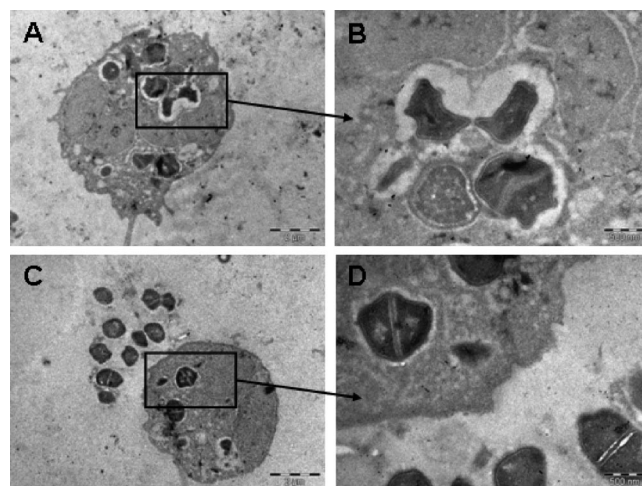

FIG. 2. Electron microscopy of peritoneal wash after staphylococcal infection showing both intracellular and extracellular bacteria. Panel B is an enlargement of panel A, and panel D is an enlargement of panel C.

the tubes were centrifuged at $300 \times g$ and 25°C for 10 min. The extracellular amount of bacteria in the supernatant was quantified.

The pellet was resuspended in Hanks balanced salt solution (H-9269; Sigma-Aldrich Inc.) with 100 μg/ml of lysostaphin (L-7386; Sigma-Aldrich Inc.). The suspension was incubated at room temperature for 7 min to kill the remaining extracellular bacteria. A cell-free bacterial suspension was run in parallel as a control for extracellular killing, and a 6-$\log_{10}$-unit decrease in colony counts was recorded for these controls. The supernatant from the lysostaphin-treated samples was also cultured as a control for extracellular killing. The lysostaphin was removed by washing the samples four times with 2.0 ml fresh Hanks balanced salt solution. The pellet was finally resuspended in 1.5 ml of cold sterile water for cell lysis, and the intracellular bacterial count was quantified.

WBC counts were obtained and light microscopy was performed during critical steps in the separation procedure.

Statistical methods. Normality tests (D'Agostino and Pearson omnibus normality test and probability plots) were performed with all data sets. Some data were log transformed to obtain a normal distribution. In general, the data sets were $\log_{10}$ normally distributed. Parametric tests were performed with the $\log_{10}$-transformed data.

The mean colony counts among the treatment groups were compared by one-way analysis of variance, followed by the Tukey-Kramer multiple-comparison test (mutual comparison of treatment groups) or Dunnett's multiple-comparison test (comparison of results for the treatment groups to those for the control group). The paired t test was used to compare the means of matched groups. A P value of <0.05 was considered significant. For analysis of dose-effect relationships, the Hill equation (variable slope) was employed. The Prism program (version 5; GraphPad Software Inc., San Diego, CA) was used for illustration and statistical analysis.

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}s) corresponding to the free drug (fC_{max}) (Table 1) were applied, and 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 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).

In vivo model. Intracellular infection by *S. aureus* E19977 was documented by light and electron microscopy. On the basis of the findings of light microscopy, the proportion of macrophage-like cells found to be infected was estimated to be $>60\%$ after 6 h of infection.

The cells harvested from the peritoneums of the mice were fragile. They were sensitive to, e.g., agitation, turbulence, and fast temperature drops.

Figure 2 displays electron micrographs of the peritoneal wash after 6 h of staphylococcal infection and shows bacteria both intra- and extracellularly. The majority of the intracellular bacteria seemed to be located in vacuoles (Fig. 2A to D). Some apparently spacious vacuoles are seen in Fig. 2A and B, and some more closely spaced vacuoles are observed in Fig. 2C and D. As displayed in Fig. 2B and D, it seems plausible that *S. aureus* can divide intracellularly.

The number of granulocytes increased over time in the peritoneum after inoculation of *S. aureus* E19977. The numbers of granulocytes/ml (mean ± standard deviation or range) in the peritoneal wash after inoculation were 6.13×10^5 (range, 3.0×10^5 to 1.2×10^6 [$n = 4$]) at 0 h, $1.07 \times 10^6 \pm 3.47 \times 10^5$ ($n = 20$) at 2 h, and $2.49 \times 10^6 \pm 5.88 \times 10^5$ ($n = 27$) at 6 h. The increased numbers of granulocytes from 2 h to 6 h was significant ($P < 0.0001$).

The median total bacterial counts were $10^{5.5}$ CFU/ml (interquartile [IQ] range, $10^{5.4}$ to $10^{5.8}$ CFU/ml; $n = 20$) 2 h after inoculation and $10^{7.4}$ CFU/ml [IQ range, $10^{7.1}$ to $10^{7.5}$ CFU/ml;

$n = 28$) 6 h after inoculation if the mice were untreated. The inocula injected ranged from $10^{7.7}$ to $10^{8.1}$ CFU/ml.

The cell-free bacterial suspension used as a control for extracellular killing by lysostaphin revealed that sedimentation of the bacteria also occurred during centrifugation. After centrifugation, a significant decrease in the bacterial count of up to 1 $\log_{10}$ unit was observed (mean $\log_{10}$ difference, 0.85; 95% confidence interval, 0.74 to 0.95; $P < 0.0001$; $n = 16$).

The rate of appearance of false-positive intracellular bacteria during the separation assay was shown to be low; uninfected WBCs were harvested from the mouse peritoneum after a sterile immune response. Concentrations of 10^7, 10^6, and 10^5 WBCs/ml were all spiked with 10^7, 10^6, 10^5, and 10^4 extracellular CFU/ml; and each WBC-CFU combination was tested by the intra- and extracellular separation assay.

False-positive intracellular bacteria occurred, and this occurrence was most dependent on the amount of spiked bacteria. The occurrence of false-positive findings was also, to some extent, dependent on the amount of cells. For samples with 10^7 WBCs/ml, 1.620% ± 0.007% of the spiked bacteria were counted as intracellular during the separation assay. For samples containing 10^6 WBCs/ml, 0.518% ± 0.002% were counted as intracellular, and for samples containing 10^5 WBCs/ml, 0.080% ± 0.001% were counted as intracellular. Microscopy of the cells after the separation procedure revealed that the false-positive "intracellular" bacteria were almost all extracellular bacteria trapped between the cell surfaces and were not extracellular bacteria forced into the cells. This trapping apparently protected the bacteria from the lysostaphin treatment and from washout, and they were thereby counted as part of the intracellular pool.

Tests with five different antibiotics. To verify the new in vivo setup, five antibiotics with theoretical differences in intracellular penetration and antibacterial properties were tested in the model. The antibiotics tested were DCX, CXM, AZM, GEN, and RIF. Mice were treated 2 h after bacterial challenge with single doses expected to yield simulated human C_{max}s in mice (Table 1) (group size, $n = 16$). The mice were sampled 4 h after treatment onset. Untreated control groups were included and were sampled 2 and 6 h after bacterial challenge.

The changes in the numbers of CFU/ml after 4 h of treatment i.p. (total, extracellular, and intracellular) are shown in Fig. 3A to I.

The bacterial counts in the peritoneums of untreated mice increased 1.94 $\log_{10}$ units in total, 3.65 $\log_{10}$ units intracellularly, and 1.88 $\log_{10}$ 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:

$$\underset{\text{NS}}{\text{DCX}} = \underset{\text{NS}}{\text{CXM}} = \underset{***}{\text{RIF}} > \underset{\text{NS}}{\text{GEN}} = \text{AZM}$$

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:

$$\underset{***}{\text{RIF}} > \underset{\text{NS}}{\text{DCX}} = \underset{***}{\text{CXM}} > \underset{**}{\text{GEN}} > \text{AZM}$$

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:

$$\underset{\text{NS}}{\text{DCX}} = \underset{***}{\text{CXM}} > \underset{*}{\text{RIF}} > \underset{\text{NS}}{\text{GEN}} = \text{AZM}$$

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 $\log_{10}$ CFU/ml ($\Delta\log_{10}$ 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 $\Delta\log_{10}$ 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.

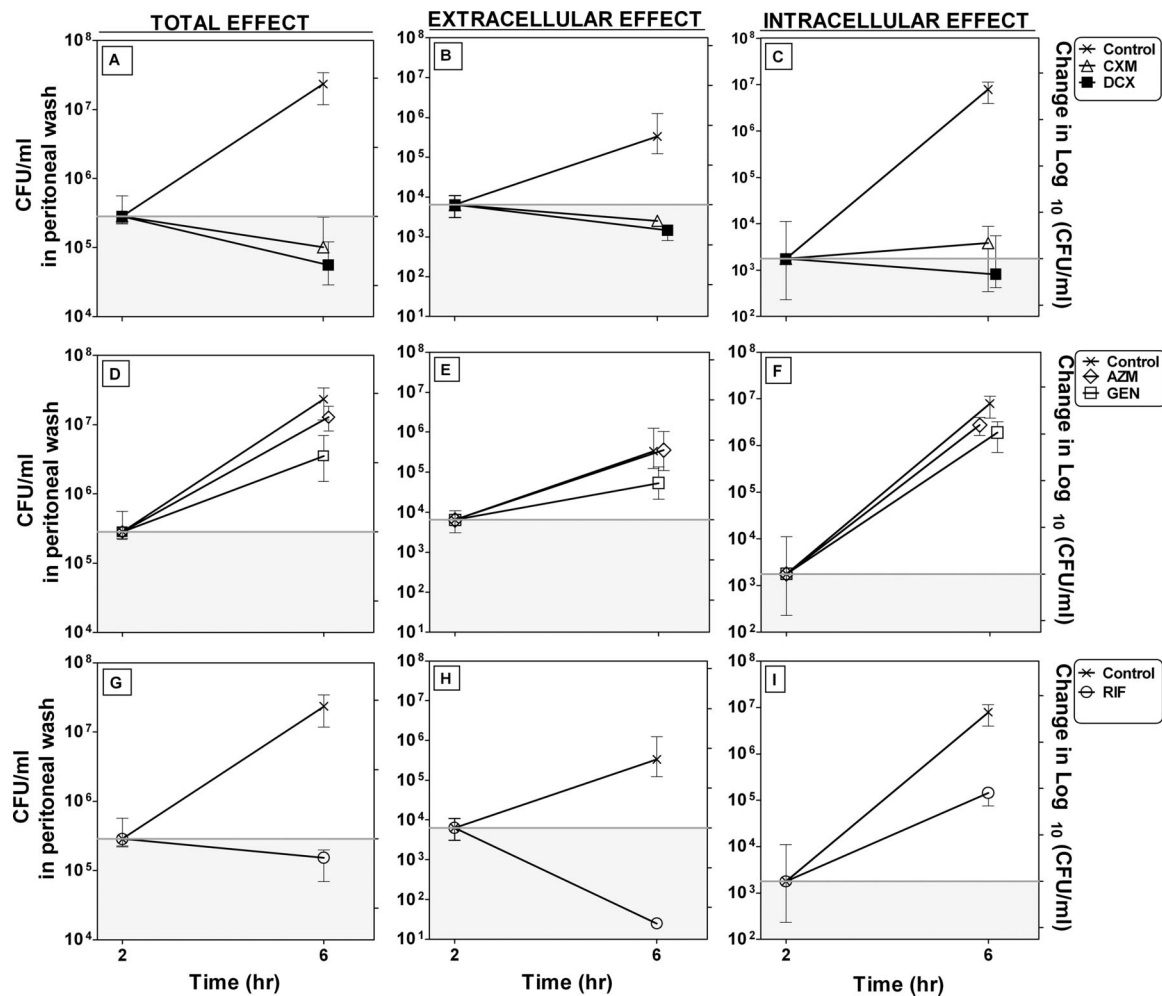The $\Delta\log_{10}$ CFU/ml correlated to the dose given ($\log_{10}$ mg/

FIG. 3. Bacterial counts in peritoneums of mice after 4 h of treatment (from 2 to 6 h of infection) with a single dose (Table 1) of AZM, GEN, RIF, CXM, and DCX 2 h after bacterial challenge. The graph shows the change in CFU/ml (median ± IQ range; $n = 8$). Data for the control groups at 2 h and 6 h are the medians ± IQ ranges for all studies ($n = 11$). (A to C) Changes in the total, intracellular, and extracellular numbers of CFU/ml, respectively, for the β-lactams DCX and CXM; (D to F) changes in the total, intracellular, and extracellular numbers of CFU/ml, respectively, for GEN and AZM; (G to I) changes in the total, intracellular and extracellular numbers of CFU/ml, respectively, for RIF. The left ordinate displays the actual numbers of CFU/ml, and the right ordinate displays the changes in the $\log_{10}$ numbers of CFU/ml compared to the amount of bacteria before the start of treatment. The gray areas display decreased colony counts compared to the starting bacterial count, and the white areas display increased colony counts compared to the starting bacterial count. Note that the scales for each compartment (total, extracellular, and intracellular) are different.

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
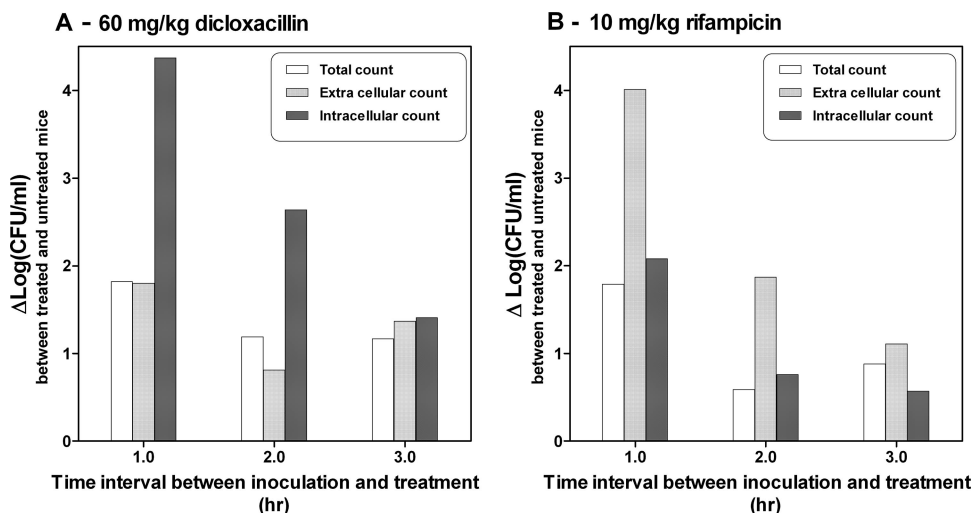
FIG. 4. Difference in colony counts ($\Delta\log_{10}$ CFU/ml) between the treated and untreated mice in relation to the time of treatment after inoculation (1, 2, and 3 h). The mice treated with DCX received 60 mg/kg, and the mice treated with RIF received 10 mg/kg. The $\Delta\log_{10}$ CFU/ml was based on the numbers of CFU/ml in the untreated mice infected for the same duration (5, 6, and 7 h, respectively). All treated mice received treatment for 4 h.

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 $\log_{10}$ unit after 19 h of treatment was estimated for these mice.

In the extracellular compartment, a reduction of approximately 2.5 $\log_{10}$ 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 $\log_{10}$ units appeared during the 19 h of treatment. For the mice receiving only one dose, a reduction of less than 1 $\log_{10}$ 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 $\log_{10}$ unit. Treatment with RIF resulted in an increase in the colony count of more than 1 $\log_{10}$ compared to the bacterial level at the start of treatment for the first 4 h; this was followed by a decrease of 1 $\log_{10}$ unit for the following 15 h of treatment, 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 C_{max} (human doses). Similar to our findings for AZM, they reported decreases in counts of less than 1 $\log_{10}$ unit both intra- 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.

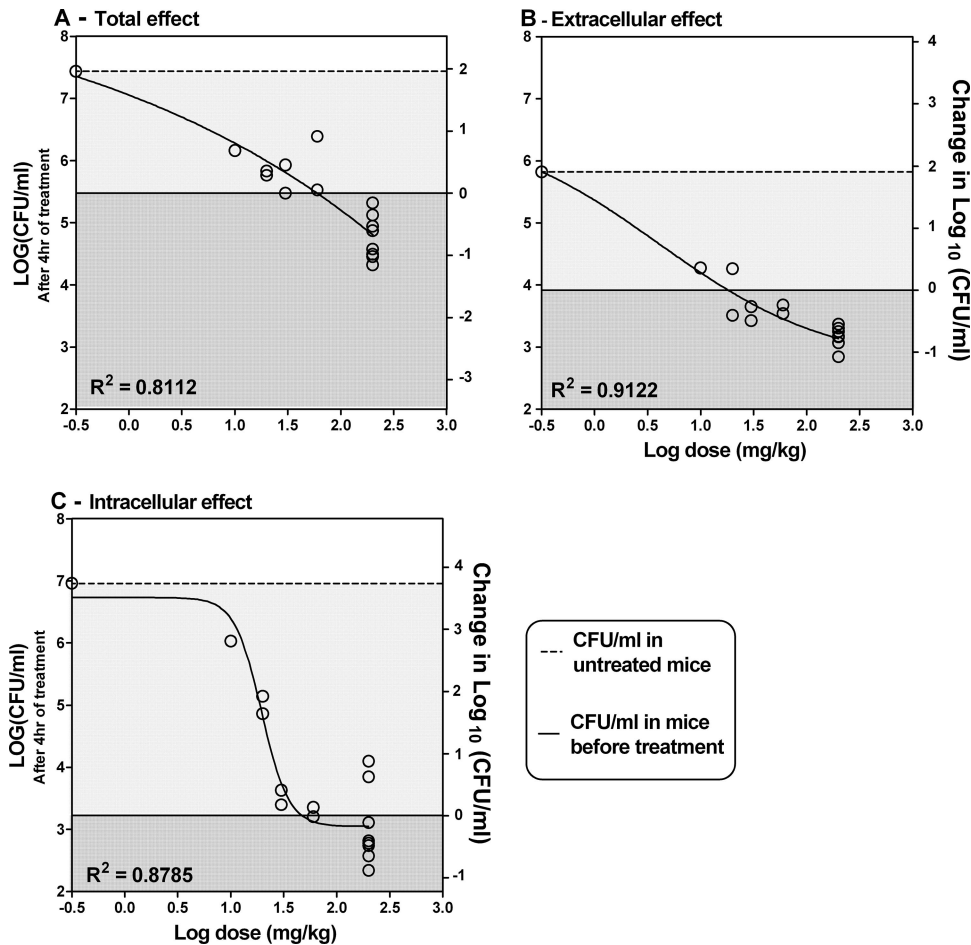(ii) The separation assay induced false-positive intracellular

FIG. 5. Dose-response curves for DCX showing the change in the numbers of $\log_{10}$ CFU/ml in the peritoneal wash in mice after 4 h of treatment in relation to dose. (A) Change in the total count; (B) change in extracellular count; (C) change in intracellular count. The left ordinate displays the actual numbers of $\log_{10}$ CFU/ml, and the right ordinate displays the changes in the numbers of $\log_{10}$ compared to the amount of bacteria before the start of treatment. The dark gray areas display decreases in colony counts compared to the starting level of bacteria, and the light gray areas display increases in colony counts compared to the starting level of bacteria. The median for the untreated control group ($n = 12$) forms a part of the dose-response curve, displayed as a small concentration (0.3 mg/kg).

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
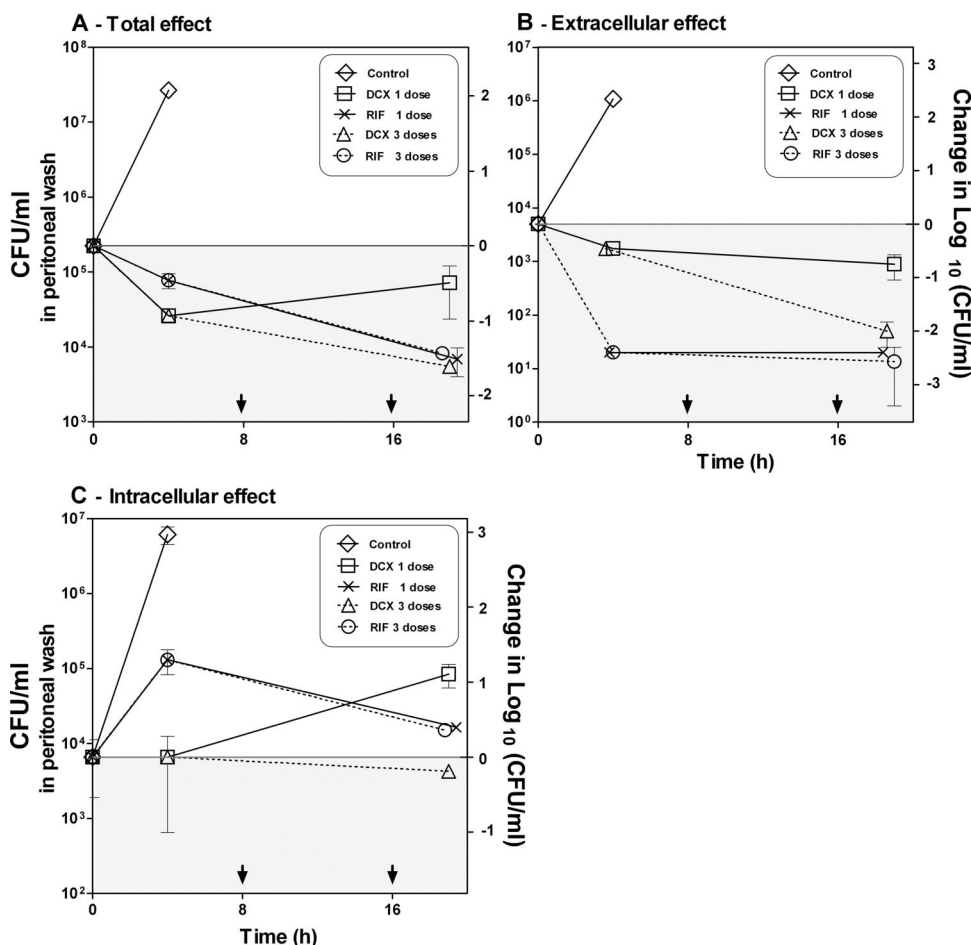
FIG. 6. In vivo time-kill curves showing the change in the numbers of CFU/ml in the peritoneums of mice (mean ± range, $n = 2$) compared to the number of doses of DCX (200 mg/kg) and RIF (10 mg/kg). (A) Change in the total count; (B) change in extracellular count; (C) change in intracellular count. The left ordinate displays the actual numbers of CFU/ml, and the right axis displays the changes in the numbers of $\log_{10}$ CFU/ml compared to the amount of bacteria before the start of treatment. The gray areas display decreased colony counts compared to the starting bacterial count, and the white areas display increased colony counts compared to the starting bacterial count. The arrows at the abscissa indicate the times of dosing the second and third doses of DCX and RIF.

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 Bambeke 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 *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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