ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2003, p. 2283–2292 Vol. 47, No. 7
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.7.2283–2292.2003

Quantitative Analysis of Gentamicin, Azithromycin, Telithromycin, Ciprofloxacin, Moxifloxacin, and Oritavancin (LY333328) Activities against Intracellular *Staphylococcus aureus* in Mouse J774 Macrophages

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Received 19 August 2002/Returned for modification 20 February 2003/Accepted 17 April 2003

Using J774 macrophages, the intracellular activities of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) against *Staphylococcus aureus* (strain ATCC 25923) have been quantitatively assessed in a 24-h model. *S. aureus* was positively localized in phagolysosomes by confocal and electron microscopy, and extracellular growth was prevented with 0.5 mg of gentamicin/liter (1× MIC) in controls. When tested at extracellular concentrations equivalent to their maximum concentrations in human serum, all antibiotics except azithromycin caused a significant reduction of the postphagocytosis inoculum within 24 h, albeit to markedly different extents (telithromycin [2 mg/liter], 0.60 log; ciprofloxacin [4.3 mg/liter], 0.81 log; gentamicin [18 mg/liter], 1.21 log; moxifloxacin [4 mg/liter], 1.51 log; oritavancin [25 mg/liter], 3.49 log). Intracellular activities were not systematically related to drug accumulation (apparent cellular-to-extracellular concentration ratios in infected cells: ciprofloxacin, 3.2; gentamicin, 6.8; telithromycin, 8.7; moxifloxacin, 13.4; azithromycin, 50; oritavancin, 348). Intracellular activity was not directly correlated to extracellular activity as measured in broth. Conditions of pH 5 (i.e., mimicking that of phagolysosomes) markedly reduced the activity of gentamicin, azithromycin, and telithromycin (≥32×) and fairly extensively reduced that of ciprofloxacin and moxifloxacin (≥4×) but did not affect oritavancin activity. We conclude that the cellular accumulation of antibiotics is not the only parameter to take into account for intracellular activity but that local environmental conditions (such as pH) and other factors can also prove critical.

Staphylococcus aureus, a ubiquitous pyogenic bacterium, is causing severe infections in humans as well as in animals (63). *S. aureus* adheres to and easily invades nonprofessional as well as professional phagocytes (1, 33). In the latter cells, *S. aureus* tends to be restricted to the phagolysosomal compartment, where it largely escapes destruction and survives in a semiquiescent state for prolonged periods (29, 52). These intraphagocytic forms are considered responsible for the well-known recurrent character of staphylococcal infections as well as for the many failures of apparently appropriate antibiotic treatments (16, 27, 36). The main and most current treatment option for *S. aureus* infections is the administration of a β-lactam resistant to β-lactamases (often combined with an aminoglycoside and/or rifampin) (61). Clindamycin and fusidic acid are second-line antibiotics for treatment of these infections. Glycopeptides, oxazolidinones, or synergistins are recommended for multiresistant strains only (47). Yet it is usually recognized that β-lactams are poorly active against the intracellular organisms because of their lack of cellular accumulation (56, 59). A number of models have therefore been developed to assess the potential of other antibiotics, such as rifampin, clindamycin, or glycopeptides (2, 12, 34), against intracellular *S. aureus*. Antibiotics with known cellular accumulation such as macrolides (23, 38, 48, 60), synergistins (17), or fluoroquinolones (3, 4, 10, 46, 50) have also been studied. While providing useful information, these models have so far been used to examine only short periods of incubation and have not fully taken into account (i) the slow rate of intracellular accumulation of some antibiotics (see reference 57 for comments) and (ii) the reduced growth rate of intracellular *S. aureus* in comparison with that of bacteria in broth or other more favorable media (29). Moreover, contamination by bacteria growing extracellularly has often proven difficult to control (36).

We present here data obtained with a model of *S. aureus*-infected J774 mouse macrophages in which the intracellular growth of the bacteria and the influence of antibiotics has been monitored for 24 h. These cells were selected because they are quite permissive towards a variety of intracellular infectious agents, allowing detailed analysis of the effects of antibiotics without too much interference from the host-derived mechanisms of defense. We selected commonly used drugs from three different classes of antibiotics on the basis of their contrasting behaviors concerning pharmacodynamics, cellular accumulation, and distribution properties. Thus, we chose (i) the aminoglycoside gentamicin, which is a drug characterized by a marked, intense concentration-dependent bactericidal activity (5) and a poor cellular accumulation but a preferential accumulation in lysosomes (reviewed in reference 56); (ii) ciprofloxacin and moxifloxacin, which are examples of fluoroquinolones that, like aminoglycosides, show a marked concentration-dependent bactericidal effect (5) but accumulate quickly in cells and distribute in the cytosol (10); and (iii) azithromycin and telithromycin (two macrolides), which are

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TABLE 1. MICs and MBCs of the antibiotics used in the present study for *S. aureus* ATCC 25923 in broth at pH 7.3, pH 6, and pH 5[a]

Antibiotic	Concn (mg/liter)						
	pH 7.3		pH 6		pH 5		C_{max}[b]
	MIC	MBC	MIC	MBC	MIC	MBC	
Gentamicin	0.5	2	4	32	16	64	18
Azithromycin	0.5	8	32	64	512	512	0.4
Telithromycin	0.06	2	0.25	8	4	8	2
Ciprofloxacin	0.125	1	0.5	2	1	2	4.3
Moxifloxacin	0.06	0.06	0.06	0.25	0.25	1	4
Oritavancin	0.25	1	0.25	1	0.25	1	25

[a] For comparison, the table shows also C_{max} values. These concentrations were also the maximal concentrations tested in the present study, except in the case of azithromycin, for which the maximal concentration tested was 10 mg/liter.

[b] Concentrations corresponding to the peak concentrations of the respective antibiotics in serum after administration of currently used and/or approved doses for humans, as indicated in references 26 (gentamicin), 24 (azithromycin), 42 (telithromycin), 6 (ciprofloxacin), and 54 (moxifloxacin) and D. K. Braun et al., Clin. Microbiol. Infect. **7**(Suppl. 1)**:**P434, 2001.

essentially bacteriostatic and time-dependent antibiotics (5), accumulate in cells to a large extent, and are largely localized in acidic vacuoles (9, 40). We added oritavancin (LY333328 [15]), a newly developed glycopeptide which shows an intense, concentration-dependent bactericidal activity (8), after we discovered that this antibiotic accumulates to high levels in macrophages (this study). All drugs were used throughout the present study at clinically pertinent concentrations to allow for chemotherapeutically meaningful comparisons. Dose effects were correlated with cellular accumulation to delineate the intracellular pharmacodynamic properties of each drug in comparison with what was known or could be observed of their activities towards extracellular bacteria.

MATERIALS AND METHODS

Cells. We used J774 macrophages, a continuous reticulosarcoma cell line of murine origin, which were maintained exactly as previously described (53, 57). These cells are permissive towards a large number of intracellular bacteria. Viability upon exposure to antibiotics (at the maximal concentration tested and for up to 24 h) was assessed by measuring the release of lactate dehydrogenase in the supernatant at the end of the experiments (less than 10% of the total cell content).

Bacterial strain and susceptibility testing. *S. aureus* strain ATCC 25923 was used for all experiments. MICs were determined according to the recommendations of the U.S. National Committee for Clinical Laboratory Standards (1999). Minimum bactericidal concentrations (MBCs) were defined as the lowest concentration of each drug causing >99% reduction in growth. MICs and MBCs were measured (i) without adjustment of the broth pH (standard values; measured pH, 7.3) and (ii) in broth adjusted to pH 6 and pH 5 to mimic the pH conditions of endosomes and lysosomes, respectively.

Time and dose-kill curve studies with broth. Bacteria exhibiting logarithmic growth were resuspended at a density of 10^6 CFU per ml in Mueller-Hinton broth. The number of viable bacteria was determined after incubation at 37°C with antibiotics for suitable times (up to 24 h) by plate assays with appropriately diluted samples. We checked that the amount of carried-over antibiotic, taking into account the dilution of samples and the amount plated on the dish (50 μl for 15 ml of agar), was insufficient to impair bacterial growth (the final concentration of each antibiotic being each time severalfold lower than its MIC).

Setting up of a 24-h model of intracellular infection. Bacterial cultures exhibiting logarithmic growth were centrifuged at 14,000 rpm (5415 centrifuge; Gerätebau Eppendorf GmbH, Engelsdorf, Germany) for 4 min, and the pelleted bacteria were resuspended in RPMI 1640 supplemented with 10% fresh human serum (obtained from healthy volunteers as pooled samples and stored in aliquots at −70°C until use) and incubated for 30 min at 37°C to allow for opsonization. Bacteria were then adjusted to a concentration of 3.3×10^5 CFU per ml of culture medium on the basis of measurement of absorbance at 620 nm with a preestablished calibration curve. This suspension was then used to replace the culture medium of macrophages to yield an initial bacterium-to-macrophage ratio of 0.5, and phagocytosis was allowed to occur at 37°C for 1 h. Macrophages were then washed twice with prewarmed phosphate-buffered saline (PBS). Different protocols were then tested for the ability to support intracellular growth over a 24-h period without contamination of the extracellular medium (as assessed by optic microscopy and by plating samples from culture medium on tryptic soy agar). In a first approach, infected cells were exposed to either gentamicin (50 mg/liter) or lysostaphin (0.4 unit/ml) for 1 h at 37°C after phagocytosis before being washed and returned to gentamicin- or lysostaphin-free medium. Bacteria were seen to contaminate the extracellular milieu after approximately 5 h, causing a marked acidification and subsequent cell death. In a second approach, cell sheets were subjected every 4 h to extensive washing followed by 1 h of incubation with gentamicin (50 mg/liter) before being returned to gentamicin-free medium. Although this procedure allowed the sterility of the extracellular medium to be maintained for up to 24 h, it caused variable and difficult-to-control cell loss (at least 30%, as assessed on the basis of determinations of protein levels). In a third approach, cells were exposed continuously to lysostaphin (0.4 units/ml) during the whole 24-h postphagocytosis period. This also allowed maintenance of the sterility of the extracellular medium, but cell lysates (20 μl [approximately 4 μg of cell protein]) plated on *Micrococcus luteus*-seeded agar showed a definite inhibition zone that was not seen in controls, indicating that significant amounts of lysostaphin had been internalized. Moreover, we noted a rather important irreproducibility of the infection (based on the enumeration of cell-associated CFU), suggesting that lysostaphin was interfering with the assay. In a fourth approach, infected cells were incubated continuously with 0.5 mg of gentamicin/liter (i.e., the gentamicin MIC in broth). This prevented all extracellular bacterial growth up to 24 h. In contrast to what was seen with lysostaphin, (i) no inhibition of *M. luteus* growth was seen with lysates of cells collected after 24 h of incubation with this concentration of gentamicin (lower limit of detection for gentamicin, 0.25 mg/liter); (ii) infection and intracellular bacterial growth were obtained in a reproducible fashion (approximately 1.5 $\log_{10}$ increase in CFU in 24 h; see below); and (iii) the number of cells was also kept constant and reproducible from one experiment to another (941 ± 116

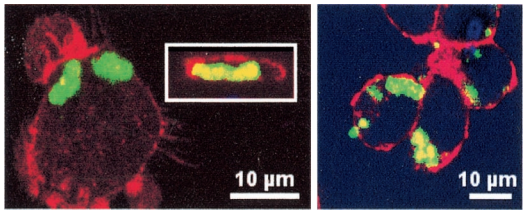

FIG. 1. Confocal microscopy of J774 macrophages observed 5 h after phagocytosis of opsonized *S. aureus*. Cell actin (located mostly on the inner face of the pericellular membrane) was labeled with rhodamine-phalloidin (red signal), and bacteria were labeled with FITC (green signal). (Left panel) Incubation was performed in the absence of any antibiotic (in the inset, a vertical section of the cell shown in the main panel is depicted; actin surrounds the bacteria, demonstrating its intracellular localization). (Right panel) Incubation performed in the presence of 0.5 mg of gentamicin/liter. No difference was seen with cells incubated without gentamicin.

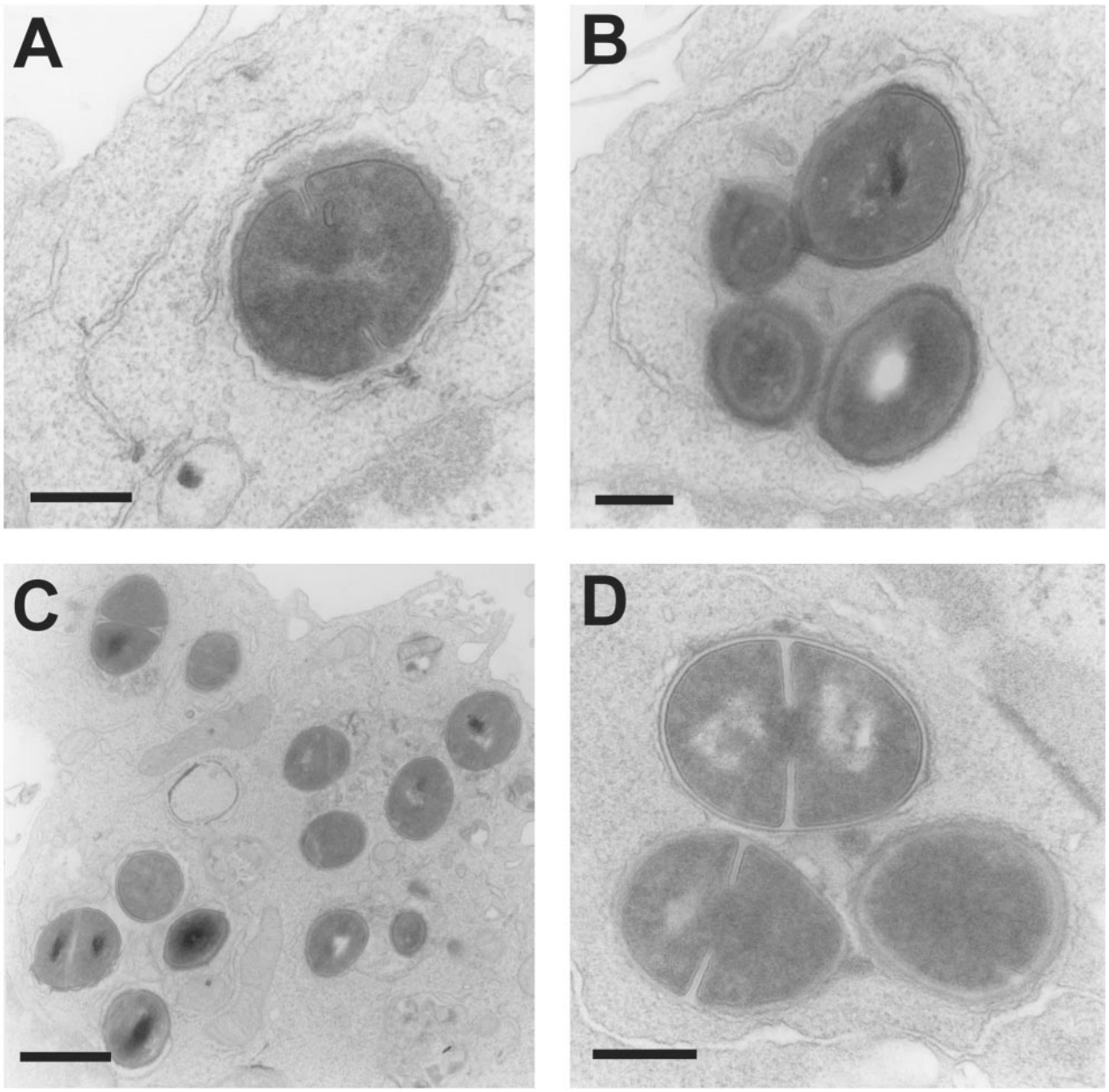

FIG. 2. Electron microscopy of J774 macrophages fixed 1 h (A and B) or 24 h (C and D) after phagocytosis of opsonized *S. aureus*. In both cases, incubation was carried out in the presence of 0.5 mg of gentamicin/liter. Bacteria appeared isolated (A) or sometimes in clusters (B) at 1 h after phagocytosis, without evidence of damage but with no sign of division. In contrast, most bacteria observed at 24 h were in the active process of division. In both cases, all bacteria were seen in membrane-bounded structures with no evidence of transfer to cytosol. Bars are 0.3 μm (A, B, and D) and 1 μm (C).

μg of cell protein/dish). This fourth protocol was then used as the standard method for all conditions in which no antibiotic was being tested (controls). When infected cells were exposed to the tested antibiotics, however, the addition of gentamicin at its MIC during the incubation was omitted. We established indeed that (i) all antibiotics under investigation were providing adequate protection against the extracellular growth of *S. aureus* when present at an extracellular concentration equal to or larger than their MICs, making their combination with gentamicin superfluous, and that (ii) the addition of gentamicin (0.5 mg/liter) only marginally modified the results obtained for these antibiotics in its absence.

Intracellular activity of antibiotics. Cells were collected by washing with PBS (three times) and scraping from the culture dish with a Teflon spatula followed by resuspension in 5 ml of sterile water. This suspension was then subjected to vigorous shaking for 30 seconds (Vortex-2; Scientific Industries, Inc., Bohemia, N.Y.). Aliquots (50 μl) were then plated on tryptic soy agar after suitable dilution for determination of CFU by colony counting after 24 h of incubation at 37°C. In parallel, samples were subjected to sonication (Labsonic L; B. Braun Biotech International GmbH, Melsungen, Germany) (15 s at 100 W) to achieve homogeneity and were then used for protein determination (32) with bovine serum albumin as a standard. All results were then calculated as CFU per milligram of cell protein.

Determination of cellular antibiotic accumulation. Accumulation studies were performed following the standard procedure described in previous publications by Carlier et al., Carryn et al., and Seral et al. (9–11, 13, 53). Briefly, cells (infected or uninfected) were incubated with antibiotics for 24 h. The medium was then decanted, and the cell sheets were quickly washed three times with

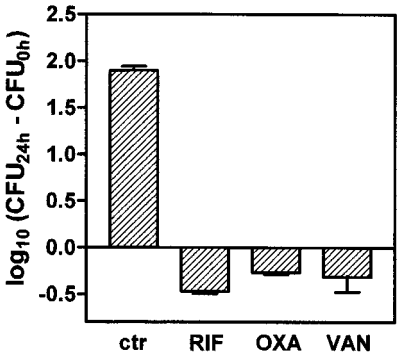

FIG. 3. Variations in the number of CFU per milligram of protein (± SD; $n = 3$) collected from J774 macrophages after 24 h of incubation postphagocytosis compared to that seen with the original postphagocytosis inoculum. ctr, control cells incubated with 0.5 mg of gentamicin/liter; RIF, cells incubated with rifampin (4 mg/liter); OXA, cells incubated with oxacillin (6 mg/liter); VAN, cells incubated with vancomycin (50 mg/liter). These concentrations correspond to peak concentrations in serum observed for patients after administration of conventional doses of oxacillin and vancomycin (14, 22) and to the low side of the range of peak concentrations in serum observed for rifampin (21). They also represent values 250 fold (rifampin) and 50 fold (oxacillin and vancomycin) larger than the MIC (in broth) of the corresponding antibiotic for the strain of *S. aureus* used in the present study.

ice-cold PBS (this effectively removes all of the antibiotic not tightly cell bound, and we checked for the absence of detectable antibiotic in the final wash). Cells were then collected by scraping in 1 ml of distilled water and subjected to sonication to achieve homogeneity. Azithromycin, telithromycin, and gentamicin were assayed by a microbiological assay exactly as described previously (53) (we checked in previous studies with azithromycin that this bioassay gave consistent results compared with radiolabeled and high-performance liquid chromatography-based assays [13]). Ciprofloxacin and moxifloxacin were measured by a fluorometric assay (13, 41, 51) with excitation and emission wavelengths (λ_{ex} and λ_{em}) set at 275 and 450 nm and at 298 and 504 nm, respectively. In preliminary experiments, we checked that this assay method gave values that consistently matched those determined with a microbiological assay. Oritavancin was assayed by scintillation counting using ^{14}C-labeled drug. Since oritavancin diffuses very poorly in agar, no direct comparison could be made between the values obtained by the radiochemical assay and those from a conventional microbiological disk-plate assay. Using *Enterococcus faecalis* ATCC 29212, we therefore devised a broth-based kill curve assay yielding a linear relationship between the log of the variation of the number of CFU and the concentration in a narrow but usable range (0.08 to 0.25 mg/liter). This method allowed us to confirm that the radiochemical assay measured bioactive drug levels.

All cell drug contents were systematically expressed by reference to the protein content of the corresponding samples (32). The apparent cellular-to-extracellular concentration ratio was then calculated by using a conversion factor of 3.8 μl of cell volume per mg of cell protein. This factor was adopted on the basis of a series of detailed volume/protein ratio measurements using the urea/sucrose partition method (44). The method provides direct information concerning the aqueous volume accessible to diffusible solutes. It is based on the same principle and provides the same information as the [^{3}H]water/[^{14}C]inulin (or [^{14}C]polyethylene glycol) partition method applied to cells pelleted through a silicone oil barrier, as used by many other investigators.

Morphological studies. Confocal microscopy was performed with *S. aureus* labeled with fluorescein-isothiocyanate (FITC) [5-(((2(carbohydrazino)methyl)-thio)acetyl)amino-fluorescein; Molecular Probes, Eugene, Oreg.]. Bacteria were incubated overnight with 0.5 mg of FITC/ml in Mueller-Hinton broth followed by sedimentation at 14,000 rpm for 1 min at 4°C and washing in PBS. Infection was carried out at a bacterium/macrophage ratio of approximately 50 (to allow for visualization of a sufficiently large number of bacteria), but all other conditions were otherwise unchanged. At appropriate times, cells were washed, fixed in 3.7% formaldehyde in PBS for 15 min at room temperature, permeabilized by exposure to Triton X-100 (0.2%), and stained for actin with 1.7×10^{-7} M rhodamine phalloidin (Molecular Probes). After washing, specimens were dried and mounted in 2.5% 1,4-diacylbicyclo(2,2,2)octane (Dabco; Sigma Chemical Co., St. Louis, Mo.) in Mowiol (Calbiochem-Novabiochem GmbH, Bad Soden, Germany). Observations were made under oil immersion conditions with a 40× or 63× objective mounted on an MRC1024 confocal microscope (Bio-Rad Laboratories, Richmond, Calif.). Images were digitally recorded with a Focus Graphics image recorder and used for direct computer-assisted reproduction with an ink-jet photo printer. For electron microscopy, infection of macrophages was carried out as described above but with unlabeled bacteria. Cells were fixed and prepared as described previously (57).

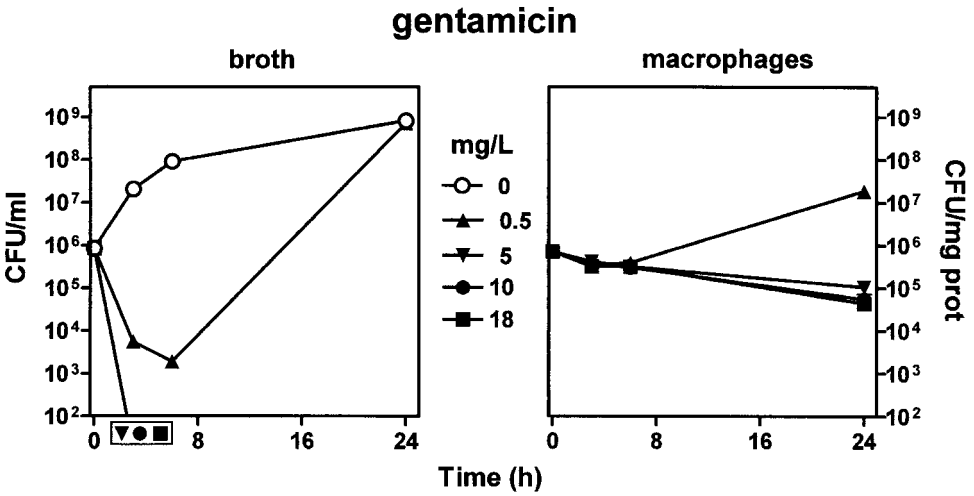

FIG. 4. Variations in the number of CFU upon incubation for up to 24 h with increasing concentrations of gentamicin. (Left panel) Results for bacteria in broth (initial pH, 7.3). (Right panel) Results for bacteria collected from J774 macrophages (after phagocytosis). The lowest drug concentration (0.5 mg/liter) corresponds to the MIC (in broth) of gentamicin for the strain of *S. aureus* used in this study; the highest concentration (18 mg/liter) corresponds to the C_{max} observed for patients after the administration of conventional doses of gentamicin (26). Each point corresponds to the mean value for three independent determinations. SD values were calculated, but the corresponding bars are smaller than the symbols in many cases. For the results obtained with broth, symbols placed outside of the bottom of the graph indicate samples in which no or only a few colonies were observed (i.e., numbers were too close to the lowest limit of detection [10^2 CFU/ml] to be fully reliable). Cells incubated with 0.5 mg of gentamicin/liter served as controls for all subsequent experiments.

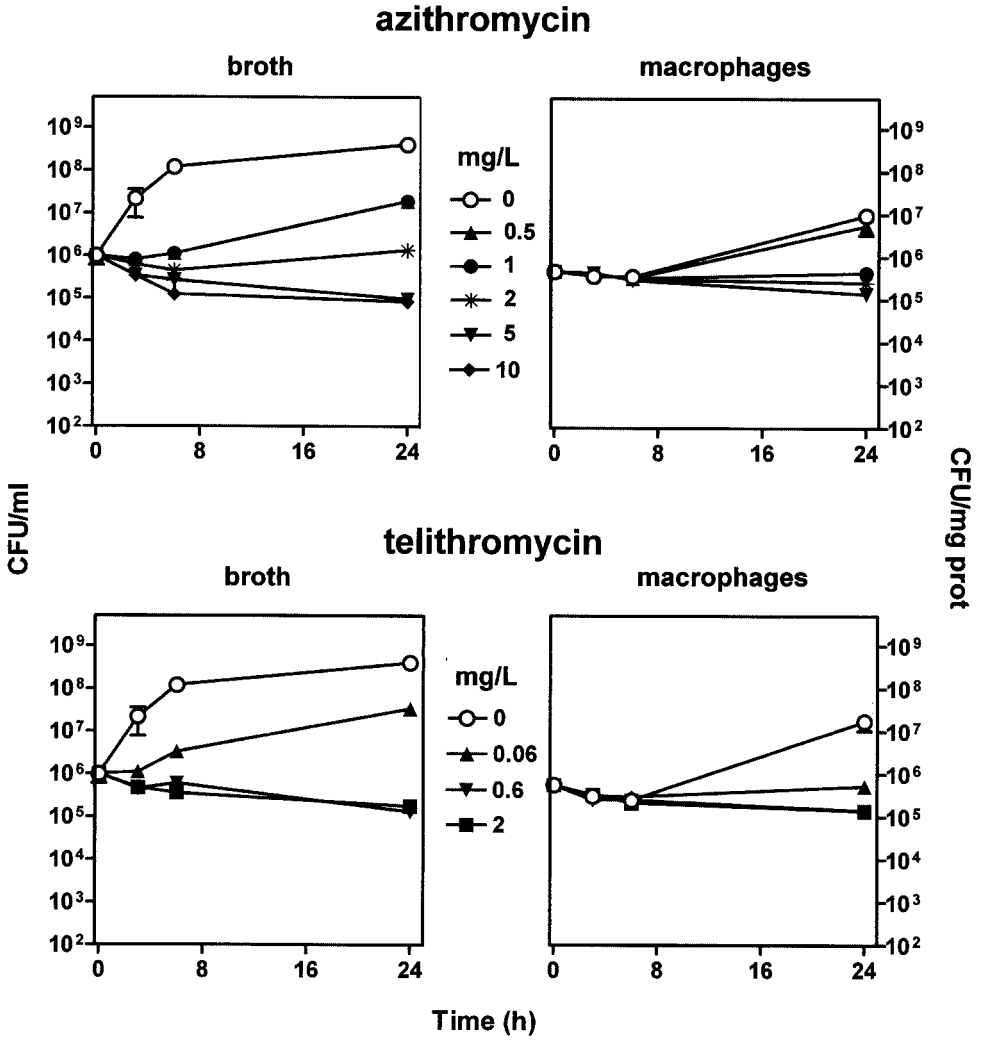

FIG. 5. Variations in the number of CFU upon incubation for up to 24 h with increasing concentrations of azithromycin (top two panels) or telithromycin (bottom two panels). (Left two panels) Results for bacteria in broth (initial pH, 7.3). (Right two panels) Results for bacteria collected from J774 macrophages (after phagocytosis). The lowest drug concentrations investigated (0.5 mg/liter for azithromycin, 0.06 mg/liter for telithromycin) correspond to the MICs (in broth) of the drugs for the strain of *S. aureus* used in this study; note that for azithromycin, the peak concentration observed for patients receiving a conventional dose (500 mg) did not exceed 0.4 mg/liter (24); for telithromycin, the highest concentration investigated (2 mg/liter) corresponds to the C_{max} observed for patients after administration of a conventional dose (800 mg) (42). Each point corresponds to the mean value for three independent determinations. SD values were calculated, but the corresponding bars are smaller than the symbols in many cases. Note that cells incubated in the absence of azithromycin or telithromycin were maintained in the presence of 0.5 mg of gentamicin/liter to prevent extracellular growth of *S. aureus*.

Antibiotics. The following antibiotics were obtained as pure substances for microbiological standards from their corresponding manufacturers: azithromycin (dihydrate salt; potency, 94.4%) from Pfizer Inc., Groton, Conn.; telithromycin (potency, 99.3%) from Aventis Pharma, Romainville, France; ciprofloxacin (potency 85%) and moxifloxacin (potency 99.8%) from Bayer AG, Wuppertal, Germany; oritavancin (as LY333328; potency, 80.6%) and [^{14}C]oritavancin (specific activity, 3.5 μCi/mg) from Eli Lilly & Co., Indianapolis, Ind. Gentamicin was procured as Geomycin (the commercial products registered for clinical use in Belgium and supplied by GlaxoSmithKline Belgium on behalf of Schering-Plough Belgium).

Other reagents. Unless stated otherwise, all other reagents were of analytic grade and were purchased from E. Merck AG (Darmstadt, Germany).

Statistical analysis. Unless specified otherwise, all data points presented were obtained from experiments made in triplicate and results are shown as means ± standard deviations (SD). When appropriate, the statistical significance of the differences observed between treated groups and controls, or between pertinent groups, was analyzed with the Student *t* test and Prism version 2.01 software (GraphPad Software, San Diego, Calif.).

RESULTS

Susceptibility testing. The MICs and MBCs at neutral and acid pH are shown in Table 1 for the main antibiotics studied. As anticipated, acid pH markedly reduced the activity of all antibiotics (especially that of azithromycin and telithromycin), with the notable exception of oritavancin, which was as active at pH 7.4 and pH 5 (the latter being taken as the value prevailing in phagolysosomes [45]).

Characterization of the model. A detailed intracellular growth kinetics experiment allowed us to determine that phagocytosed *S. aureus* stayed apparently quiescent for about 8 to 12 h and then started to multiply exponentially to reach almost 2 $\log_{10}$ growth over the next 12 h. Confocal and electron microscopy was used to ascertain the localization of the bac-

teria. As shown in Fig. 1 and 2, *S. aureus* clearly appeared intracellular and restricted to the vacuolar apparatus. In parallel, we checked for the presence of small colony variants, a phenotype that can be induced by the intracellular environment (1, 62). These were not detected upon close examination of the colonies obtained from cell samples. The activity of three antibiotics commonly used in the treatment of staphylococcal infections (rifampin, oxacillin, and vancomycin) was assessed at fixed extracellular concentrations (corresponding to the maximum concentration of drug in serum (C_{max}) for humans; Table 1) for 24 h. Only a modest reduction of the original inoculum (0.2 to 0.3 $\log_{10}$) was seen, whereas controls showed approximately 2 $\log_{10}$ growth (Fig. 3).

Extracellular and intracellular activities: time and concentration dependency. The influence of time and concentrations of all antibiotics on bacteria growing in broth in comparison with that on intracellular bacteria was then systematically investigated. Incubation was carried out for 24 h at drug concentrations from the MIC (as determined in broth at pH 7.3) to multiples thereof and up to the C_{max} (except for azithromycin, for which another scale had to be chosen since its C_{max} was below its MIC [see values in the corresponding figure]). Gentamicin (Fig. 4) displayed a fast-developing and profound bactericidal activity on bacteria in broth at all concentrations exceeding its MIC. Against intracellular bacteria, gentamicin was ineffective at its MIC but became slightly and slowly bactericidal at 5 mg/liter (10 × its MIC) with, however, no improvement at higher concentrations (up to 18 mg/liter). This pattern did not result from a slow drug accumulation, since it was also observed when cells had been preincubated with gentamicin (at 18 mg/liter) for 24 h prior to phagocytosis (the incubation of the cells being made in the continuous presence of gentamicin at 18 mg/liter after phagocytosis and washing of the extracellular bacteria).

Figure 5 shows that azithromycin and telithromycin were essentially bacteriostatic against bacteria in broth (with only a marginal bactericidal effect and with no influence seen at concentrations above 10 times the MIC). Both antibiotics exerted only a modest effect on intracellular bacteria, with a maximal decrease in bacterial counts of approximately 0.6 $\log_{10}$ at extracellular concentrations of 10 times their MIC or above.

Figure 6 shows that ciprofloxacin and moxifloxacin exhibited a marked and fast-developing bactericidal effect against bacteria in broth which was maximal at 10 times the MIC for both drugs (the steepness of the curves prevented us from analyzing in greater detail the concentration dependency of this bacterial killing in the time frame chosen). In contrast, ciprofloxacin exerted only a slowly developing and poorly concentration-dependent bactericidal effect towards intracellular *S. aureus* (0.8 $\log_{10}$ reduction at 24 h at 10 times the MIC). Moxifloxacin showed also a slowly developing bactericidal effect towards intracellular *S. aureus*, but this effect was markedly concentration dependent (reaching a 1.5 $\log_{10}$ reduction in CFU compared to that seen with the original inoculum for cells incubated with the largest concentration tested [C_{max}, 4 mg/liter]).

Figure 7 shows that oritavancin displayed an extremely fast and profound bactericidal effect on bacteria in broth (with bacterial counts falling below the detection level in less than 2 h). Intracellular bacteria were almost unaffected by oritavancin when cells were incubated with a drug concentration equal to 1× MIC in broth. Yet in contrast with all other antibiotics examined so far, larger extracellular concentrations of oritavancin caused a marked reduction in CFU which developed on both a time- and concentration-dependent manner, with the drug becoming clearly bactericidal at an extracellular concentration of 10 mg/liter (40 times its MIC). At its C_{max} (25 mg/liter), oritavancin caused a 1.5 $\log_{10}$ reduction in CFU in 6 h and a 3.5 $\log_{10}$ decrease (0.03% survival) after 24 h.

Accumulation of antibiotics. Table 2 shows the values obtained at 24 h with cells incubated with an extracellular drug concentration equivalent to the C_{max} (except for telithromycin and azithromycin, for which higher concentrations needed to be used [6 and 10 mg/liter, respectively] because of the insensitivity of the corresponding microbiological assay). We also compared infected and uninfected cells in the same experiment. Ciprofloxacin, gentamicin, telithromycin, and moxifloxacin achieved cellular-to-extracellular concentration ratios ranging from only 3.2 to 11.4, whereas azithromycin reached an approximately 50-fold accumulation and oritavancin peaked at an approximately 340-fold accumulation. No statistically significant difference was seen between infected and uninfected cells.

DISCUSSION

The present study was one of the first systematic attempts to examine the activity of different classes of antibiotics against *S. aureus* in macrophages in long-term (24 h) experiments. It was designed to allow a direct comparison between extra- and intracellular activities against a single well-characterized strain of *S. aureus* and was conducted at drug extracellular concentrations that are directly clinically relevant.

The morphological observations indicated that we were dealing with a truly phagolysosomal infection, in contrast to what takes place in epithelial cells where *S. aureus* can gain access to the cytosol (1, 28). The bacteria were therefore expected to become exposed to nonspecific cell defense mechanisms, which probably explains the very characteristic lag period (about 12 h) before they eventually started actively multiplying. This delay in growth has also been observed in P388D1 murine macrophage-like cells (35) and in CFT-1 epithelial cells (28) and was not due the presence of small colony variants in detectable amounts. The model needed the continuing presence of gentamicin in the medium (in the absence of other antibiotics) to prevent the extracellular growth of the bacteria released from macrophages 6 to 8 h after phagocytosis. These bacteria must indeed be of intracellular origin, since otherwise growth would have taken place much earlier (gentamicin has only a short postantibiotic effect towards *S. aureus* [18]). This use of gentamicin in controls could be criticized on the basis that the drug can impair the intracellular growth of *S. aureus*, thereby reducing the difference seen between controls (made in the presence of gentamicin) and antibiotic-treated cells. The model is nevertheless correct if activity is defined as a reduction in CFU in comparison with the original inoculum. Our analysis will, therefore, be limited to the examination of this parameter.

The main and probably most critical finding made here is that the intracellular activities observed were considerably lower than would be expected on the basis of their activity in

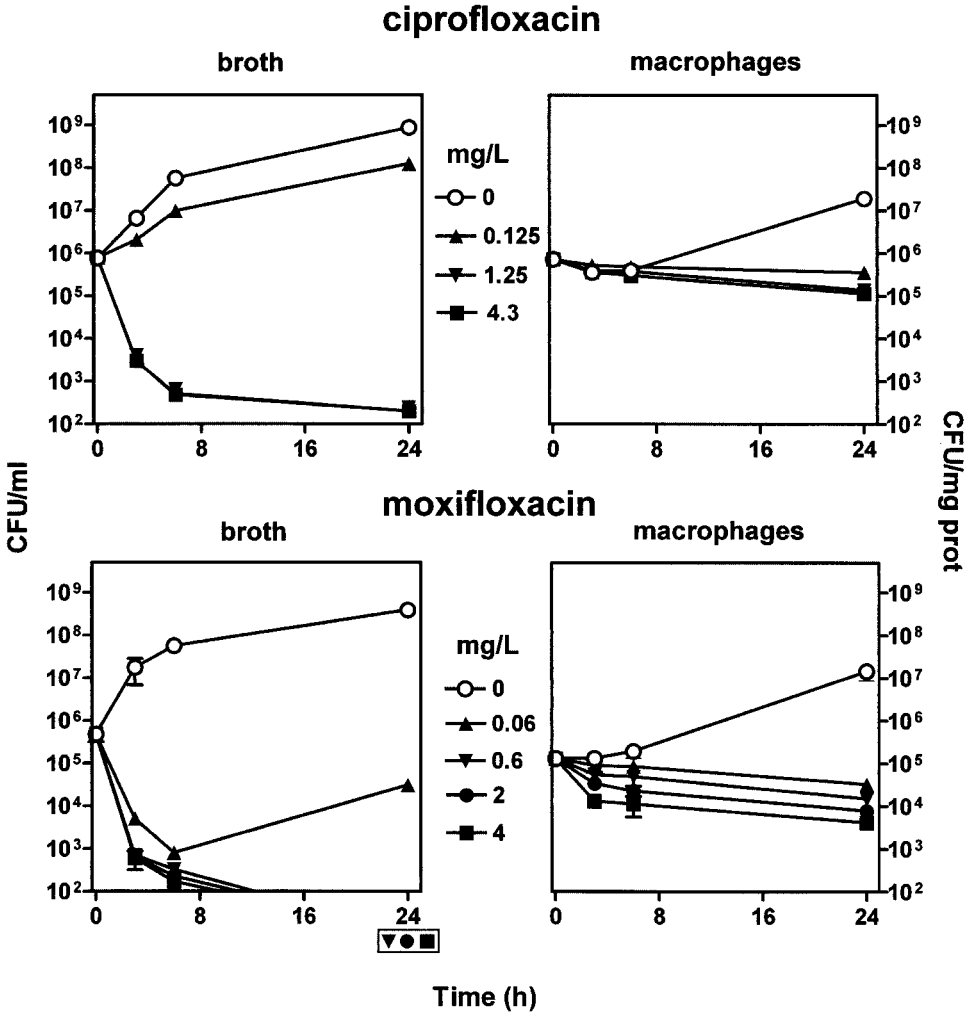

FIG. 6. Variations in the number of CFU upon incubation for up to 24 h with increasing concentrations of ciprofloxacin and moxifloxacin. (Left two panels) Results for bacteria in broth (initial pH, 7.3). (Right two panels) Results for bacteria collected from J774 macrophages (after phagocytosis). The lowest drug concentrations investigated (0.125 mg/liter for ciprofloxacin, 0.06 mg/liter for moxifloxacin) correspond to the MICs (in broth) of the drugs for the strain of *S. aureus* used in this study; the highest concentrations (4.3 mg/liter for ciprofloxacin, 4 mg/liter for moxifloxacin) correspond to the C_{max} observed for patients after administration of the currently recommended doses of each of these two fluoroquinolones (reference 54 and U.S. ciprofloxacin package insert, Bayer Corporation, West Haven, Conn.). Each point corresponds to the mean value for three independent determinations. SD values were calculated, but the corresponding bars are smaller than the symbols in many cases. Symbols placed outside of the bottom of the lower left graph indicate samples in which no or only a few colonies were observed (i.e., numbers were too close to the lowest limit of detection [10^2 CFU/ml] to be fully reliable). Note that cells incubated in the absence of ciprofloxacin or moxifloxacin were maintained in the presence of 0.5 mg of gentamicin/liter to prevent extracellular growth of *S. aureus*.

broth and the levels of their cellular accumulation. This finding is particularly impressive when examining the activity of bactericidal, concentration-dependent antibiotics such as gentamicin, fluoroquinolones, or oritavancin. It has long been suggested that intracellular accumulation of antibiotics would be conducive to intracellular activity (56, 59). Yet several authors have now offered experimental evidence that there is no simple and direct correlation between accumulation and activity for antibiotics like fluoroquinolones (3, 25, 43, 46, 50, 64) or macrolides (25, 39, 48, 49). We have confirmed this conclusion and extend it to other antibiotics. This lack of or decreased expression of the activity of antibiotics in the intracellular milieu is probably multifactorial. For bacteriostatic antibiotics such as macrolides, it can simply be argued that these drugs reach their maximal antibacterial effect at a relatively low concentration and that accumulation at higher levels is without therapeutic benefit. For macrolides and aminoglycosides, we also see that acid pH conditions markedly decrease their activity (alkalinization of phagolysosomes markedly increases the intracellular activity of amikacin [35]). The activity of fluoroquinolones is, however, less affected by acid pH, and that of oritavancin is not affected at all.

The rate of uptake and the subcellular localization could also, a priori, play a major role. The uptake rate is, however, probably not a limiting factor in the present long-term model. Macrolides and aminoglycosides concentrate in lysosomes of uninfected cells (9, 40, 55), whereas fluoroquinolones are found in the soluble fraction of cell extracts (10) and are

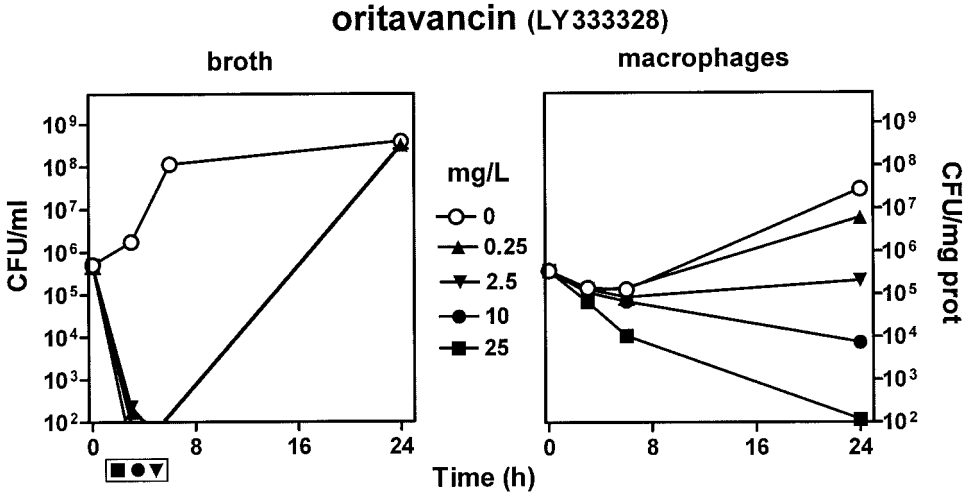

FIG. 7. Variations in the number of CFU upon incubation for up to 24 h with increasing concentrations of oritavancin. (Left two panels) Results for bacteria in broth (initial pH, 7.3). (Right two panels) Results for bacteria collected from J774 macrophages after phagocytosis. The lowest drug concentration investigated (0.25 mg/liter) corresponds to the MIC (in broth) of oritavancin for the strain of *S. aureus* used in this study, and the highest concentration investigated (25 mg/liter) corresponds to the C_{max} observed for patients after administration of the dose currently used in the clinical development program of this antibiotic (D. K. Braun et al., Abstracts of the 11th European Congress of Clinical Microbiology and Infectious Diseases, Clin. Microbiol. Infect., **7**[Suppl. 1]**:**P434, 2001). Each point corresponds to the mean value of three independent determinations. SD values were calculated, but the corresponding bars are smaller than the symbols in many cases. Symbols placed outside of the bottom of the lower left graph indicate samples in which no or only a few colonies were observed (i.e., numbers were too close to the lowest limit of detection [10^2 CFU/ml] to be fully reliable). Note that cells incubated in the absence of oritavancin were maintained in the presence of 0.5 mg of gentamicin/liter to prevent extracellular growth of *S. aureus.*

considered to be highly diffusible. The subcellular localization of oritavancin is not known, but vancomycin was found to slowly accumulate in the lysosomes of renal cells (7) and preliminary data suggest that oritavancin also localizes in lysosomes (F. Van Bambeke et al., unpublished data). Thus, localization per se may not be predictive of activity. Another and perhaps more important parameter which needs to be considered is the binding of the antibiotics to proteins or other intracellular constituents. We unfortunately lack direct information in this context, especially concerning infected cells. Yet we know that macrolides and aminoglycosides which are sequestered in lysosomes are likely to bind to phospholipids through electrostatic interactions (31, 58) and to be therefore largely unavailable. The same phenomenon could take place for oritavancin, which is a basic compound. Concerning fluoroquinolones, no molecular explanation has been given so far concerning the mechanism of their accumulation in cells but available data (10, 19) suggest that it results from loose (nonspecific?) binding to cell constituents. Finally, parameters related to the bacteria may also be critical. The intracellular milieu may indeed be directly responsible for the modulation of metabolism towards a stage of decreased sensitivity. Moreover, the slower multiplication rate of intracellular bacteria may play a major role (6, 19, 20, 30). Slow growth, however, does not seem to adversely affect the activity of oritavancin (37).

TABLE 2. Accumulation of antibiotics in macrophages incubated for 24 h with the concentrations of antibiotics indicated (these concentrations correspond to the C_{max} value as defined in Table 1 except for those for azithromycin and telithromycin)

Antibiotic	Extracellular concn (mg/liter)	Cellular-to-extracellular concn ratio[a]	
		Noninfected cells	Infected cells
Gentamicin	18	6.3 ± 0.7	6.8 ± 1.7
Azithromycin	10	45.0 ± 5.1	50.0 ± 7.5
Telithromycin	6	8.6 ± 1.2	8.7 ± 1.1
Ciprofloxacin	4.3	3.2 ± 0.6	3.2 ± 0.3
Moxifloxacin	4	11.4 ± 0.8	13.4 ± 0.4
Oritavancin	25	336.3 ± 13.6	343.8 ± 10.6

[a] Calculated from the determinations of the drug content per milligram of cell protein and using a conversion factor of 3.8 μl/mg of cell protein. All values are means of three determinations ± SD. In statistical analysis (Student's *t* test), all differences between infected and uninfected cells were nonsignificant ($P > 0.05$) except those seen with moxifloxacin, for which the *P* value was 0.02.

Whatever the various mechanisms explaining the loss of activity of antibiotics against intracellular *S. aureus*, the type of model developed here may prove useful for comparisons of drugs in this context. Indeed, it clearly appears that gentamicin, macrolides, and ciprofloxacin are largely ineffective, raising questions about the true usefulness of these antibiotics for acting upon and for eradicating intracellular forms of *S. aureus* in vivo. Conversely, moxifloxacin and (to a greater degree) oritavancin appear to be very effective. Our data, therefore, open interesting perspectives and suggest useful animal and humans trials with these drugs. Our results also show that intracellular activity of new antibiotics should be experimentally addressed early on during the selection and development process.

ACKNOWLEDGMENTS

Jean-Michel Michot determined the volume/cell protein ratio of J774 macrophages as used in this paper. F. Renoird-Andries provided skillful help for the electron microscopy studies, and M. C. Cambier provided skillful help for cell culture maintenance.

C.S. was Chercheur post-doctoral of the Belgian Fonds de la Recherche Scientifique Médicale (fellowship 3.4.549.00). F.V.B. is Chercheur Qualifié of the Belgian Fonds National de la Recherche Scientifique. This work was supported by the Belgian Fonds de la Recherche Scientifique Médicale (grant 3.4.549.00). We thank Eli Lilly Benelux, Brussels, Belgium, for a grant-in-aid and Eli Lilly & Co., Indianapolis, Ind., for providing us with ^{14}C-labeled oritavancin. We thank the other manufacturers for the kind gift of their corresponding antibiotics.

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