

## Pharmacodynamic Evaluation of the Intracellular Activities of Antibiotics against *Staphylococcus aureus* in a Model of THP-1 Macrophages

Maritza Barcia-Macay, Cristina Seral,<sup>†</sup> Marie-Paule Mingeot-Leclercq, Paul M. Tulkens, and Françoise Van Bambeke\*

Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, B-1200 Brussels, Belgium

Received 17 August 2005/Returned for modification 30 October 2005/Accepted 8 December 2005

The pharmacodynamic properties governing the activities of antibiotics against intracellular *Staphylococcus aureus* are still largely undetermined. Sixteen antibiotics of seven different pharmacological classes (azithromycin and telithromycin [macrolides]; gentamicin [an aminoglycoside]; linezolid [an oxazolidinone]; penicillin V, nafcillin, ampicillin, and oxacillin [ $\beta$ -lactams]; teicoplanin, vancomycin, and oritavancin [glycopeptides]; rifampin [an ansamycin]; and ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin [quinolones]) have been examined for their activities against *S. aureus* (ATCC 25923) in human THP-1 macrophages (intracellular) versus that in culture medium (extracellular) by using a 0- to 24-h exposure time and a wide range of extracellular concentrations (including the range of the MIC to the maximum concentration in serum [ $C_{\max}$ ; total drug] of humans). All molecules except the macrolides caused a net reduction in bacterial counts that was time and concentration/MIC ratio dependent (four molecules tested in detail [gentamicin, oxacillin, moxifloxacin, and oritavancin] showed typical sigmoidal dose-response curves at 24 h). Maximal intracellular activities remained consistently lower than extracellular activities, irrespective of the level of drug accumulation and of the pharmacological class. Relative potencies (50% effective concentration or at a fixed extracellular concentration/MIC ratio) were also decreased, but to different extents. At an extracellular concentration corresponding to their  $C_{\max}$ s (total drug) in humans, only oxacillin, levofloxacin, garenoxacin, moxifloxacin, and oritavancin had truly intracellular bactericidal effects (2-log decrease or more, as defined by the Clinical and Laboratory Standards Institute guidelines). The intracellular activities of antibiotics against *S. aureus* (i) are critically dependent upon their extracellular concentrations and the duration of cell exposure (within the 0- to 24-h time frame) to antibiotics and (ii) are always lower than those that can be observed extracellularly. This model may help in rationalizing the choice of antibiotic for the treatment of *S. aureus* intracellular infections.

*Staphylococcus aureus*, which often causes chronic or relapsing diseases (68), is reported to persist as an opportunistic intracellular organism both in vitro and in vivo (8, 10, 18, 30, 31, 34, 39). Antibiotic treatments should therefore be optimized not only toward the extracellular forms of *S. aureus* but also toward the intracellular forms of *S. aureus* to avoid creating a niche where bacteria may persist, cause cell alterations, and possibly, be selected for resistance if they are exposed to subtherapeutic concentrations (1). A large body of literature on the activities of antibiotics against intracellular *S. aureus* in various cellular models is available (see references 54, 66, 67, and 70 for reviews). Yet, many of these studies yield contradictory results, and we still lack a clear understanding of which parameters are truly critical for the expression of antibiotic activity in the intracellular milieu (11). In a previous study, we measured the activities of selected antibiotics characterized by a fair to high level of cellular accumulation against intracellular *S. aureus* in a model of unstimulated murine J774 macrophages

(57). We observed that cellular accumulation was only partially and nonconsistently predictive of activity. In a subsequent pilot study, performed with human THP-1 macrophages, we also noted that  $\beta$ -lactams, which notoriously do not accumulate in cells, actually showed significant activity against intracellular *S. aureus* when their extracellular concentration was brought to a sufficiently high but still clinically meaningful level (36). This triggered us to broaden and systematize our approach. For this purpose, we selected typical representatives of seven classes of antibiotics with known activities against *S. aureus* and included in commonly used guidelines for the handling of staphylococcal infections. We concentrated our effort on THP-1 macrophages because these cells present many of the characteristics of human monocytes while forming a homogeneous and reproducible population (6). THP-1 macrophages have been successfully used in various studies aimed at characterizing the interactions between *S. aureus* and macrophages in a clinical context (19, 28, 49) and to analyze the potential relationship between the accumulation of antibiotics in cells and intracellular activity (48). In contrast to many other models, however, we explored a large array of extracellular concentrations (including the range observed in the serum of patients receiving conventional doses) and used incubation times up to 24 h. Our purpose, indeed, was to analyze the pharmacodynamic parameters governing the activities of these antibiotics against intra-

\* Corresponding author. Mailing address: Unité de Pharmacologie Cellulaire et Moléculaire, Université Catholique de Louvain, UCL 7370 Avenue E. Mounier 73, B-1200 Brussels, Belgium. Phone: 32-2-764.73.78. Fax: 32-2-764.73.73. E-mail: vanbambeke@facm.ucl.ac.be.

<sup>†</sup> Present address: Department of Clinical Microbiology, University Hospital "Lozano Blesa," Zaragoza, Spain.

cellular forms of *S. aureus* in terms of both the concentration/MIC ratio and the time of exposure. This approach was thought to be necessary to enable us to draw pharmacologically as well as clinically meaningful conclusions.

(Parts of this study were presented at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 14 to 17 September 2003 [M. Barcia-Macay, C. Seral, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke, Abstr. 43rd Intersci. Conf. Antimicrob Agents Chemother., abstr. A-1174, 2003], and at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington D.C., 30 October to 2 November 2004 [M. Barcia-Macay, C. Seral, M. P. Mingeot-Leclercq, P. M. Tulkens, and F. Van Bambeke, Abstr. 44th Intersci. Conf. Antimicrob Agents Chemother., abstr. A1488, 2004].)

#### MATERIALS AND METHODS

**Bacterial strain, susceptibility testing, and time and dose-kill curve studies in extracellular medium.** *S. aureus* (strain ATCC 25923, fully susceptible) was used for all experiments. All conditions for measurement of the MICs (at pH 7.3 and 5.0) and the minimal bactericidal concentrations (MBCs) were exactly the same as those described earlier (57). Dose-kill curve studies were performed as described previously (57), with the following modifications: (i) RPMI 1640 medium supplemented with 10% fetal calf serum rather than broth was systematically used to measure the extracellular activities to better mimic a true extracellular environment, and (ii) enumeration of colonies (for determination of CFU) was performed with an automated detector (14). All samples (diluted as needed) were prepared in a final volume of 1 ml, of which 50  $\mu$ l was used to seed 8.2-cm-diameter petri dishes containing 12.5 ml of nutrient agar. In the present study, we validated the method by assessing (i) the linearity of the instrument response for counting from 3 to 2,000 colonies per dish ( $R^2 = 0.997$ ;  $n = 220$ ) and (ii) the intraday reproducibilities for samples that yielded 3 to 1,500 colonies/dish (the observed standard deviation [SD] for 10 repeated assays with the same sample was from 0.6 to 1.7 times the theoretical value [which is equal to the square root of  $n$ , where  $n$  is the value of the actual counts, assuming a Poisson type of distribution], with no trend toward less reproducibility for samples with low counts). Samples with counts more than 1,500 colonies/dish tended to give lower reproducibilities due to the fusion of colonies and larger dilutions were then used. The lowest limit of detection was set at 3 counts/plate (actual SD, 1.1 for 10 repeated assays with the same sample). This lowest value corresponded to 60 CFU in the original samples (if it was undiluted) and to a 4.2-log decrease in the numbers of CFU from a typical initial inoculum of  $10^6$  bacteria per ml (variation, from 4.1 to 4.4 when an SD value of 1.1 at the level of the determination was considered). All samples yielding less than three colonies were arbitrarily considered to have a 5-log decrease from the typical original inoculum, and this value was used in all illustrations and calculations.

**Cells, cell cultures, and intracellular infection.** Human THP-1 macrophages were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum exactly as described in a previous publication (55). Infection was performed as described earlier (36), based on a model with murine J774 macrophages (57). Phagocytosis was initiated at a bacterium-macrophage ratio of 4:1, and after removal of unphagocytosed and adherent *S. aureus* cells, the inoculum was typically  $1 \times 10^6$  to  $2 \times 10^6$  bacteria per mg of cell protein, as for J774 macrophages (57). At the end of the experiments, the cells were collected by centrifugation, resuspended in phosphate-buffered saline, and centrifuged again to further remove adherent bacteria. The cells were then processed for CFU counting as described above with the same upper and lowest limits of detection. The typical initial intracellular inoculum of approximately  $10^6$  bacteria per mg of cell protein was diluted in approximately 1 ml after cell collection and processing. The protein concentrations were measured in parallel, as described previously (13). Previous studies showed that the amounts of antibiotic that could be carried over from the cells into the final assay mixture were too low to interfere significantly with CFU determinations, given the high dilution of the cell content during sample preparation (12, 55). Extracellular contamination was assessed in pilot studies by collecting the culture fluid at the end of the observation period, mixing it with all media used to wash the corresponding cell samples, and enumerating the CFU after plating and incubation on Trypticase soy agar.

**Determination of cellular antibiotic accumulation.** Accumulation studies were performed as described in previous publications (12, 47), but the majority of

antibiotics were assayed by a microbiological method (disk diffusion method with *Bacillus subtilis* ATCC 6633 as the test organism and antibiotic medium 11 adjusted to pH 8 for gentamicin; disk diffusion method with *Micrococcus luteus* ATCC 9341 and antibiotic medium 2 adjusted to pH 8 for macrolides; and disk diffusion method with *Micrococcus luteus* ATCC 9341 and antibiotic medium 2 adjusted to pH 7 for  $\beta$ -lactams, rifampin, linezolid, vancomycin, and teicoplanin). For each of these antibiotics, the assay method was checked for linearity (telithromycin, 0.03 to 4 mg/liter; azithromycin, 0.05 to 4 mg/liter; ampicillin, 0.21 to 10 mg/liter; nafcillin, 1.1 to 120 mg/liter; oxacillin, 1.6 to 30 mg/liter; penicillin V, 0.04 to 10 mg/liter; rifampin, 0.2 to 15 mg/liter; linezolid, 12 to 190 mg/liter; vancomycin, 2.7 to 150 mg/liter; teicoplanin, 2.9 to 250 mg/liter; gentamicin, 0.6 to 240 mg/liter) and for reproducibility (coefficient of variation, <10%). Ciprofloxacin, moxifloxacin, and levofloxacin concentrations were measured by fluorimetry (12, 56), and garenoxacin and oritavancin concentrations were measured by radiometry by using  $^{14}$ C-labeled drugs (41, 64). In pilot studies we checked that these assays detected genuine, bioactive drug. The cell concentration of each drug was expressed by reference to the protein content of the corresponding samples, with a conversion factor of 5  $\mu$ l of cell volume per mg of cell protein used to calculate the apparent cellular drug accumulation (12, 47).

**Morphological studies.** Infection was carried out at a bacterium-macrophage ratio of approximately 8 to allow visualization of a sufficiently large number of bacteria, with all other conditions similar to those described in the general protocol. The cells were fixed and prepared as described previously (47, 63).

**Antibiotics.** Whenever possible, antibiotics were obtained as microbiological standards from their corresponding manufacturers: azithromycin (dihydrate salt; potency, 94.4%) from Pfizer Inc., Groton, CT; telithromycin (potency, 99.3%) from Aventis, Romainville, France; ciprofloxacin (potency, 85%) and moxifloxacin (potency, 91%) from Bayer AG, Wuppertal, Germany; oxacillin (potency, 85%) from Bristol-Myers Squibb Co., Syracuse, NY; unlabeled garenoxacin (potency, 79%) and  $^{14}$ C-labeled garenoxacin (specific activity, 0.80 MBq/mg) from the Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT; and unlabeled oritavancin (as LY333328; potency, 80.6%) and [ $^{14}$ C]oritavancin (labeled on the chloro-biphenyl side chain; specific activity, 3.5  $\mu$ Ci/mg) from Eli Lilly & Co., Indianapolis, IN. Ampicillin and penicillin V were purchased from Sigma-Aldrich-Fluka (St. Louis, MO). The other antibiotics were procured as the commercial products registered in Belgium for parenteral use from their respective marketing authorization holders or resellers (gentamicin as Geomycin and vancomycin as Vancocin from Glaxo-SmithKline; rifampin as Rifadine, levofloxacin as Tavanic, and teicoplanin as Targocid from Aventis; and linezolid as Zyvoxid from Pfizer).

**Other reagents.** Unless stated otherwise, all other reagents were of analytical grade and were purchased from E. Merck AG (Darmstadt, Germany) or Sigma-Aldrich-Fluka. Cell culture or microbiology media were from Invitrogen (Paisley, Scotland) and Difco (Sparks, MD).

**Curve fittings and statistical analyses.** Curve fittings were done with GraphPad Prism (version 4.02) software for Windows (GraphPad Prism Software, San Diego, CA), and statistical analyses were performed with XLSTAT Pro (version 7.5.2; Addinsoft SARL, Paris, France).

## RESULTS

**Susceptibility testing.** Table 1 shows the MICs (measured at pH 7.3 and 5.0 to mimic the extracellular and phagolysosomal environments, respectively) and the MBCs for the *S. aureus* strain used in this study. Except for azithromycin, all MICs measured at pH 7.3 were considerably lower than the peak concentration in serum ( $C_{\max}$  [total drug; see Table 1 for estimates of the fraction of free drug at that concentration in human serum]) commonly observed in patients receiving conventional dosages of the corresponding antibiotics. Lowering of the pH to 5.0 had contrasting effects on MICs, with a marked increase (more than eightfold) for gentamicin and the macrolides, a moderate increase (two- to eightfold) for linezolid and the quinolones, no or little change for the glycopeptides, and a modest (1 to 2 dilutions) but reproducible decrease for the  $\beta$ -lactams and rifampin. The MBCs were close to the MICs measured at pH 7.3 ( $\leq 2$ -dilution difference) for gentamicin, rifampin, the  $\beta$ -lactams, oritavancin, and the quin-

TABLE 1. MICs and MBCs of antibiotics under study against *S. aureus* ATCC 25923 compared to the  $C_{max}^a$

Antibiotic <sup>b</sup>	MIC (mg/liter)		MBC (mg/liter)	Dosage and route <sup>c</sup>	Human $C_{max}$ (mg/liter) (Total drug)	% Free drug	Reference(s)
	pH 7.3	pH 5.0					
Azithromycin	0.5	512	8	500 mg p.o.	0.5	88	22
Telithromycin	0.06	4	2	800 mg p.o.	2	30	44, 71
Gentamicin	0.5	16	2	6 mg/kg i.v.	18	80	26, 43
Linezolid	2	4	32	600 mg i.v.	21	69	5
Penicillin V	0.015	<0.015	0.06	500 mg p.o.	6.3	20	4, 50
Nafcillin	0.25	0.06	1	1,000 mg i.v.	40	3	4, 27
Ampicillin	0.06	0.03	0.25	1,000 mg i.v.	47.6	85	4, 50
Oxacillin	0.125	0.06	0.25	500 mg i.v.	63	10	4, 50
Teicoplanin	0.25	0.5	64	12 mg/kg i.v.	100	10	20
Vancomycin	1	1	16	15 mg/kg i.v.	50	45–90	20
Oritavancin	0.25	0.25	1	3 mg/kg i.v.	25	10	9, 65
Rifampin	0.0075	0.002	0.03	600 mg i.v.	18	10–20	4, 29
Ciprofloxacin	0.125	1	1	750 mg p.o.	4.3	63	32, 33
Levofloxacin	0.125	1	0.125	400 mg p.o.	4	62–76	21
Garenoxacin	<0.03	0.125	0.03	400 mg p.o.	4	25	23 <sup>d</sup>
Moxifloxacin	0.06	0.25	0.06	400 mg p.o.	4	52	58, 59

<sup>a</sup> Commonly observed maximal concentration in serum (and estimated percentage of free drug) after intravenous or oral administration of conventional doses to humans, based on the references indicated.

<sup>b</sup> The molecules are ranked by pharmacological classes, with each class appearing by order of its mean level of intracellular activity (as shown in Fig. 5).

<sup>c</sup> p.o., oral; i.v., intravenous.

<sup>d</sup> Reference for protein binding: A. Bello, D. Hollenbaugh, D. A. Gajjar, L. Christopher, and D. M. Grasela, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. A-45, 2001.

olones (except ciprofloxacin). In contrast, the macrolides and linezolid had higher MBCs (equal to or higher than their corresponding  $C_{max}$ s). Vancomycin, teicoplanin, and ciprofloxacin showed intermediate behaviors, with their MBCs being considerably higher than their MICs but still lower than their  $C_{max}$ s.

**Validation of the intracellular model.** We first examined whether the accumulation of antibiotics in THP-1 cells was consistent with their known behavior in other cell types (see reference 11 for a review). Whenever possible, the extracellular concentration was set at the  $C_{max}$  of the drug (as defined in Table 1), but poor assay sensitivity forced us to use higher concentrations for a number of molecules. The 24-h time point was selected since this corresponded to the maximal duration of our studies with infected cells. Data are presented in Table 2. Linezolid,  $\beta$ -lactams, and gentamicin showed no or only modest accumulation (from 0.5- to 4.4-fold). The quinolones, vancomycin, and teicoplanin reached slightly higher levels (5- to 10-fold). Rifampin, azithromycin, and telithromycin achieved higher levels (17- to 38-fold); and oritavancin accumulated up to almost 150-fold.

We then characterized the course of the infection of THP-1 cells by the strain of *S. aureus* that we used (Fig. 1). In the absence of antibiotic, the number of bacteria collected from cells (after the washing procedure) increased almost at the same rate as that for bacteria incubated in complete culture medium in the absence of cells (extracellular infection). The medium of the infected cells, however, showed visible acidification at 24 h (compared to the medium of the uninfected cells), with the number of viable bacteria in low speed super-

TABLE 2. Cellular accumulation factor of antibiotics in THP-1 cells after 24 h of incubation at a fixed extracellular concentration

Antibiotic <sup>a</sup>	Cellular accumulation <sup>b</sup>	Extracellular concn (mg/liter)
Azithromycin	37.8 ± 1.3	5 <sup>c</sup>
Telithromycin	27.9 ± 1.3	2 <sup>d</sup>
Gentamicin	4.4 ± 0.1	250 <sup>c</sup>
Linezolid	0.5 ± 0.0	250 <sup>c</sup>
Penicillin V	1.2 ± 0.1	150 <sup>c</sup>
Nafcillin	2.6 ± 0.1	400 <sup>c</sup>
Ampicillin	1.0 ± 0.1	150 <sup>c</sup>
Oxacillin	4.0 ± 0.1	250 <sup>c</sup>
Teicoplanin	7.4 ± 0.2	150 <sup>c</sup>
Vancomycin	6.3 ± 0.1	100 <sup>c</sup>
Oritavancin	148.0 ± 12.0	25 <sup>d</sup>
Rifampin	17.6 ± 0.9	50 <sup>c</sup>
Ciprofloxacin	5.1 ± 0.1	4.3 <sup>d</sup>
Levofloxacin	7.0 ± 0.6	4 <sup>d</sup>
Garenoxacin	9.1 ± 0.3	4 <sup>d</sup>
Moxifloxacin	7.6 ± 0.3	4 <sup>d</sup>

<sup>a</sup> The molecules are ranked by pharmacological classes, with each class appearing by order of its mean level of intracellular activity (as shown in Fig. 5).

<sup>b</sup> Apparent cellular concentration-to-extracellular concentration ratio, based on a cell volume of 5  $\mu$ l per mg of cell protein (12,47).

<sup>c</sup> A concentration larger than the  $C_{max}$  was used because of a lack of sensitivity of the microbiological assay.

<sup>d</sup> Concentration corresponding to the  $C_{max}$  (as defined in Table 1).

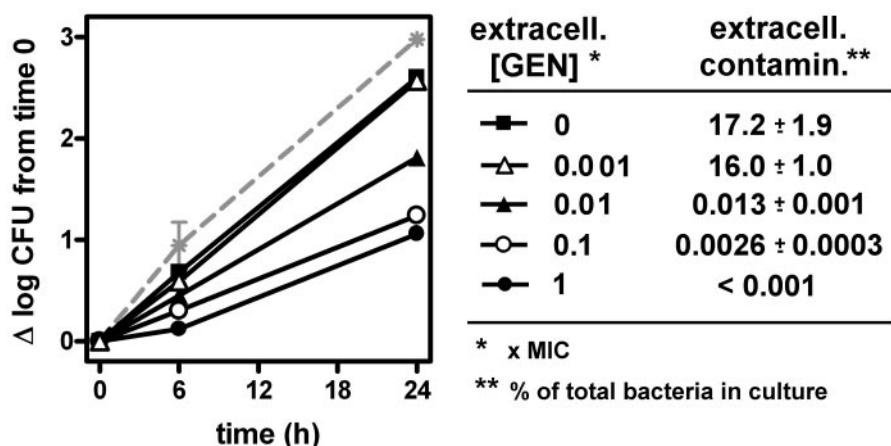


FIG. 1. Kinetics of growth of *S. aureus* (i) in THP-1 macrophages exposed to increasing extracellular concentrations of gentamicin (solid lines) or (ii) in culture medium in the absence of antibiotic (dotted gray line). The graph shows the change in the number of CFU ( $\Delta \log$  CFU; means  $\pm$  SDs;  $n = 3$ ; most SD bars are smaller than the symbols), starting from an initial inoculum of approximately  $10^6$  bacteria per mg of protein (intracellular) or per ml of medium (extracellular). The table shows the contamination (contamin.) of the culture medium by *S. aureus* at the end of the experiment. extracell., extracellular; GEN, gentamicin.

natants and washing media amounting to approximately 17% of the total sample content (medium plus cells). Based on previous experience with J774 macrophages (57), gentamicin was added to the culture medium to prevent this contamination. As shown in Fig. 1, a gentamicin concentration as low as 0.01 its MIC reduced the extracellular contamination to an almost negligible level, while it still allowed a marked increase in the number of cell-associated CFU (to about 65% of what was seen without antibiotic). A further increase in the extracellular concentration of gentamicin to its MIC allowed extracellular contamination to go to undetectable levels, but with a further decrease in the cell-associated CFU, demonstrating interference with the intracellular multiplication of the bacteria. Yet, optical and electron microscopy of cells incubated for 24 h with gentamicin at the MIC still revealed the presence of actively multiplying bacteria within membrane-bound structures, consistent with intraphagolysosomal localization (see Fig. 6). Because of all those uncertainties in the true level of intracellular growth of *S. aureus* and the potential impact of even low concentrations of gentamicin, intracellular activities were therefore examined and expressed not as the difference from the controls but in terms of variations of the cell-associated CFU from the original, postphagocytosis inoculum. Finally, careful examination of the bacterial cultures obtained from cell samples exposed to gentamicin or to other antibiotics (see below) failed to identify so-called small-colony variants.

**Kinetics of antibacterial effects at a fixed, large concentration ( $C_{max}$ ).** Time-kill curves were obtained for eight molecules selected on the basis of (i) their increasing MBC/MIC ratios (from 1 [moxifloxacin] to 33 [telithromycin]; see Table 1) when they were tested in broth and (ii) their increasing levels of cellular accumulation (apparent cellular-concentration-to-extracellular-concentration ratio from less than 1 [linezolid] to about 150 [oritavancin] in uninfected cells; see Table 2). The results are shown in Fig. 2. By first considering the extracellular activities, it appears that gentamicin, rifampin, and oritavancin acted very fast, with bacterial counts reaching the limit of detection within 6 h or less, whereas linezolid and telithromy-

cin, although they were tested at concentrations equal or close to their MBCs, were only slowly and poorly bactericidal. Oxacillin, vancomycin, and moxifloxacin reached the limit of detection upon prolonged incubation. There was thus only a poor correlation between the rates and extents of killing and the MBC/MIC ratios. By next considering the intracellular bacteria, overall decreases in the rates and extents of killing of

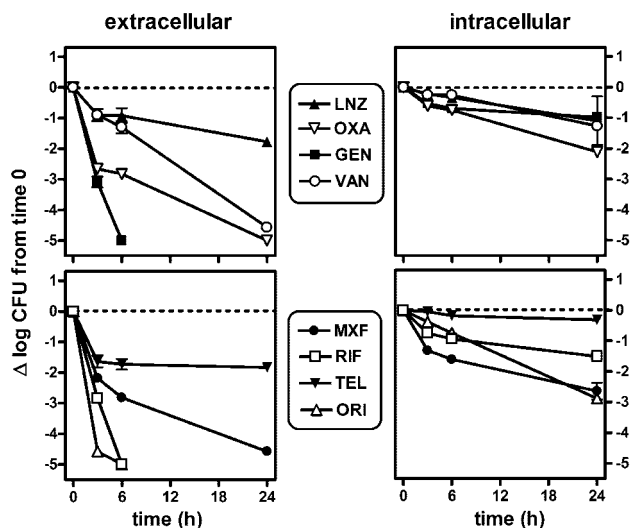


FIG. 2. Influence of time on the rate and the extent of the activities of the antibiotics against extracellular and intracellular *S. aureus* upon incubation at a fixed extracellular concentration. The graphs show the change in the number of CFU ( $\Delta \log$  CFU; means  $\pm$  SDs;  $n = 3$ ; most SD bars are smaller than the symbols) per ml of culture medium (extracellular) or in THP-1 macrophages (intracellular) per mg of cell protein. The molecules are listed in order from the upper to the lower panel by increasing level of cellular accumulation (as determined in uninfected cells; Table 2). Each antibiotic was added at a concentration corresponding to its  $C_{max}$  in humans (total drug, as defined in Table 1). LNZ, linezolid; OXA, oxacillin; GEN, gentamicin; VAN, vancomycin; MXF, moxifloxacin; RIF, rifampin; TEL, telithromycin; ORI, oritavancin.

intracellular bacteria compared to those of extracellular bacteria were observed, but some antibiotics were more affected than others. Only oritavancin, moxifloxacin, and oxacillin achieved bactericidal effects (as defined by a 2-log decrease from the original inoculum) at 24 h. Rifampin and gentamicin, which were highly bactericidal toward extracellular bacteria, did not reach this limit (and their intracellular activities were actually close to those of vancomycin and linezolid). Telithromycin was essentially bacteriostatic. There was no correlation between intracellular activity and cellular accumulation among the eight drugs tested.

**Kinetics and influence of concentration on antibacterial effects in the MIC- $C_{max}$  range.** Six molecules were then selected from among the bactericidal drugs to examine the influence of concentration on the rate and extent of killing (Fig. 3). By first considering extracellular activities, the extent of killing was significantly concentration dependent for all drugs over the range of concentrations investigated. The rate of killing also increased with concentration for all drugs except rifampin, for which a low concentration (but still above the MIC) caused the antibiotic activity to plateau after 6 h. By next considering the intracellular activities, both the rate and the extent of killing of intracellular bacteria were considerably reduced compared to those of extracellular bacteria; but significant concentration-dependent effects were still observed with respect to both of these parameters for vancomycin, oxacillin, and oritavancin and with respect to the extent of killing for moxifloxacin and rifampin. For gentamicin, an increase in the extracellular concentration from 5 to 18 mg/liter (10- to 36-fold the MIC) was without significant effect at 6 h but caused a modest, albeit statistically significant, increase in activity at 24 h, the extent of which remained, however, very limited.

These experiments were then repeated with all drugs included in this study but were limited to the examination of the 24-h time point and to three critical concentrations (the MIC, 10 times the MIC, and  $C_{max}$ , except for rifampin, in view of its very low MIC [see the Fig. 4 legend for the concentrations of rifampin used]). The results are shown in Fig. 4 in a synoptic fashion for ease of direct comparison of the results between molecules and, for each molecule, between its extracellular and intracellular levels of activity. The data show that (i) the macrolides were always bacteriostatic toward both extracellular and intracellular bacteria, whichever concentration was tested; (ii) the largest discrepancy between extracellular and intracellular activities occurred for gentamicin; and (iii) oxacillin (among the four penicillins tested), levofloxacin, garenoxacin, and moxifloxacin (among the four quinolones tested) and oritavancin were bactericidal toward intracellular bacteria (and the level of activity was in that order) but had to be used at concentrations close to or equal to their  $C_{max}$ s to achieve such an effect. There was, again, no simple correlation between intracellular bactericidal effects and the MBC/MIC ratios or the levels of cellular accumulation (as measured in uninfected cells).

**Wide range of concentration-effect relationships (pharmacological comparisons).** Four molecules (oxacillin, gentamicin, moxifloxacin, and oritavancin) were selected to obtain full pharmacological dose-response curves based on (i) their demonstrated dose-effect relationships in the MIC- $C_{max}$  range and (ii) their contrasting behaviors with respect to their intracellu-

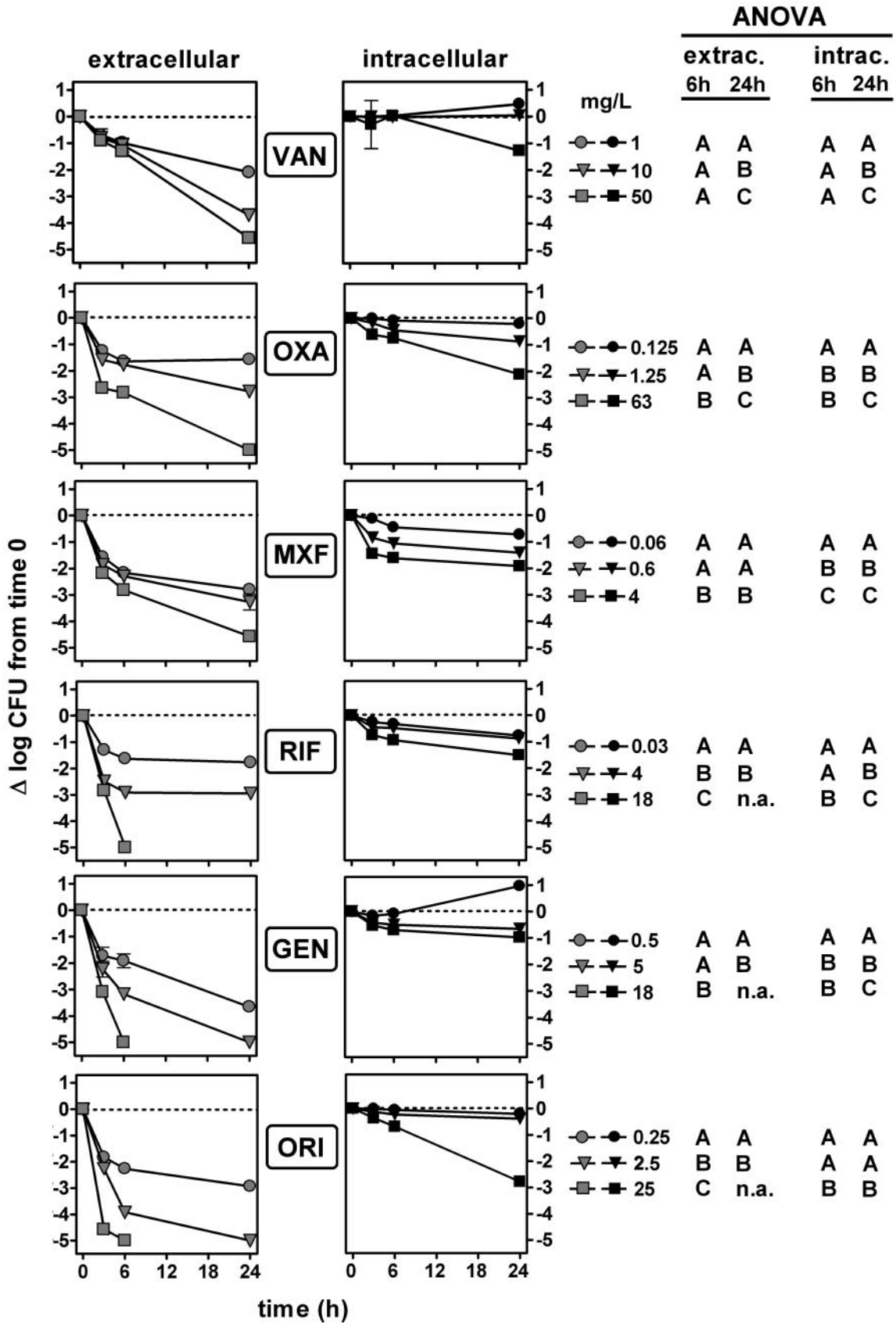
lar activity/extracellular activity ratios. Figure 5 shows the results, with the regression parameters and a detailed statistical analysis presented in Table 3. Against extracellular bacteria, all four drugs displayed similar relative potencies (50% effective concentrations [ $EC_{50}$ s]) (53) and static concentrations at about their MICs and 0.3 their MICs, respectively. Their relative efficacies (maximum effects [ $E_{max}$ s]), however, were significantly different (oxacillin < moxifloxacin < oritavancin  $\approx$  gentamicin). Against intracellular bacteria, all four drugs had significant decreases in their relative efficacies ( $E_{max}$ ), but these decreases were roughly similar ( $E_{max}$  against intracellular bacteria/ $E_{max}$  against extracellular bacteria ratios, 0.42 [minimum] to 0.64 [maximum]; because we could not reliably assess inoculum decreases larger than 4.2 log and arbitrarily set all larger values to 5, these ratios may actually be overestimated for highly bactericidal antibiotics such as oritavancin and gentamicin). In contrast, the relative potencies ( $EC_{50}$ s) were very differentially affected, with oxacillin and moxifloxacin showing no significant change compared to their corresponding potencies against extracellular bacteria, whereas marked decreases (9- to 14-fold) in potency (indicated by an increase in  $EC_{50}$ ) were noted for gentamicin and oritavancin. This partially translated into an increase in the static concentrations of about 2-, 4-, 7-, and 17-fold for moxifloxacin, oxacillin, gentamicin, and oritavancin, respectively.

**Morphological studies.** Electron microscopy (Fig. 6) was used to examine the morphological changes of phagocytosed *S. aureus* after exposure to the antibiotics in order to ascertain that the decreases in the numbers of CFU seen in our experiments were associated with visible changes in the number and/or morphology of the bacteria. Oxacillin and oritavancin were selected for use in this study, since both are reported to act on cell wall biosynthesis and/or the cell wall structure, making their potential action on the bacteria more easily recognizable. In the absence of these antibiotics (but in the presence of gentamicin at the MIC, to fully avoid extracellular contamination), phagocytosed bacteria were darkly stained (Fig. 6A), often actively multiplying, and surrounded by a thick cell wall (Fig. B and C). In cells incubated with oxacillin, a large number of intracellular bacteria appeared as ghosts with a rarefied cytoplasmic material (Fig. 6D and E) or with large, electron-lucent vacuoles (Fig. 6F). Ghosts were also commonly observed in infected cells incubated with oritavancin (Fig. 6G), with profiles often showing granular material sometimes apposed on the periphery of the bacterial body (Fig. 6H).

## DISCUSSION

The data presented in this paper underline three main properties of antibiotics in relation to their intracellular activities that may not have been sufficiently detected in previous studies because of an insufficient duration of exposure and the investigation of a limited range of concentrations. The model used here has specifically tried to address these issues and has been validated to exclude the significant contribution of extracellular growth within the limits of the experimental setup.

A first and unanticipated property is that all classes of antibiotics tested, with the exception of the macrolides, showed significant intracellular killing when their extracellular concentration was brought to a sufficiently high level and the time of



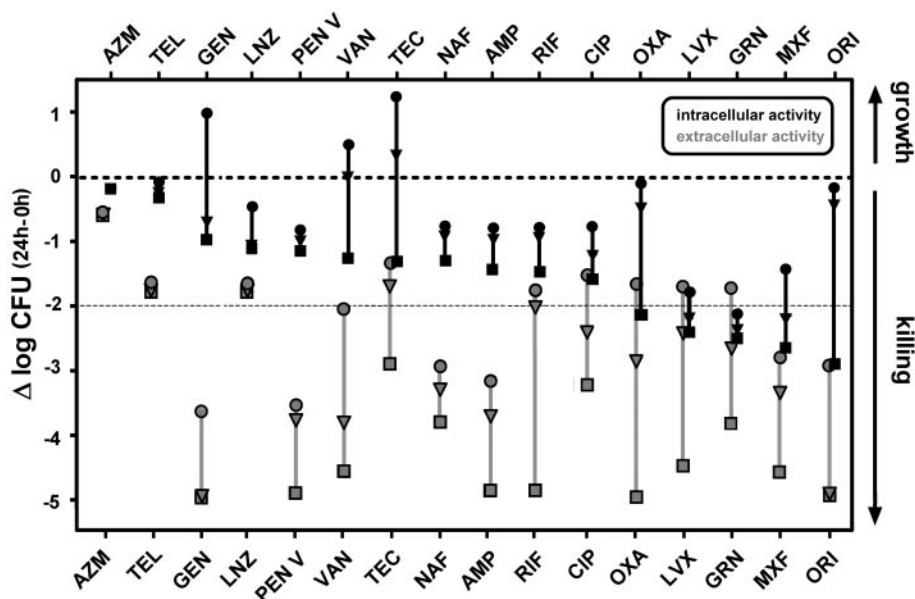


FIG. 4. Influence of concentration on the extent of antibiotic activity against extracellular and intracellular *S. aureus*. The graphs show the change in the number of CFU ( $\Delta \log \text{CFU}$ ; means  $\pm$  SDs;  $n = 3$ ; most SD bars are smaller than the symbols) per ml of culture medium (extracellular) or in THP-1 macrophages (intracellular) per mg of cell protein. Each antibiotic was added at concentrations corresponding to its MIC (circles; 4 times the MIC for rifampin), 10 times its MIC (triangles; 530 times the MIC for rifampin), or its  $C_{\text{max}}$  in humans (squares; total drug [Table 1]). Thick dotted line, static effect; thin dotted line,  $-2$ -log change (bactericidal effect, as defined by the Clinical and Laboratory Standards Institute for bacteria growing in broth). AZM, azithromycin; TEL, telithromycin; GEN, gentamicin; LNZ, linezolid; PEN V, penicillin V; VAN, vancomycin; TEC, teicoplanin; NAF, nafcillin; AMP, ampicillin; RIF, rifampin; CIP, ciprofloxacin; OXA, oxacillin; LVX, levofloxacin; GRN, garenoxacin; MXF, moxifloxacin; ORI, oritavancin.

exposure was prolonged to 24 h. For two molecules at least (oxacillin and oritavancin), we could show that the decreases in cell-associated CFU are accompanied by evidence of severe morphological alterations of the intracellular bacteria, consistent with their known modes of action (3, 25), indicating true intracellular expression of drug-related activity. This property is actually the direct consequence of two factors. The first is that all antibiotics studied here, with the exception of the macrolides, show concentration-dependent effects (for the four molecules tested in detail, we even observed typical pharmacological dose-response curves with the classical basic properties of threshold, slope, and maximal effects upon increasing concentration [53], irrespective of their specific modes of action). This definitely helps to provide an understanding of why contradictory results are reported when only narrow ranges of extracellular concentrations are explored. The second factor, which is perhaps as critical as the first one, is that all drugs, with the exception of the macrolides and, surprisingly, rifampin, showed time-dependent effects when they were tested at low multiples of their MICs. Both concentration and time

therefore appear to modulate the final response and need to be taken into account when results from different models are compared. We know that this first part of our conclusion may appear to be at variance with what has been drawn from previous studies of the pharmacodynamics of antibiotics, namely, that the activities of some drugs (most notably, the  $\beta$ -lactams) are predominantly time dependent, whereas the activities of others (most notably, the aminoglycosides and the fluoroquinolones) are mainly concentration dependent (15, 16). Our observations being what they are, we suggest that the way that the drugs appear and can be differentiated from one another in most models essentially depends on two factors, namely, (i) the value of the  $E_{\text{max}}$  parameter of the pharmacological response (maximal activity) and the concentrations at which effects approaching  $E_{\text{max}}$  are obtained and (ii) the size (how large) of the concentration range examined. (The  $E_{\text{max}}$  values shown in Table 3 are negative numbers, since they pertain to decreases in bacterial counts. Greater activity is, therefore, strictly speaking, associated with a smaller  $E_{\text{max}}$ . Since this is rather counterintuitive, we use the term “maximal activity” throughout this

FIG. 3. Influence of concentration on the rate and the extent of the activities of antibiotics against extracellular and intracellular *S. aureus*. The graphs show the change in the number of CFU ( $\Delta \log \text{CFU}$ ; means  $\pm$  SDs;  $n = 3$ ; most SD bars are smaller than the symbols) per ml of culture medium (extracellular) or in THP-1 macrophages per mg of cell protein (intracellular). Molecules are ordered by increasing bactericidal potential, as determined by their MIC/MBC ratios in broth (Table 1). Except for rifampin, each antibiotic was tested at three increasing concentrations corresponding to its MIC (circles), 10 times the MIC (triangles), and the  $C_{\text{max}}$  in humans (squares; total drug); rifampin was used at 4 (circles) and 530 (triangles) times the MIC and at the  $C_{\text{max}}$  in humans (squares; total drug). Dotted line, static effect. For analysis of variance (ANOVA), the same letter indicates no statistically significant difference between values; different letters indicate a  $P$  value  $< 0.05$ . n.a., not applicable (below the detection level); extrac., extracellular; intrac., intracellular; VAN, vancomycin; OXA, oxacillin; MXF, moxifloxacin; RIF, rifampin; GEN, gentamicin; ORI, oritavancin.

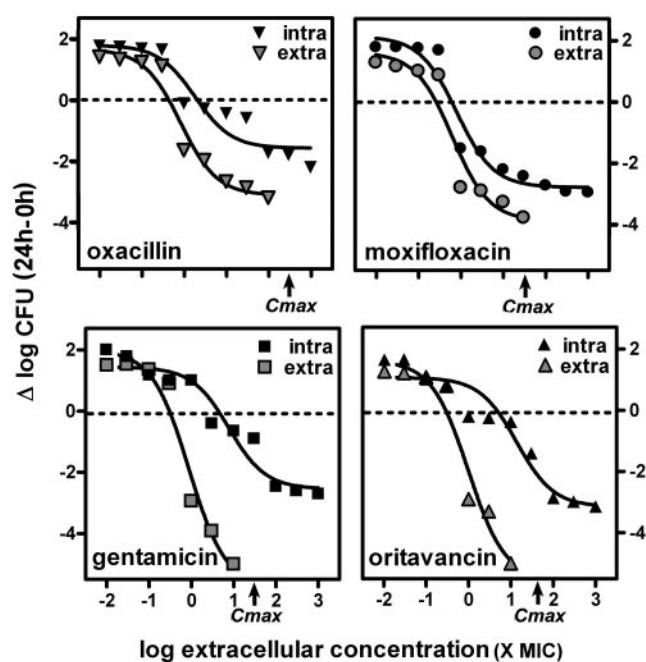


FIG. 5. Dose-response curves of four selected antibiotics against extracellular (extra) and intracellular (intra) *S. aureus*. The graphs show the change in the number of CFU ( $\Delta \log \text{CFU}$ ; means  $\pm$  SD;  $n = 3$ ; most SD bars are smaller than the symbols) per ml of culture medium (extracellular; closed gray symbols) or in THP-1 macrophages (intracellular; closed black symbols) per mg of cell protein. Dotted line, static effect. The sigmoidal function was used (Hill coefficient = 1 [42]); goodness of fit and regression parameters are shown in Table 3). Vertical arrow,  $C_{\text{max}}$  in humans (total drug; Table 1).

discussion.) This explains why the activities of some drugs may have mainly been considered concentration dependent if they were examined within a concentration range that is close to their  $\text{EC}_{50}$ , whereas the activities of others, tested over a higher concentration range, are essentially reported as time dependent. In Fig. 5 we show where the  $C_{\text{max}}$  of each drug in humans would fall on the abscissa to help delineate what could be the microbiologically and clinically meaningful range of concentrations to be considered ( $\text{MIC}-C_{\text{max}}$ ). In this context, gentamicin and oritavancin, which have higher maximal activities than oxacillin against extracellular bacteria within this  $\text{MIC}-C_{\text{max}}$  range, will be expected to be markedly influenced by the concentration within that range. Conversely, oxacillin, the  $C_{\text{max}}$  of which (as defined in Table 1) is much larger than its MIC and which has a weaker maximal effect, will be expected to be less influenced by the concentration, which in turn will make time a more predominant parameter. Moxifloxacin has an intermediate behavior, with its activity being largely concentration dependent when it is evaluated with concentrations close to its MIC but with its activity becoming less concentration dependent when it is tested at concentrations close to its reported  $C_{\text{max}}$  in humans. Since the maximal activities against intracellular *S. aureus* are systematically lower than those against extracellular bacteria, the impact of the concentration on bacterial survival is accordingly less marked, which makes gentamicin and oritavancin behave more like oxacillin within the  $\text{MIC}-C_{\text{max}}$  range of extracellular concentrations.

This will increase the impact of the time during which the bacteria are exposed to the antibiotic and suggests that the activities of all these antibiotics actually appear to be mainly time dependent. Extrapolation of our data for the categorization of the activities of the drugs as concentration or time dependent in vivo cannot, however, be done without caution. A first uncertainty relates to the effective availability of the antibiotics in blood and extracellular fluids, which can be severely impaired by binding to proteins or other biological constituents. As a help to the reader, however, we have provided in Table 1 an estimation of the percentage of free drug in human serum for each  $C_{\text{max}}$  used in our study. If it is assumed that it is only the free drug that drives activity, one could surmise that the clinically meaningful concentration range of antibiotics that are highly protein bound will shift toward lower values, making the activities of most of them more and more concentration dependent as their effective concentrations approach the  $\text{EC}_{50}$ s. Unfortunately, the model used here does not easily lend itself to a pertinent evaluation of even this simple effect of serum protein binding, because (i) the serum concentration is low, resulting in only weak and limited binding of antibiotics that are usually reported to be highly protein bound (36); (ii) this concentration cannot be markedly changed without causing cell death, thereby preventing most concentration-effect studies; and (iii) the serum is of bovine and not human origin. A second uncertainty is whether the results obtained with a constant concentration over a 24-h period are predictive of what may be observed in vivo with fluctuating concentrations, as will be the case unless drugs are administered by continuous infusion. This will need to be specifically addressed in future studies. However, recent data from a study examining the pharmacodynamics of erythromycin against intracellular *Legionella pneumophila* by the use of both static and kinetic models failed to reveal significant differences in behavior related to the type of exposure (60).

A second property that appears from the comparative analysis of the dose-effect is that intracellular activities consistently remain lower than the extracellular ones, whether one considers what can be obtained at any given extracellular concentration or the maximal achievable effects (the  $E_{\text{max}}$  parameter; because we could not reliably assess inoculum decreases greater than 4.2 log, the intracellular  $E_{\text{max}}$ /extracellular  $E_{\text{max}}$  ratios observed for drugs highly bactericidal toward extracellular bacteria may actually be underestimated). This property was seen for all molecules studied and is probably more related to bacterial or cellular parameters than to drug pharmacodynamic or pharmacokinetic ones. The present study offers no insight into the underlying mechanism. However, we know that *S. aureus* cells phagocytosed by macrophages sojourn and thrive in phagolysosomes (35, 52). We may reasonably suggest that the metabolic changes triggered by the exposure of bacteria to this specific environment and to an acid pH in particular (46) could play a critical role (69). Alternatively, it is possible that those bacteria that apparently remain insensitive to antibiotics are physically protected from direct contact with the drugs. These hypotheses need to be addressed in future work but may face the difficulty of the specific analysis of what may concern only a small, albeit significant, part of the original inoculum. Thus, we could not directly examine the role of the so-called small-colony variants, which have been linked to per-



TABLE 3. Pertinent regression parameters<sup>a</sup> (with confidence intervals) and statistical analysis of the dose-response curves illustrated in Fig. 5

Antibiotic <sup>b</sup>	Extracellular <sup>c</sup>				Intracellular <sup>d</sup>				<i>P</i> value <sup>e</sup>
	$E_{\max}^f$ (CI) <sup>g</sup>	EC <sub>50</sub> <sup>h</sup> (CI)	$C_{\text{static}}^i$	$R^2$	$E_{\max}$ (CI)	EC <sub>50</sub> (CI)	$C_{\text{static}}$	$R^2$	
Oxacillin	-3.13 (-3.81 to -2.44)a;A	0.84 (0.34 to 2.04)a;A	0.45	0.966	-1.58 (-2.20 to -0.96)a;B	1.85 (0.51 to 6.53)a;A	2.09	0.909	<0.001
Moxifloxacin	-3.86 (-5.22 to -2.51)b;A	0.64 (0.16 to 2.56)a;A	0.27	0.936	-2.77 (-3.31 to -2.22)b;B	0.81 (0.34 to 1.9)1b;A	0.63	0.956	<0.001
Gentamicin	-5.76 (-7.89 to -3.62)c;B	0.88 (0.28 to 2.78)a;A	0.30	0.969	-2.54 (-3.22 to -1.86)b;B	7.73 (2.86 to 20.9)c;B	2.09	0.943	<0.001
Oritavancin	-5.55 (-8.03 to -3.07)c;B	1.00 (0.25 to 4.0)1a;A	0.29	0.956	-3.15 (-4.04 to -2.57)c;B	13.77 (4.48 to 42.4)c;B	4.79	0.927	<0.001

<sup>a</sup> By use of all data points from antibiotic concentrations of 0.01 to 1,000 times the MIC. Data for samples without antibiotics were not used since there was evidence of an overestimation of the true value of the intracellular counts when the extracellular concentration of antibiotic was lower than 0.01 the MIC (see Fig. 1). Statistical analyses were performed as follows: analysis per column (one-way analysis of variance by the Tukey test for multiple comparisons between each parameter for all drugs), data with different lowercase letters are significantly different from each other ( $P < 0.05$ ); analysis per row (unpaired, two-tailed  $t$  test between corresponding parameters of extracellular and intracellular activities), data with different uppercase letters are significantly different from each other ( $P < 0.05$ ); global analysis (analysis of covariance) by the Tukey test for multiple comparisons, for extracellular versus intracellular concentrations, the curves for each antibiotic are compared between these two conditions; for global analysis between drugs, all four curves of extracellular or intracellular activities are compared between drugs.

<sup>b</sup> By analysis of covariance, there was no significant difference between drugs.

<sup>c</sup> In complete cell culture medium where the original inoculum (time zero) was  $1.01 \pm 0.20 \times 10^6$  CFU/ml ( $n = 3$ ).

<sup>d</sup> Original (postphagocytosis) inoculum (time zero),  $1.91 \pm 0.18 \times 10^6$  CFU/mg protein ( $n = 3$ ).

<sup>e</sup>  $P$  values were determined by analysis of covariance and are for the extracellular concentration versus the intracellular concentration.

<sup>f</sup> CFU decrease (in log<sub>10</sub> units) at 24 h from the corresponding original inoculum, as extrapolated for the antibiotic concentration at infinity; counts of less than 3 colonies/dish were considered below the detection level.

<sup>g</sup> CI, confidence interval.

<sup>h</sup> Concentration (in multiples of the MIC) causing a reduction of the inoculum half-way between the initial ( $E_0$ ) and the maximal ( $E_{\max}$ ) values, as obtained from the Hill equation (by using a slope factor of 1; SD of log EC<sub>50</sub> values, 0.163 [minimum] to 0.241 [maximum]).

<sup>i</sup> Concentration (in multiples of the MIC) resulting in no apparent bacterial growth (the number of CFU was identical to that in the original inoculum), as determined by graphical interpolation.

sistent and relapsing infections (51), since we failed to detect them in significant numbers in our experimental conditions. Likewise, it may prove difficult to determine to what extent a small subpopulation of all intracellular bacteria are sojourning in poorly accessible compartments.

A third property, and probably the most critical one, to be

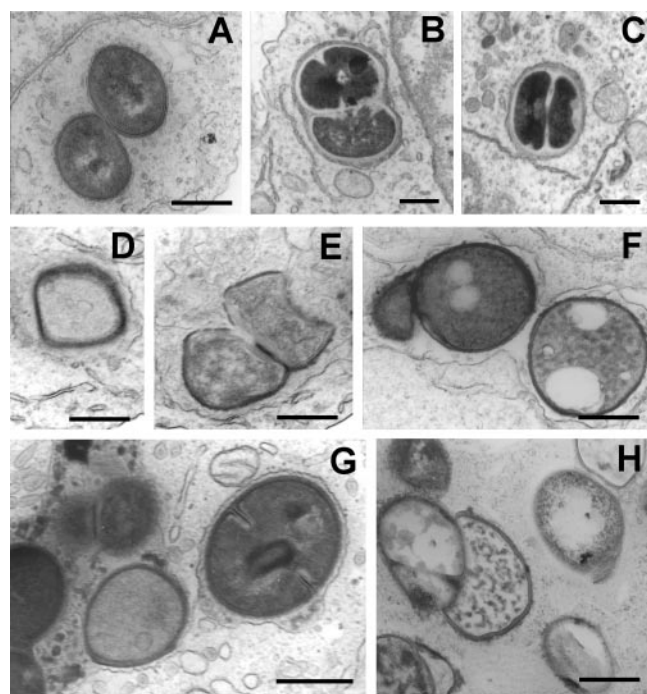


FIG. 6. Electron microscopic appearance of serum-opsonated *S. aureus* in THP-1 macrophages 24 h after phagocytosis. (A to C) Control cells (gentamicin was added at its MIC to prevent extracellular growth); (D to F) cells exposed to oxacillin; (G and H) cells exposed to oritavancin (both at their  $C_{\max}$ s in humans [total drug; Table 1]). Bars, 0.5  $\mu$ m.

considered in drug selection is the fact that the relative potencies (as measured by the EC<sub>50</sub> parameter) of some molecules are markedly decreased against intracellular bacteria compared with those against extracellular bacteria. Gentamicin and oritavancin appear to be the most affected, even though both drugs primarily concentrate in lysosomes and related vacuoles (62, 64), where *S. aureus* is thought to localize. These vacuoles are acidic, which will markedly decrease the activities of aminoglycosides (as is well known and which has been confirmed here for the strain of *S. aureus* used). In this context, it is interesting that alkalization of lysosomes has been associated with improved intracellular activities of aminoglycosides (38). Yet, the activity of oritavancin is unaffected by acidity (as shown in a previous publication [64] and confirmed here), which indicates that effects other than pH, such as binding to intralysosomal constituents, need to be taken into consideration. In a broader context, a lack of true bioavailability and the defeating effect of the local physicochemical conditions on activity probably explain why cell accumulation per se is not necessarily predictive of intracellular efficacy for most antibiotics. This even appears to be the case for drugs with apparent large bioavailabilities, such as the fluoroquinolones. Indeed, fluoroquinolones show considerably less activity than is anticipated from their level of cellular accumulation, as demonstrated here and in other recent studies (2, 48, 56). Macrolides may also suffer from the same effects, but their bacteriostatic character is probably the most critical determinant in their lack of an intracellular killing effect. Conversely, the bactericidal effects of  $\beta$ -lactams against intracellular *S. aureus* when these compounds are used at large extracellular concentrations, as seen here for oxacillin and in previous studies with ampicillin and meropenem (36), not only could be due to the fact that these drugs may reach intracellular concentrations that eventually reach far above their MICs but could also be due to the production of cellular factors that enhance their activities (37, 45, 67).

Our results with linezolid and rifampin require attention,

since both drugs are usually recommended for the treatment of difficult-to-treat staphylococcal infections, but they failed to demonstrate significant intracellular bactericidal effects in our study. This observation is actually not surprising for linezolid, which is essentially bacteriostatic and which does not accumulate in macrophages. Conversely, the weak intracellular activity of rifampin, also seen in murine macrophages (57), was more puzzling since its activity is concentration dependent and its MIC was one of the lowest among those of all drugs tested, especially at acidic pH. A key factor here could be that the activity of rifampin, while it is marked after 3 to 6 h, does not progress over time thereafter, showing the importance of taking this parameter into account when different antibiotics are compared.

The present study used only one strain of fully susceptible *S. aureus*, which may be considered a major limitation for extrapolation of the findings of this study to clinical situations. Actually, the strain studied here has been widely used for the evaluation of the in vitro activities of new antibiotics in broth (61) as well as in phagocytes (24). The choice of a unique, well-characterized strain was actually essential for addressing the question of antibiotic intracellular activity per se and avoiding the blurring of the results because of other factors that can modulate the intracellular response to antibiotics, such as virulence and variations in the expression of resistance mechanisms. Given this caveat and pending further studies with clinical strains, the data presented in this paper may provide unambiguous pharmacological support to the use of new quinolones (7, 17) or oritavancin (40), as an alternative to  $\beta$ -lactams (68), for the treatment of recurrent *S. aureus* infections, provided that sufficient extracellular concentration/MIC ratios are obtained for a sufficient period of time. These conditions may not be obtainable for more toxic drugs such as aminoglycosides or conventional glycopeptides and will not be met with bacteriostatic antibiotics. We also suggest that in vitro models are useful for the appropriate design of animal and clinical studies aimed at evaluating the efficacies of antibiotics against intracellular pathogens, provided that they are made as relevant to the in vivo situation as possible in terms of the drug concentration and the duration of exposure.

#### ACKNOWLEDGMENTS

We thank F. Renoird and M. C. Cambier for expert technical assistance. We thank all the manufacturers for their kind gifts of the corresponding antibiotics.

C.S. was a postdoctoral fellow and F.V.B. is a chercheur qualifié of the Belgian Fonds National de la Recherche Scientifique. This work was supported by the Belgian Federal Science Policy Office (research project P5/33; research action P5); the Belgian Fonds de la Recherche Scientifique Médicale (grants 3.4549.00 and 9.4.549.04 F) and grants-in-aids from Eli Lilly Benelux s.a., Brussels, Belgium; Pfizer s.a., Brussels, Belgium; and Bristol-Myers Squibb Company, Wallingford, CT.

#### REFERENCES

- Alexander, E. H., and M. C. Hudson. 2001. Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. *Appl. Microbiol. Biotechnol.* **56**:361–366.
- Al Nawas, B., and P. M. Shah. 1998. Intracellular activity of ciprofloxacin and moxifloxacin, a new 8-methoxyquinolone, against methicillin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **41**:655–658.
- Allen, N. E., and T. I. Nicas. 2003. Mechanism of action of oritavancin and related glycopeptide antibiotics. *FEMS Microbiol. Rev.* **26**:511–532.
- Amsden, G. W., and J. J. Schentag. 1995. Tables of antimicrobial agent pharmacology, p. 492–529. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, New York, N.Y.
- Anonymous. 2005. Zyvox US full prescribing information. [http://www.pfizer.com/pfizer/download/uspi\\_zyvox.pdf](http://www.pfizer.com/pfizer/download/uspi_zyvox.pdf). Pharmacia & Upjohn, Division of Pfizer Inc, New York, N.Y. (last updated, May 2005; last accessed, 18 July 2005).
- Auwerx, J. 1991. The human leukemia cell line, THP-1: a multifaceted model for the study of monocyte-macrophage differentiation. *Experientia* **47**:22–31.
- Berrington, A. W., R. J. Koerner, J. D. Perry, H. H. Bain, and F. K. Gould. 2001. Treatment of *Staphylococcus aureus* endocarditis using moxifloxacin. *Int. J. Med. Microbiol.* **291**:237–239.
- Bishayi, B., and M. Sengupta. 2003. Intracellular survival of *Staphylococcus aureus* due to alteration of cellular activity in arsenic and lead intoxicated mature Swiss albino mice. *Toxicology* **184**:31–39.
- Braun, D. K., J. K. Chien, D. S. Farlow, D. L. Phillips, M. W. Wasilewski, and M. L. Zeckel. 2001. Oritavancin (LY333328): a dose-escalation safety and pharmacokinetics study in patients. *Clin. Microbiol. Infect.* **7**:P434.
- Brouillette, E., G. Grondin, L. Shkreta, P. Lacasse, and B. G. Talbot. 2003. In vivo and in vitro demonstration that *Staphylococcus aureus* is an intracellular pathogen in the presence or absence of fibronectin-binding proteins. *Microb. Pathog.* **35**:159–168.
- Carryn, S., H. Chanteux, C. Seral, M. P. Mingeot-Leclercq, F. Van Bambeke, and P. M. Tulkens. 2003. Intracellular pharmacodynamics of antibiotics. *Infect. Dis. Clin. N. Am.* **17**:615–634.
- Carryn, S., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens. 2002. Comparative intracellular (THP-1 macrophage) and extracellular activities of beta-lactams, azithromycin, gentamicin, and fluoroquinolones against *Listeria monocytogenes* at clinically relevant concentrations. *Antimicrob. Agents Chemother.* **46**:2095–2103.
- Carryn, S., F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens. 2003. Activity of beta-lactams (ampicillin, meropenem), gentamicin, azithromycin and moxifloxacin against intracellular *Listeria monocytogenes* in a 24 h THP-1 human macrophage model. *J. Antimicrob. Chemother.* **51**:1051–1052.
- Carryn, S., S. Van de Velde, F. Van Bambeke, M. P. Mingeot-Leclercq, and P. M. Tulkens. 2004. Impairment of growth of *Listeria monocytogenes* in THP-1 macrophages by granulocyte macrophage colony-stimulating factor: release of tumor necrosis factor-alpha and nitric oxide. *J. Infect. Dis.* **189**:2101–2109.
- Craig, W. A. 2003. Basic pharmacodynamics of antibacterials with clinical applications to the use of beta-lactams, glycopeptides, and linezolid. *Infect. Dis. Clin. N. Am.* **17**:479–501.
- Craig, W. A., and S. C. Ebert. 1990. Killing and regrowth of bacteria in vitro: a review. *Scand. J. Infect. Dis. Suppl.* **74**:63–70.
- Eckart, R. E., D. R. Hoshenthal, and J. T. Fishbain. 2000. Response of complicated methicillin-resistant *Staphylococcus aureus* endocarditis to the addition of trovafloxacin. *Pharmacotherapy* **20**:589–592.
- Ellington, J. K., M. Harris, L. Webb, B. Smith, T. Smith, K. Tan, and M. Hudson. 2003. Intracellular *Staphylococcus aureus*. A mechanism for the indolence of osteomyelitis. *J. Bone Joint Surg. Br.* **85**:918–921.
- Fan, H., G. Teti, S. Ashton, K. Guyton, G. E. Tempel, P. V. Halushka, and J. A. Cook. 2003. Involvement of G(i) proteins and Src tyrosine kinase in TNFalpha production induced by lipopolysaccharide, group B streptococci and *Staphylococcus aureus*. *Cytokine* **22**:126–133.
- Feketi, R. 2000. Vancomycin, teicoplanin, and the streptogramins: quinupristin and dalbapristin, p. 382–392. *In* G. E. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, Philadelphia, Pa.
- Fish, D. N., and A. T. Chow. 1997. The clinical pharmacokinetics of levofloxacin. *Clin. Pharmacokinet.* **32**:101–119.
- Foulds, G., R. M. Shepard, and R. B. Johnson. 1990. The pharmacokinetics of azithromycin in human serum and tissues. *J. Antimicrob. Chemother.* **25**(Suppl. A):73–82.
- Gajjar, D. A., A. Bello, Z. Ge, L. Christopher, and D. M. Grasela. 2003. Multiple-dose safety and pharmacokinetics of oral garenoxacin in healthy subjects. *Antimicrob. Agents Chemother.* **47**:2256–2263.
- Garcia, I., A. Pascual, S. Ballesta, C. del Castillo, and E. J. Perea. 2003. Accumulation and activity of cethromycin (ABT-773) within human polymorphonuclear leucocytes. *J. Antimicrob. Chemother.* **52**:24–28.
- Giesbrecht, P., T. Kersten, H. Maidhof, and J. Wecke. 1998. Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin. *Microbiol. Mol. Biol. Rev.* **62**:1371–1414.
- Gilbert, D. N. 2000. Aminoglycosides, p. 307–336. *In* G. E. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, Philadelphia, Pa.
- Glew, R. H., and R. C. Moellering, Jr. 1979. Effect of protein binding on the activity of penicillins in combination with gentamicin against enterococci. *Antimicrob. Agents Chemother.* **15**:87–92.
- Gotoh, M., Y. Takamoto, K. Kurosaka, J. Masuda, M. Ida, A. Satoh, E. Takayama, K. Kojima-Aikawa, Y. Kobayashi, and I. Matsumoto. 2005. An-

- nexins I and IV inhibit *Staphylococcus aureus* attachment to human macrophages. *Immunol. Lett.* **98**:297–302.
29. **Grosset, J., L. Chauvelot-Moachon, and J. P. Giroud.** 1988. Antituberculeux, p. 1553–1573. *In* J. P. Giroud, G. Mathé, and G. Meyniel (ed.), *Pharmacologie clinique, bases de la thérapeutique*. Expansion Scientifique Française, Paris, France.
  30. **Hebert, A., K. Sayasith, S. Senechal, P. Dubreuil, and J. Lagace.** 2000. Demonstration of intracellular *Staphylococcus aureus* in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. *FEMS Microbiol. Lett.* **193**:57–62.
  31. **Hess, D. J., M. J. Henry-Stanley, E. A. Erickson, and C. L. Wells.** 2003. Intracellular survival of *Staphylococcus aureus* within cultured enterocytes. *J. Surg. Res.* **114**:42–49.
  32. **Hoffken, G., H. Lode, C. Prinzing, K. Borner, and P. Koeppe.** 1985. Pharmacokinetics of ciprofloxacin after oral and parenteral administration. *Antimicrob. Agents Chemother.* **27**:375–379.
  33. **Israel, D., J. G. Gillum, M. Turik, K. Harvey, J. Ford, H. Dalton, M. Towle, R. Echols, A. H. Heller, and R. Polk.** 1993. Pharmacokinetics and serum bactericidal titers of ciprofloxacin and ofloxacin following multiple oral doses in healthy volunteers. *Antimicrob. Agents Chemother.* **37**:2193–2199.
  34. **Jett, B. D., and M. S. Gilmore.** 2002. Host-parasite interactions in *Staphylococcus aureus* keratitis. *DNA Cell Biol.* **21**:397–404.
  35. **Kapral, F. A., and M. G. Shayegani.** 1959. Intracellular survival of staphylococci. *J. Exp. Med.* **110**:123–128.
  36. **Lemaire, S., F. Van Bambeke, M. P. Minget-Leclercq, and P. M. Tulkens.** 2005. Activity of three  $\beta$ -lactams (ertapenem, meropenem and ampicillin) against intraphagocytic *Listeria monocytogenes* and *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **55**:897–904.
  37. **Matsumoto, Y., A. Ikemoto, Y. Wakai, F. Ikeda, S. Tawara, and K. Matsumoto.** 2001. Mechanism of therapeutic effectiveness of cefixime against typhoid fever. *Antimicrob. Agents Chemother.* **45**:2450–2454.
  38. **Maurin, M., and D. Raoult.** 1994. Phagolysosomal alkalization and intracellular killing of *Staphylococcus aureus* by amikacin. *J. Infect. Dis.* **169**:330–336.
  39. **Mempel, M., C. Schnopp, M. Hojka, H. Fesq, S. Weidinger, M. Schaller, H. C. Korting, J. Ring, and D. Abeck.** 2002. Invasion of human keratinocytes by *Staphylococcus aureus* and intracellular bacterial persistence represent haemolysin-independent virulence mechanisms that are followed by features of necrotic and apoptotic keratinocyte cell death. *Br. J. Dermatol.* **146**:943–951.
  40. **Mercier, R. C., and L. Hrebickova.** 2005. Oritavancin: a new avenue for resistant gram-positive bacteria. *Expert Rev. Anti. Infect. Ther.* **3**:325–332.
  41. **Michot, J. M., C. Seral, F. Van Bambeke, M. P. Minget-Leclercq, and P. M. Tulkens.** 2005. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. *Antimicrob. Agents Chemother.* **49**:2429–2437.
  42. **Motulsky, H. J.** 2005. Analyzing data with GraphPad Prism. <http://www.graphpad.com>. GraphPad Software Inc., San Diego, Calif. (last updated, 1999; last accessed, 2 August 2005).
  43. **Myers, D. R., J. DeFehr, W. M. Bennet, G. A. Porter, and G. D. Olsen.** 1978. Gentamicin binding to serum and plasma proteins. *Clin. Pharmacol. Ther.* **23**:356–360.
  44. **Namour, F., D. H. Wessels, M. H. Pascual, D. Reynolds, E. Sultan, and B. Lenfant.** 2001. Pharmacokinetics of the new ketolide telithromycin (HMR 3647) administered in ascending single and multiple doses. *Antimicrob. Agents Chemother.* **45**:170–175.
  45. **Nielsen, S. L., and F. T. Black.** 1998. The effect of dicloxacillin and fusidic acid on the extracellular and intracellular killing of *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **42**:221–226.
  46. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA* **75**:3327–3331.
  47. **Ouadrhiri, Y., B. Scoreaux, Y. Sibille, and P. M. Tulkens.** 1999. Mechanism of the intracellular killing and modulation of antibiotic susceptibility of *Listeria monocytogenes* in THP-1 macrophages activated by gamma interferon. *Antimicrob. Agents Chemother.* **43**:1242–1251.
  48. **Paillard, D., J. Grellet, V. Dubois, M. C. Saux, and C. Quentin.** 2002. Discrepancy between uptake and intracellular activity of moxifloxacin in a *Staphylococcus aureus*-human THP-1 monocytic cell model. *Antimicrob. Agents Chemother.* **46**:288–293.
  49. **Peck, O. M., H. Fan, G. E. Tempel, G. Teti, P. V. Halushka, and J. A. Cook.** 2004. *Staphylococcus aureus* and lipopolysaccharide induce homologous tolerance but heterologous priming: role of interferon-gamma. *Shock* **21**:254–260.
  50. **Phillippon, A., G. Paul, F. Brunet, and J. P. Giroud.** 1988. Pénicillines, p. 1295–1329. *In* J. P. Giroud, G. Mathé, and G. Meyniel (ed.), *Pharmacologie clinique, bases de la thérapeutique*. Expansion Scientifique Française, Paris, France.
  51. **Proctor, R. A., J. M. Balwit, and O. Vesga.** 1994. Variant subpopulations of *Staphylococcus aureus* as cause of persistent and recurrent infections. *Infect. Agents Dis.* **3**:302–312.
  52. **Rogers, D. E., and R. Tompsett.** 1952. The survival of staphylococci within human leukocytes. *J. Exp. Med.* **95**:209–230.
  53. **Ross, E. M., and T. Kenakin.** 2001. Pharmacodynamics: mechanism of drug action and the relationship between drug concentration and effect, p. 31–43. *In* J. Hardman and L. E. Limbird (ed.), *Goodman & Gilman's the pharmacological basis of therapeutics*. McGraw-Hill Medical Publishing Division, New York, N.Y.
  54. **Schwab, J. C., and G. L. Mandell.** 1989. The importance of penetration of antimicrobial agents into cells. *Infect. Dis. Clin. N. Am.* **3**:461–467.
  55. **Scoreaux, B., Y. Ouadrhiri, G. Anzalone, and P. M. Tulkens.** 1996. Effect of recombinant human gamma interferon on intracellular activities of antibiotics against *Listeria monocytogenes* in the human macrophage cell line THP-1. *Antimicrob. Agents Chemother.* **40**:1225–1230.
  56. **Seral, C., M. Barcia-Macay, M. P. Minget-Leclercq, P. M. Tulkens, and F. Van Bambeke.** 2005. Comparative activity of quinolones (ciprofloxacin, levofloxacin, moxifloxacin and garenoxacin) against extracellular and intracellular infection by *Listeria monocytogenes* and *Staphylococcus aureus* in J774 macrophages. *J. Antimicrob. Chemother.* **55**:511–517.
  57. **Seral, C., F. Van Bambeke, and P. M. Tulkens.** 2003. Quantitative analysis of gentamicin, azithromycin, telithromycin, ciprofloxacin, moxifloxacin, and oritavancin (LY333328) activities against intracellular *Staphylococcus aureus* in mouse J774 macrophages. *Antimicrob. Agents Chemother.* **47**:2283–2292.
  58. **Stass, H., A. Dalhoff, D. Kubitz, and U. Schuhly.** 1998. Pharmacokinetics, safety, and tolerability of ascending single doses of moxifloxacin, a new 8-methoxy quinolone, administered to healthy subjects. *Antimicrob. Agents Chemother.* **42**:2060–2065.
  59. **Sullivan, J. T., M. Woodruff, J. Lettieri, V. Agarwal, G. J. Krol, P. T. Leese, S. Watson, and A. H. Heller.** 1999. Pharmacokinetics of a once-daily oral dose of moxifloxacin (Bay 12–8039), a new enantiomerically pure 8-methoxy quinolone. *Antimicrob. Agents Chemother.* **43**:2793–2797.
  60. **Tano, E., O. Cars, and E. Lowdin.** 2005. Pharmacodynamic studies of moxifloxacin and erythromycin against intracellular *Legionella pneumophila* in an in vitro kinetic model. *J. Antimicrob. Chemother.* **56**:240–242.
  61. **Tome, J. P., M. G. Neves, A. C. Tome, J. A. Cavaleiro, M. Soncin, M. Magaraggia, S. Ferro, and G. Jori.** 2004. Synthesis and antibacterial activity of new poly-S-lysine-porphyrin conjugates. *J. Med. Chem.* **47**:6649–6652.
  62. **Tulkens, P., and A. Trouet.** 1978. The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. *Biochem. Pharmacol.* **27**:415–424.
  63. **Tyteca, D., P. Van Der Smissen, M. Mettlen, F. Van Bambeke, P. M. Tulkens, M. P. Minget-Leclercq, and P. J. Courtoy.** 2002. Azithromycin, a lysosomotropic antibiotic, has distinct effects on fluid-phase and receptor-mediated endocytosis, but does not impair phagocytosis in J774 macrophages. *Exp. Cell Res.* **281**:86–100.
  64. **Van Bambeke, F., S. Carryn, C. Seral, H. Chanteux, D. Tyteca, M. P. Minget-Leclercq, and P. M. Tulkens.** 2004. Cellular pharmacokinetics and pharmacodynamics of the glycopeptide antibiotic oritavancin (LY333328) in a model of J774 mouse macrophages. *Antimicrob. Agents Chemother.* **48**:2853–2860.
  65. **Van Bambeke, F., Y. Van Laethem, P. Courvalin, and P. M. Tulkens.** 2004. Glycopeptide antibiotics: from conventional molecules to new derivatives. *Drugs* **64**:913–936.
  66. **van den Broek, P. J.** 1991. Activity of antibiotics against microorganisms ingested by mononuclear phagocytes. *Eur. J. Clin. Microbiol. Infect. Dis.* **10**:114–118.
  67. **van den Broek, P. J., L. F. Buys, H. Mattie, and R. van Furth.** 1986. Comparison of the effect of phenoxymethylpenicillin, cloxacillin, and flucloxacillin on *Staphylococcus aureus* phagocytosed by human monocytes. *J. Antimicrob. Chemother.* **17**:767–774.
  68. **Waldvogel, F. A.** 2000. *Staphylococcus aureus*, p. 2069–2092. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and practice of infectious diseases*. Churchill Livingstone, Philadelphia, Pa.
  69. **Weinrick, B., P. M. Dunman, F. McAleese, E. Murphy, S. J. Projan, Y. Fang, and R. P. Novick.** 2004. Effect of mild acid on gene expression in *Staphylococcus aureus*. *J. Bacteriol.* **186**:8407–8423.
  70. **Yancey, R. J., M. S. Sanchez, and C. W. Ford.** 1991. Activity of antibiotics against *Staphylococcus aureus* within polymorphonuclear neutrophils. *Eur. J. Clin. Microbiol. Infect. Dis.* **10**:107–113.
  71. **Zhanel, G. G., A. K. Wierzbowski, P. Hisanaga, and D. J. Hoban.** 2004. The use of ketolides in treatment of upper respiratory tract infections. *Curr. Infect. Dis. Rep.* **6**:191–199.