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Drug Effects on Intracellular Mycobacteria Determined by Mass Spectrometric Analysis of the Na^+-to-K^+ Ratios of Individual Bacterial Organisms

MONIKA WIESE AND ULRICH SEYDEL*

Research Center Borstel, Center for Medicine and Biosciences, Division of Biophysics, D-23845 Borstel, Germany

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The successful establishment of a drug screening system for intracellular cultivable and noncultivable mycobacteria based on the mass spectrometric determination of bacterial viability is described. To compare drug efficacies on intra- and extracellular mycobacteria, the mycobacteria were subjected to drug treatment either after phagocytosis by the mouse macrophage cell line RAW 264.7 or in cell-free medium. After reisolation, their viability was monitored by analyzing the intrabacterial sodium-to-potassium ratios for a limited number of individual organisms. This approach offers a reliable and quick tool for monitoring the influence of intracellular growth and of additional permeation barriers on intracellular drug efficacy and will thus provide useful information for the rational development and testing of optimized antimycobacterial drugs. In particular, the methodology is applicable to the noncultivable species *Mycobacterium leprae*, because the mass spectrometric analysis of the intrabacterial sodium-to-potassium ratio allows the determination of bacterial viability independent from their ability to multiply in vitro. Because of the improved metabolic activity of intracellularly growing *M. leprae* compared with that of extracellularly growing *M. leprae*, the spectrum of antileprosy drugs that can be tested in vitro could even be extended to those interfering with DNA replication and cell division.

Diseases caused by mycobacterial infections are important sources of morbidity and mortality throughout the world (2). Whereas the prevalence of leprosy continues to decline, tuberculosis and opportunistic infections caused by *Mycobacterium avium* have reemerged during the past few years because of the increasing prevalence of human immunodeficiency virus infections and the development of multidrug resistance (5). Therefore, new, effective drugs are urgently needed. For rational drug design, it must be considered that pathogenic mycobacteria like *Mycobacterium tuberculosis* and *Mycobacterium leprae* are facultatively or obligate intracellularly growing organisms which survive and multiply in unstimulated macrophages of the infected host. From their ability to survive intracellularly, several consequences arise for the efficacy of antibiotics against these bacteria. On the one hand, phagocytic membrane systems (cell membrane and the phagosomal membrane) represent additional permeation barriers for the drugs, and drugs can be exported from the host cells by active mechanisms, i.e., specific (3) or nonspecific pumps which are located in the cell membrane of mammalian cells and which mediate multidrug resistance (11). On the other hand, the metabolic activity of phagocytosed bacteria may be enhanced in comparison with that of the same species growing in a cell-free medium. This is to be expected, for example, for the obligate intracellularly growing organism *M. leprae*. For the cultivable species *M. avium* (24), and recently also for *M. tuberculosis* (22), the synthesis of additional cell envelope layers and several new protein products (8, 11, 23) in response to the intracellular habitat have been reported. With the aim of elucidating the influence of the factors mentioned above on the activities of antimycobacterial drugs, we developed a reliable and quick system for testing their intracellular antibacterial activities.

So far, the effects of drugs on cultivable mycobacteria have been determined mainly in cell-free media (4, 25, 27, 33), but they have also been determined in phagocytosis systems with macrophages isolated from humans or mice (6, 20, 26, 32). In the latter experiments, drug-induced impairment was monitored by growth or plating experiments with reisolated bacteria. This approach, however, is rather time- and labor-consuming, especially in the case of slowly growing mycobacteria, and may be subject to several experimental errors such as bacterial clumping and loss of infected cells during incubation (34). For the noncultivable species *M. leprae*, this approach is not at all practicable.

Here we report the results of experiments in which the intracellular survival of *M. leprae* and *M. tuberculosis* was monitored in the absence and the presence of drugs by the mass spectrometric determination of the intrabacterial sodium-to-potassium ratio (Na^+-to-K^+ ratio) for a limited number of individual bacterial organisms. This technique allows for the quick and precise quantification of the percentage of viable organisms within a given bacterial population without the need for cultivation experiments and is routinely used in our laboratory to investigate the effects of drugs not only on the noncultivable organism *M. leprae* ex vivo (10) or in vitro (36) but also on cultivable mycobacteria (12, 29). The rationale behind this approach is the ability of all unimpaired living cells, bacteria as well as eucaryotes, to accumulate potassium and exclude sodium by energy-demanding processes, resulting in the generation of transmembrane ion gradients which are used as energy stores for several central metabolic processes, e.g., transport of nutrients (18). Dead or impaired cells are unable to maintain these transmembrane concentration gradients. Therefore, the intrabacterial Na^+-to-K^+ ratio is the smallest for unimpaired bacterial organisms and increases in proportion to the degree of impairment up to a maximum value for

* Corresponding author. Mailing address: Research Center Borstel, Center for Medicine and Biosciences, Division of Biophysics, Parkallee 10, D-23845 Borstel, Germany. Phone: 49-(0)4537-188232. Fax: 49-(0)4537-188632.

completely impaired organisms, which equals that for the extrabacterial environment. For bacteria inside an intact phagosome, this maximum value is defined by the ratio of the cation concentrations of the extracellular fluid, i.e., the cell culture medium, and for bacteria in a cell-free system, this maximum value is defined by the ratio of the concentrations of the bacterial growth medium. Details of the technique and the mass spectrometric determination of viability have been described previously (36). For cultivable bacteria, we showed that the physiological states (viabilities) determined by mass spectrometric analysis of the cation ratios agreed well with those determined by established microbiological techniques (17, 30, 37) and, in the case of *M. lepraemurium*, with those determined by the mouse footpad assay (13).

In this report we describe the successful establishment of a phagocytosis system for *M. leprae* and *M. tuberculosis* by using the mouse macrophage cell line RAW 264.7 as host cells and report the results of the first experiments demonstrating the applicability of the system to in vitro drug screening based on the mass spectrometric determination of bacterial viability.

MATERIALS AND METHODS

Source and isolation of *M. leprae.* *M. leprae*-infected armadillo tissue was supplied by J. Kazda (Research Center Borstel), frozen in liquid nitrogen, and stored at −80°C until use. To prevent the bacteria from damage by additional freezing-thawing cycles, they were immediately isolated and used after thawing. The procedure used to isolate *M. leprae* was a protocol modified from that of Dhople and Storrs (9). Briefly, it included the following steps: removal of fat; homogenization in 0.05 M phosphate-buffered saline (PBS); removal of intact tissue via centrifugation (200 × *g*, 10 min, 4°C); decontamination of *M. leprae* from the remaining tissue by treatment with 450 U of collagenase (Sigma, Deisendorf, Germany), 125 U of chymotrypsin (Boehringer, Mannheim, Germany), and 40 U of trypsin (Boehringer) for 30 min at 20°C in PBS; washing in PBS; and decontamination of other bacterial genera with an *N*-acetyl-L-cysteine (NALC)–NaOH mixture (final NaOH concentration, 1%; 50 min; 20°C) as described by Kubica et al. (16). To diminish clumping, the bacteria were treated for 30 intervals of 1 min each in a sonifier water bath (Bandelin Sonorex RV 100, 35 kHz, 160 W) during the decontamination procedure. Decontamination was stopped by the addition of distilled water (1:10), and the bacteria were harvested by centrifugation and were resuspended in PBS. Repeated application of enzyme treatment or NaOH decontamination to already isolated *M. leprae* did not affect their initial intrabacterial Na^+-to-K^+ ratios. Therefore, an effect of the relatively crude isolation procedure on the viability of *M. leprae* could be excluded.

Bacterial cultivation and treatment in cell-free medium. Stock cultures of *M. tuberculosis* H37Ra were maintained on Lowenstein-Jensen medium (Difco Laboratories, Detroit, Mich.) at room temperature; for extracellular drug screening, modified Dubos broth (Difco) containing 0.01% (wt/vol) sodium nitrate and 1% fetal calf serum (FCS) was inoculated with the same bacterial density used in the cellular system (typically, 1×10^7 to 3×10^7 bacteria per ml) from a preliminary culture grown in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Life Technologies GmbH, Berlin, Germany) in the presence of 1% FCS (Seromed; Biochrom KG, Berlin, Germany) and was cultivated at 33°C. In the case of *M. leprae*, aliquots of the bacterial suspensions were used to inoculate Middlebrook 7H9 (Difco) supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (Difco) and 0.5 ml of malachite green (1% [wt/vol]) per 100 ml of medium (modified Middlebrook 7H9) (total cell number, 3×10^7 bacteria per ml). The cultures were incubated at 33°C in airtight, closed tubes without shaking. The sterility of the bacterial cultures was checked by inoculation of blood and chocolate agar (Merck) incubated for several weeks and Lowenstein-Jensen slants incubated for 2 months and by acid-fast staining.

Clarithromycin, ethambutol, fusidic acid, gentamicin, minocycline, and rifampin were obtained from Sigma, ofloxacin was obtained from Hoechst (Frankfurt am Main, Germany), and rifabutin was obtained from Farmitalia (Freiburg im Breisgau, Germany). The drugs were added as stock solutions to the main cultures immediately after inoculation.

Cultivation, infection, and treatment of cell lines. Murine and human macrophage cell lines were cultivated at 33°C in 5% CO_2 with 100% humidity in 24-well plates. The cell lines RAW 264.7, THP-1, and U937 were cultivated in IMDM, which was supplemented with 2×10^{-5} M mercaptoethanol in the case of THP-1 cells, and which contained, in addition, 1 mM pyruvate and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) in the case of U937. The cell lines Mono Mac 6 and J774 were cultivated in RPMI 1640 with L-glutamine (Gibco). The concentration of FCS added to the media ranged from 0 to 10% and is indicated for each experiment. To prevent contamination with fungi, the media usually contained 2.5 μg of amphothericin B (Sigma) per ml. Cell cultures for infection experiments with *M. leprae*, but not those for infection experiments with *M. tuberculosis*, contained, in addition, 50 μg of ampicillin per ml to suppress the growth of bacterial contaminants. Before infection, the cells were usually grown to the stationary phase. Freshly isolated *M. leprae* or *M. tuberculosis* organisms obtained from the precultures used for extracellular drug screening were added in a 50- to 100-fold (*M. leprae*) or a 10- to 30-fold (*M. tuberculosis*) excess relative to the cellular densities, unless indicated otherwise. Infected cultures were incubated overnight and were washed thoroughly with medium to remove possibly existing extracellular bacteria. Subsequently, cell culture media containing the respective test antibiotics were added and were replenished every 3 days.

In some experiments, the number of phagocytosed bacteria was estimated from acid-fast-stained cytopreparations, in which a minimum of 100 cells per sample were classified according to the number of intracellular bacteria detected by light microscopy (total magnification, ×1,000; oil immersion) in different categories (number of phagocytosed bacteria per cell: 0, 1 to 10, 11 to 30, 31 to 100, 101 to 300, and 301 to 1,000). From the frequency distributions, the percentage of infected cells (infection rate) and the mean number of bacteria per infected cell (bacterial load) were calculated.

Mass spectrometric determination of bacterial viability. (i) Reisolation and preparation of bacteria. For the mass spectrometric analysis of their Na^+-to-K^+ ratios, phagocytosed bacteria must be reisolated from their host cells via cellular lysis and further purified. For this, culture medium was replaced by 200 μl of distilled water and the cells were reincubated for 30 min at 33°C. Subsequently, 10 μl of Tween solution (10% [vol/vol]) and 200 μl of a 5-fold (*M. leprae*) or 20-fold (*M. tuberculosis*) dilution of the NALC–NaOH mixture defined under the section Source and Isolation of *M. leprae* were added, and the samples were stirred thoroughly for 3 min with a magnetic stirrer and transferred to Eppendorf tubes filled with distilled water to dilute the NaOH concentration. In the case of *M. leprae*, cellular lysis could be further improved by sonification for 3 min in a water bath sonifier. Any possibly remaining intact cells as well as cell debris were removed by low-speed centrifugation (200 × *g*, 10 min, 4°C), and the bacteria were harvested by high-speed centrifugation (10,000 × *g*, 5 min, 4°C) and washed twice with distilled water to remove extrabacterial cations. For bacteria cultivated in a cell-free medium, only the last two washing steps were necessary for a sufficient purification. After washing, one drop of the concentrated bacterial suspension was transferred to a Formvar-coated copper mesh and excess fluid was drained off with tissue paper to achieve a widespread distribution of bacteria, allowing the evaporation of a single cell with each laser shot in the laser microprobe mass analyzer.

(ii) Mass spectrometric measurements and data evaluation. The mass spectrometric instrumentation, the laser microprobe mass analyzer (LAMMA 500; Leybold AG, Cologne, Germany), has been described in detail elsewhere (14). Briefly, in the LAMMA instrument a high-energy UV laser pulse is focused through the objective of a light microscope onto the sample to be analyzed and, in our experiments, evaporates one bacterial organism at one shot. The positive atomic and molecular (fragment) ions produced are registered by means of a time-of-flight mass spectrometer. The detection limits of the instrument for Na^+ and K^+ are less than 10^{-18} g out of an analyzed volume of about 1 μm^3 ($\approx 10^{-12}$ g).

The intrabacterial Na^+-to-K^+ ratios were derived from the intensities of the $^{23}Na^+$ and $^{39}K^+$ ion peaks, respectively, for 300 individual organisms per sample. From the 300 individual ratios acquired, the relative cumulative distributions of the Na^+-to-K^+ ratios within the populations and the medians (50% values) of these distributions were calculated.

The assessment of the proportion of viable organisms within a population is based on the so-called limiting value of the Na^+-to-K^+ ratio for viability (0.45 ± 0.02 [mean ± standard error of the mean]). This value has been determined in a series of experiments (n = 57) with cultivable bacteria under different experimental conditions (growth state, drug treatment, etc.), in which the distribution of the cation ratios in each bacterial population was correlated with the number of CFU derived from plating experiments. Since the upper limit of the Na^+-to-K^+ ratio of 0.45 for viable bacteria was shown to be independent of the bacterial species, the degree of drug-induced impairment, and the mode of drug action, we assumed that the limiting value is of general validity and thus is also applicable to the noncultivable species *M. leprae*. On the basis of this assumption, the percentage of viable bacteria within a population is easily defined by the percentage of bacteria with an Na^+-to-K^+ ratio of ≤0.45 (36). Because of the limited number of analyzed bacteria per sample (a few hundred), the percentage of viable organisms within a given population can be quantified exactly only in the range of between 10 and 100%. Less than 1% viable bacteria are not detectable by this technique.

RESULTS

Establishment of a suitable cell line. To test their suitabilities for use in intracellular drug screening, different cell lines of human or murine origin were cultivated in cell culture medium containing 10% FCS at 33°C. For each cell line the growth kinetics, the degree of adherence to plastic surfaces, and its ability to phagocytose *M. leprae* were tested. For the determi-

TABLE 1. Suitabilities of various cell lines for the development of an in vitro drug screening system

Cell line	Origin	Medium (+ 10% FCS)	Growth	Adherence	Phagocytosis
J774	Murine	RPMI	+	+	+
RAW 264.7	Murine	IMDM	++	+ (−)	+++
Mono Mac 6	Human	RPMI	+	−	+++
THP-1	Human	RPMI[a]	+++	−	+++[c]
U937	Human	IMDM[b]	++	−	++

[a] Containing 2×10^{-5} mercaptoethanol.
[b] Containing 1 mM pyruvate and 20 mM HEPES.
[c] Cell lysis occurred.

nation of the last parameter, cell cultures were grown to the stationary phase and were infected with a 100-fold excess of armadillo-derived *M. leprae*. The efficacy of phagocytosis was roughly classified into categories ranging from − (no phagocytosis) to +++ (most cells harboring 10 to 100 bacteria) according to the number of infected cells and the number of bacteria found intracellularly in acid-fast-stained cytopreparations. The results are presented in Table 1. Of all cell lines tested, the mouse macrophage cell line RAW 264.7 and the human cell line THP-1 showed the highest phagocytic capabilities. In contrast to THP-1 cells, which lysed rapidly upon infection, RAW 264.7 cells were not visually impaired by the phagocytosis process or the intracellular multiplication of the bacteria. Furthermore, since this cell line showed strong adherence and only a moderate growth rate, characteristics which are rather advantageous for a cellular drug screening system for slowly growing or nongrowing mycobacteria, it was used in all further experiments.

To investigate the capability of RAW 264.7 to phagocytose *M. leprae* and *M. tuberculosis* more closely, confluent monolayers of the cell lines were infected with different amounts of unimpaired *M. leprae* or *M. tuberculosis*, resulting in bacteria-to-cell ratios of between 10 and 100. At a bacteria-to-cell ratio of 100, infection was also performed with bacterial samples which had been subjected to four freeze-thaw cycles in liquid N_2, a procedure that kills *M. leprae* completely and *M. tuberculosis* partially (as determined mass spectrometrically). After phagocytosis and washing, the infection rate (the percentage of infected cells) and the bacterial load (the mean number of intracellular bacteria) were quantified from acid-fast-stained cytopreparations of the different samples. The results are depicted in Fig. 1a and b. A maximum of 90% of the cells were infected with *M. leprae* when the bacteria were added at a density exceeding the cellular density by a factor greater than 10. The maximal infection rate was independent from bacterial viability. On the average, a maximum bacterial load of 100 was observed. In contrast to the infection rate, the bacterial load increased proportionally to the amount of bacteria provided extracellularly and decreased with bacterial impairment. For *M. tuberculosis*, the rate of phagocytosis was, in general, lower than that for *M. leprae* and was proportional to the number of bacteria provided extracellularly. The maximum rate of phagocytosis did not exceed 65%, and a slight reduction was observed upon bacterial impairment. The bacterial loads, however, were almost identical to those observed for *M. leprae.*

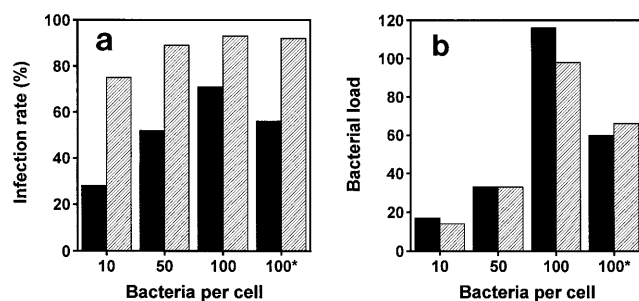

FIG. 1. Phagocytic capacity of RAW 264.7 infected with various numbers of *M. tuberculosis* (■) and *M. leprae* (▨) organisms for a phagocytosis period of 24 h. (a) Percentage of infected cells (infection rate); (b) average number of intracellular bacteria per infected cell (bacterial load); bacterial populations marked with an asterisk were killed (*M. leprae*) or heavily damaged (*M. tuberculosis*) by four freeze-thaw cycles before infection.

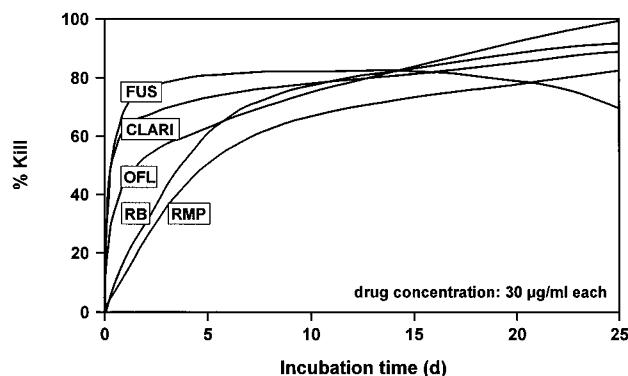

FIG. 2. Time kinetics of mass spectrometrically determined drug-induced killing of intracellular *M. leprae* (phagocytosed by RAW 264.7 cells in IMDM–10% FCS) by different antileprosy drugs (fusidic acid [FUS], clarithromycin [CLARI], ofloxacin [OFL], rifabutin [RB], and rifampin [RMP]) applied at a concentration of 30 μg/ml each. The viabilities of the treated samples are normalized to the respective untreated controls, varying between 90 and 40% during the observation period.

Since *M. tuberculosis* but not *M. leprae* tend to lyse the cells during intracellular growth, a 50- to 100-fold excess of *M. leprae* and a 10- to 30-fold excess of *M. tuberculosis* was used for infection in all further experiments.

Drug effects on *M. leprae* phagocytosed by RAW 264.7 cells. To test whether the cellular system established so far also reflects the concentration and time dependence of drug-induced impairment for those antileprosy drugs that failed to interfere with the restricted metabolism of *M. leprae* kept in cell-free media (for example, ofloxacin [36]), *M. leprae*-infected RAW 264.7 cells were incubated with differently acting drugs at various concentrations and for various durations, and drug-induced killing was monitored mass spectrometrically.

In the absence of drugs, the survival time of *M. leprae* compared with that observed for the same bacterial isolate in cell-free Middlebrook 7H9 was prolonged by a factor of about 3. Thus, in the cell-free medium, the bacteria died completely within 1 week, whereas in the cellular system the same bacterial inoculum showed a rapid decrease in viability within the first 3 days of infection; this was followed by a slight, continuous decrease within the next 3 weeks and a rapid and complete death at longer incubation times. Nevertheless, during a period of 2 to 3 weeks, viability ranged from 40 to 60%. Under the influence of several antileprosy drugs used at a concentration of 30 μg/ml, a time-dependent killing could be monitored, which was fastest for fusidic acid and clarithromycin; these were followed by rifabutin and rifampin (Fig. 2). Ofloxacin, which showed no activity against *M. leprae* in the cell-free system, induced an intermediate kinetics of impairment. At the longest treatment period (25 days), ofloxacin killed the bacte-

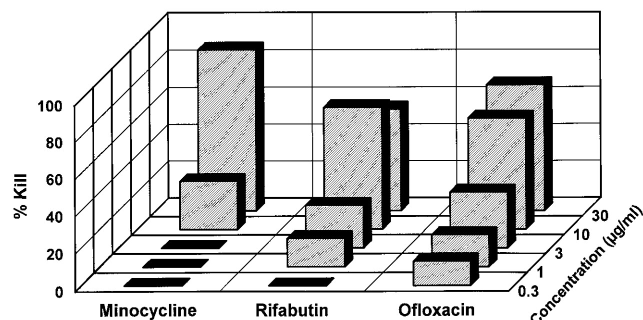

FIG. 3. Concentration dependence of mass spectrometrically determined drug-induced killing of intracellular *M. leprae* (phagocytosed by RAW 264.7 cells in IMDM–10% FCS) after 2 weeks of treatment with various concentrations of different antileprosy drugs (minocyclin, rifabutin, and ofloxacin). The viability of the untreated control was 45%.

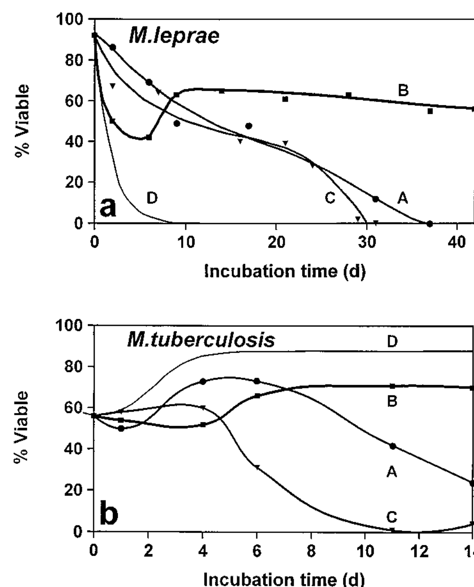

FIG. 4. Survival of phagocytosed *M. leprae* (a) and *M. tuberculosis* (b) in RAW 264.7 cells incubated in IMDM in the presence of 0% (curves A), 1% (curves B), or 10% (curves C) FCS. Curves D indicate the survival of the corresponding extracellular *M. leprae* control in modified Middlebrook 7H9 medium (a) and *M. tuberculosis* in IMDM–1% FCS (b).

ria completely. The killing rates of the other drugs varied from 90 to 70%, being the highest for rifabutin and the lowest for fusidic acid. Experiments on the concentration dependence of minocycline, rifabutin, and ofloxacin after 2 weeks of treatment (Fig. 3) revealed that at the lowest concentration (0.3 μg/ml), only ofloxacin had a slight killing effect, which increased in proportion to the drug concentration. Significant killing effects of rifabutin and minocycline were observed for concentrations ≥1 and ≥10 μg/ml, respectively.

Optimization of intracellular survival of *M. leprae* and *M. tuberculosis* by FCS adjustment. The experiments presented so far indicate that our cellular system can, in principle, be used for drug screening. The survival time of *M. leprae* in murine RAW 264.7 cells, however, was significantly shorter than that observed in initial experiments with human blood macrophages cultured in IMDM–1% type AB serum, which were performed in collaboration with E. Richter, Division of Molecular Immunology, Research Center Borstel (data not shown), and adherence of the infected cells decreased after 1 to 2 weeks of incubation. On the basis of the findings of Crowle and Poche (7) that intracellular multiplication of cultivable mycobacteria is inhibited by high serum concentrations in the cell culture medium (the observation was explained by the Fe-binding capability of serum leading to bacterial Fe deficiency), we tested the effect of serum concentration reduction on the intracellular survival of *M. leprae* and *M. tuberculosis*. Serum concentration reduction to 1 and 0%, respectively, reduced the growth kinetics of the uninfected cells but had, after an adaptation time of several days, no or only a slight influence on their viabilities (determined by trypan blue exclusion; data not shown). Moreover, serum concentration reduction also improved the adherence of the cells significantly. The influence of serum concentration reduction on the viabilities of intracellular *M. leprae* and *M. tuberculosis* is indicated in Fig. 4a and b. A reduction from 10 to 1% resulted in a significant prolongation of the intracellular survival of *M. leprae* as well as of *M. tuberculosis*, although *M. leprae* did adapt to the intracellular environment more slowly during the first days of infection. Both bacterial species died much faster in the absence than in the presence of 1% FCS. At optimal FCS concentrations in the cell culture medium, *M. leprae* populations maintained a viability of 50 to 60% (starting value of 90%) for about 6 weeks, compared with only a few days in cell-free medium. For *M. tuberculosis* the viability of intracellular bacteria was similar to that determined in the cell-free medium, IMDM–1% FCS. Unfortunately, *M. tuberculosis*-infected cells lysed after 2 weeks of cultivation. Therefore, the drugs had to be tested within a period of at most 2 weeks after infection.

Characterization of the optimized cellular system: intracellular growth of *M. leprae* and *M. tuberculosis*. To examine whether intracellular *M. leprae* and *M. tuberculosis* were able to multiply intracellularly under these optimized conditions, cells were infected with suboptimal amounts of bacteria (bacteria-to-cell ratio, 5). The total number of intracellular bacteria per vial was determined by evaluating acid-fast-stained cytopreparations at different times after infection. From the growth kinetics, the intracellular generation times of the two bacterial species were determined and compared with those observed in cell-free mycobacterial medium and in cell culture medium. As summarized in Table 2, *M. leprae* showed a slow but significant multiplication, with a generation time of about 15 days in the presence of 1% FCS and 19 days in the presence of 0% FCS. No multiplication was observed in cell culture medium or in modified Dubos medium and Middlebrook 7H9 medium. Under optimal conditions (1% FCS), *M. tuberculosis* was able to multiply intracellularly and extracellularly in modified Dubos

TABLE 2. Intra- and extracellular multiplication of *M. tuberculosis* and *M. leprae*

Growth conditions	Generation time (days)	
	M. tuberculosis	*M. leprae*
Intracellular		
RAW 264.7, IMDM–1% FCS	1.5	15
RAW 265.7, IMDM–0% FCS	2.0	19
Extracellular		
IMDM–1% FCS	2.9	∞
Modified Dubos medium	1.6	∞
Middlebrook 7H9 medium	ND[a]	∞

[a] ND, not determined.

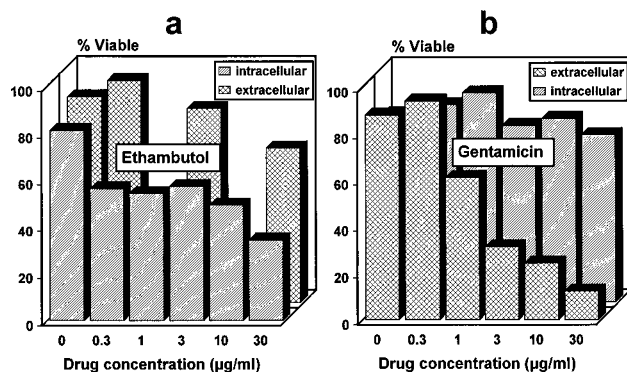

FIG. 5. Effect of ethambutol (a) and gentamicin (b) on extracellular (modified Dubos medium–1% FCS) and intracellular (RAW 264.7 cells in IMDM–1% FCS) *M. tuberculosis* after 2 weeks of treatment.

medium with the same generation time (1.5 days). Intracellular growth was reduced slightly in the absence of FCS, and in IMDM in the absence of host cells only slow extracellular growth was observed.

Drug effects on intra- and extracellular *M. tuberculosis*: influence of additional permeation barriers. To elucidate whether the efficacy of a drug was influenced by the bacterial environment (cellular versus cell-free), *M. tuberculosis* was incubated in the cellular system (under optimal conditions in the presence of 1% FCS) and, in parallel, in cell-free modified Dubos medium in the presence of ethambutol and gentamicin. The concentration dependences of impairments induced by the two drugs were monitored mass spectrometrically. As depicted in Fig. 5a and b, the activity of ethambutol against intracellular bacteria compared with its activity against extracellular organisms was slightly enhanced, whereas that of gentamicin was drastically reduced.

DISCUSSION

The mass spectrometric analysis of intrabacterial Na^+-to-K^+ ratios of individual bacterial organisms is routinely used in our laboratory to monitor the effects of drugs on bacterial viability (10, 12, 13, 17, 29, 30, 36, 37). Since this method renders information independent from the ability of the bacterial organisms to multiply in vitro, it saves time and labor in the case of slowly growing cultivable mycobacteria and, moreover, can also be used in the case of the noncultivable species *M. leprae*. So far, the method has been applied to monitoring the effects of drugs on mycobacteria grown or incubated in vitro in cell-free medium or isolated from human or animal tissue. To mimic the in vivo habitat of intracellularly growing mycobacteria and its influences on drug efficacies more closely, we then aimed at developing a cellular system in which the effects of drugs against phagocytosed mycobacteria could be monitored mass spectrometrically and compared with those against extracellular mycobacteria.

For the sake of better practicability and reproducibility, we decided to use a macrophage cell line as the host cells because they are available in unlimited numbers. Of several lines of cells of human and murine origin tested, RAW 264.7 and THP-1 revealed the highest phagocytic activities (Table 1). Since the latter cells lysed rapidly upon infection (within 12 h), RAW 264.7 cells were chosen as the most promising candidate for the development of an intracellular drug screening system. Besides its phagocytic activity, this mouse macrophage cell line exhibits several other advantageous characteristics, e.g., its adherence, which simplifies the experimental procedures, and its moderate growth, which allows for the long-term cultivation of infected monolayers and prevents overgrowth by uninfected cells. A more detailed characterization of its phagocytic capability showed that the percentage of phagocytic cells ranged between 90% for *M. leprae* and 70% for *M. tuberculosis* when the bacteria were added in a 100-fold excess. Under these conditions, infected cells harbor, on average, a maximum of 100 *M. leprae* or 115 *M. tuberculosis* organisms. These findings imply that almost all extracellularly added *M. leprae* were phagocytosed, whereas phagocytosis of *M. tuberculosis* was somewhat incomplete. This different behavior may be understood in view of the fact that mycobacteria tend to clump in cultures but not in bacterial suspensions isolated from infected tissues, and that these clumps are more resistant to phagocytosis. For both species the phagocytosis rate decreased with decreasing viability of the bacteria. In the case of *M. leprae*, however, bacterial impairment only reduced the bacterial load, whereas in the case of *M. tuberculosis* both the bacterial load and the infection rate were diminished. Our observation of reduced phagocytic uptake of dead bacteria agreed well with the hypothesis published in the literature that viable *M. tuberculosis* (1) as well as viable *M. leprae* (28) are able to support their own phagocytosis by several active mechanisms, one of which is the secretion of fibronectin-binding molecules that promote uptake via the fibronectin receptors of macrophages.

In any case, phagocytosis of unimpaired organisms of both bacterial species was sufficiently high to obtain qualitatively clean preparations of phagocytosed bacteria (i.e., free of extrabacterial cations and cell debris) for the mass spectrometric analysis of their intrabacterial Na^+-to-K^+ ratios. For this, however, relatively high bacterial densities (about 10^7/ml) had to be used in the cellular system and, to achieve comparable conditions, in the cell-free system as well. This high bacterial density may reduce the susceptibility to the effects of drugs. A further indispensible prerequisite for the mass spectrometric detection of the effects of drugs on the viability of bacteria via the measurement of the intrabacterial Na^+-to-K^+ ratios is the existence of transmembrane concentration gradients for these two cations for viable bacteria. This prerequisite should be fulfilled as long as the intracellular bacteria are surrounded by extracellular medium (high Na^+, low K^+) taken up by the phagosome and are separated from the cytoplasm of the macrophage (low Na^+, high K^+) by the phagosomal membrane. It is reported in the literature, however, that *M. leprae* (19) and virulent strains of *M. tuberculosis* (21) may occasionally escape from the phagosome into the cytoplasm of the host cells. In this case, drug-induced impairment of the bacterial organisms cannot be detected by the mass spectrometric determination of their intrabacterial Na^+-to-K^+ ratios and damaged bacteria would be incorrectly classified as viable.

To determine whether the mass spectrometric determination of bacterial viability could be applied not only to extracellular bacteria but also to intracellular bacteria, we performed a series of experiments in which intracellular *M. leprae* were exposed to a number of known antileprosy drugs at various concentrations and for different incubation times. The experiments revealed that our mass spectrometric approach did in fact reflect the time and concentration dependence of drug effects correctly. Thus, all drugs tested (fusidic acid, clarithromycin, ofloxacin, rifabutin, and rifampin) known to be effective against *M. leprae* in vivo and in mouse footpad experiments (35) were also active in our cellular system. The time kinetics of drug effects could even be correlated with the mode of action, being fastest for fusidic acid, a membrane-active drug, and slowest for rifampin, which interferes with protein synthe-

sis at the transcription level. For a drug concentration of 30 μg/ml, killing rates of between 70% (fusidic acid) and 100% (ofloxacin) were achieved after 25 days of treatment. Besides the time kinetics of drug-induced impairment, the mass spectrometric evaluation also reflected the concentration dependence of drug action on intracellular *M. leprae*, as shown for minocycline, rifabutin, and ofloxacin in Fig. 3. Ofloxacin was active at concentrations as low as 0.3 μg/ml; this was followed by rifabutin and minocycline, which induced detectable changes in viability (Na^+-to-K^+ ratios) at concentrations of ≥1 and ≥10 μg/ml, respectively. The relative efficacies of the three drugs agreed well with those observed in vivo (35). The high in vitro efficacy of ofloxacin, an inhibitor of DNA replication, however, was quite surprising, since in earlier experiments *M. leprae* incubated in cell-free Middlebrook 7H9 medium, which supports the survival but not the multiplication of this bacterial species for a limited period, was not impaired by ofloxacin, whereas cultivable mycobacterial species were killed (36). The activity of an inhibitor of DNA replication and the remarkable prolongation of the survival time of intracellular *M. leprae* compared with that of extracellular *M. leprae* (Fig. 5a) indicate that the metabolic activity of intracellular *M. leprae* is more complete than that in the cell-free system. Therefore, the cellular drug screening system also renders information about the efficacies of the increasingly important group of quinolones against *M. leprae*.

Furthermore, for *M. leprae* we observed a limited but significant multiplication in the macrophages when macrophages were infected with low bacterial numbers and incubated with a low serum concentration. The generation time of *M. leprae* was comparable to that found in mouse footpad experiments (31). Under these conditions, growth rates of intracellular *M. tuberculosis* were nearly identical to those observed extracellularly in modified Dubos medium but higher than those observed in IMDM in the absence of cells. Both *M. leprae* and *M. tuberculosis* showed optimal growth (Table 2) and maximum intracellular survival times (Fig. 5a and b) in the presence of 1% FCS in the cell culture medium. It may be hypothesized that lower FCS concentrations inhibit cell metabolism and higher FCS concentrations reduce the availability of Fe ions, which are essential for bacterial survival (7). Under optimal conditions (1% FCS), intracellular survival of up to 5 weeks for *M. leprae* and up to 2 weeks for *M. tuberculosis* was achieved. For *M. tuberculosis*, the period of intracellular multiplication was limited by the organism's tendency to cause cellular lysis, in particular when the cells were infected with a high bacterial inoculum.

After optimization, the system was used to measure the influence of the intracellular habitat on the efficacies of drugs against *M. tuberculosis*. The intra- and extracellular survivals of untreated *M. tuberculosis* were rather similar within the incubation period of 2 weeks (Fig. 5b). Under the influence of gentamicin, a strong concentration-dependent decrease in viability was observed for extracellular but not for intracellular *M. tuberculosis*. In contrast, ethambutol had a higher killing activity against intracellular bacteria than against extracellular bacteria and showed an overall effectiveness lower than that of gentamicin. The relative efficacies of the two drugs against intra- and extracellular bacteria correlate well with the results derived from uptake assays with radiolabeled substances performed by Johnson et al. (15). Those investigators observed a sevenfold accumulation of ethambutol inside uninfected alveolar macrophages but a twofold exclusion of gentamicin. These first experiments demonstrate the strong influence of the macrophage habitat on the efficacies of drugs against obligate (*M. leprae*) or facultative (*M. tuberculosis*) intracellular bacteria.

In conclusion, mass spectrometric analysis of bacterial viability via the determination of their intrabacterial Na^+-to-K^+ ratios offers a valuable tool for monitoring the effects of drugs on intra- and extracellular mycobacteria. Although the information obtained by this methodology is limited in some respects (viability must be ≥10% for an exact quantification, and the high bacterial numbers needed for reisolation may reduce sensitivity for the detection of drug effects), the technique has some important advantages over conventional microbiological techniques, in particular in the case of slowly growing or nongrowing mycobacteria. These are, for example, a high degree of reliability, quick data acquisition, and independence from in vitro multiplication. From a comparison of the actions of drugs on the viabilities of mycobacteria in cell-free and cellular systems, important information on the influence of intracellular growth and membrane-mediated effects can be obtained. This should render new possibilities for the rational development of new drugs and drug derivatives with optimized permeation properties and thus help to overcome the problem of membrane-mediated multidrug resistance in the therapy of mycobacterial infections.

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