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Mechanism of the Intracellular Killing and Modulation of Antibiotic Susceptibility of *Listeria monocytogenes* in THP-1 Macrophages Activated by Gamma Interferon

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***Listeria monocytogenes*, a facultative intracellular pathogen, readily enters cells and multiplies in the cytosol after escaping from phagosomal vacuoles. Macrophages exposed to gamma interferon, one of the main cellular host defenses against *Listeria*, become nonpermissive for bacterial growth while containing *Listeria* in the phagosomes. Using the human myelomonocytic cell line THP-1, we show that the combination of L-monomethyl arginine and catalase restores bacterial growth without affecting the phagosomal containment of *Listeria*. A previous report (B. Scorneaux, Y. Ouadrhiri, G. Anzalone, and P. M. Tulkens, Antimicrob. Agents Chemother. 40:1225–1230, 1996) showed that intracellular *Listeria* was almost equally sensitive to ampicillin, azithromycin, and sparfloxacin in control cells but became insensitive to ampicillin and more sensitive to azithromycin and sparfloxacin in gamma interferon-treated cells. We show here that these modulations of antibiotic activity are largely counteracted by L-monomethyl arginine and catalase. In parallel, we show that gamma interferon enhances the cellular accumulation of azithromycin and sparfloxacin, an effect which is not reversed by addition of L-monomethyl arginine and catalase and which therefore cannot account for the increased activity of these antibiotics in gamma interferon-treated cells. We conclude that (i) the control exerted by gamma interferon on intracellular multiplication of *Listeria* in THP-1 macrophages is dependent on the production of nitric oxide and hydrogen peroxide; (ii) intracellular *Listeria* may become insensitive to ampicillin in macrophages exposed to gamma interferon because the increase in reactive oxygen and nitrogen intermediates already controls bacterial growth; and (iii) azithromycin and still more sparfloxacin cooperate efficiently with gamma interferon, one of the main cellular host defenses in *Listeria* infection.**

Listeria monocytogenes is a facultative intracellular pathogen responsible for severe infections in humans and other animal species (14, 42). In vitro, *L. monocytogenes* can thrive inside a large variety of phagocytic and nonphagocytic cells by actively infecting them and subverting the host cell's normal defensive response (6, 11, 17, 18, 24, 26, 36). In this context, the sojourn and multiplication of *Listeria* in macrophages and monocytes probably play a key role in the persistence and/or recurrence of the infection (28). Studies with cell culture models have shown that after penetration into cells by binding through internalin A, the virulent variants of *L. monocytogenes* (i.e., those producing the hemolytic and cytolytic toxin listeriolysin O, also called hemolysin [Hly]) quickly escape from phagosomes upon acidification of this subcellular compartment (5). They multiply in the cytosol, where they acquire a propulsive motility through actin polymerization and increasing local concentration of profilin-actin-ATP complex (20, 39, 46), allowing them to spread toward adjacent cells (12, 17, 24, 32, 36, 40, 48). Variants defective in Hly, which are avirulent in mice (16, 21), invade cells but fail to reach the cytosol and to multiply therein (4). Activation of macrophages by T-cell-mediated immune response is highly critical for controlling *Listeria* infection (9, 22, 29). In particular, the induction of bactericidal macrophages by gamma interferon (IFN-γ) (7, 23) and the production of tumor necrosis factor alpha (33, 34) have been recognized as crucial events in listerial clearance. In many cases, however, these host responses are insufficient to contain the infection (2), requiring the use of antibiotics. Ampicillin (or penicillin) and gentamicin are usually considered first-choice agents (2), but these recommendations are primarily based on in vitro bacterial susceptibility testing and largely ignore the role of the intracellular forms of *Listeria* as well as the potential cooperation or antagonism between antibiotics and cytokines at the level of the macrophages. In a previous report (41), we showed that the human myelomonocytic cell line THP-1, in which virulent *L. monocytogenes* Hly^+ strains grow readily, becomes nonpermissive for bacterial growth when preexposed to IFN-γ. We also showed that IFN-γ modulates in opposite directions the susceptibility of the intracellular *L. monocytogenes* Hly^+ strain to the bactericidal activities of three classes of antibiotics of distinct pharmacological classes, namely, ampicillin (which loses all intrinsic activity), azithromycin (the activity of which remains unaffected), and sparfloxacin (the activity of which is markedly enhanced). In the present study, we examine the mechanism of these effects in light of the known influence of IFN-γ on the intracellular trafficking of *L. monocytogenes*, its stimulation of the oxygen- and nitrogen-derived reactive intermediates, and the pharmacodynamic and cellular pharmacokinetic properties of these three classes of antibiotics.

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MATERIALS AND METHODS

Bacterial strains and cultures. *L. monocytogenes* Hly^+ and Hly^- strains were obtained from P. Berche (Laboratoire de Microbiologie, Faculté de Médecine Necker, Paris, France). The wild type (Hly^+) is a type collection strain (strain EGD, serotype 1/2a, Hly-producing strain) from the Trudeau Institute (Saranac

Lake, N.Y.). Its nonhemolytic, nonvirulent variant (Hly$^-$) was obtained by insertion of the transposon Tn*1545* within the Hly structural gene of the wild-type strain (16, 17). Hly production in both strains was controlled by growth on 5% horse blood tryptic soy agar (Becton Dickinson, Erembodegem, Belgium). For use in cell culture experiments, bacteria were grown in tryptic soy broth (TSB; Becton Dickinson), harvested in log-phase growth ($\approx 10^8$ bacteria per ml), and stored in 1-ml aliquots in 20% glycerol at −80°C until required. For each experiment, a sample of the frozen stock was rapidly thawed and inoculated in 50 ml of TSB. After 18 h of incubation at 37°C, bacteria were washed once in phosphate-buffered saline (PBS) and used after an appropriate dilution in RPMI 1640 medium supplemented with 10% decomplemented (56°C, 30 min) fetal calf serum (FCS). The number of viable bacteria was determined by plating 0.1-ml aliquots of serial dilutions on tryptic soy agar. Colonies (CFU) were counted after 24 h of incubation at 37°C.

Determination of the MICs. MICs were determined in RPMI 1640–10% decomplemented FCS by the arithmetic dilution method (0.1-μg increment) and at a constant initial inoculum (10^6 bacteria per ml). The MIC was defined as the lowest concentration of each antibiotic giving no visible bacterial growth by naked-eye examination after an 18-h incubation at 37°C. MICs obtained under these conditions were 0.2 μg/ml for ampicillin, 0.4 μg/ml for azithromycin, 1.2 μg/ml for sparfloxacin, and 0.8 μg/ml for gentamicin for the *L. monocytogenes* Hly$^+$ strain and 0.3 μg/ml for ampicillin, 0.6 μg/ml for azithromycin, 2.5 μg/ml for sparfloxacin, and 0.8 μg/ml for gentamicin for the *L. monocytogenes* Hly$^-$ strain. These values were very similar to those obtained in TSB.

Time and dose-kill curve studies. The influence of the antibiotic concentration and time of exposure on bacterial killing was examined with multiplying and nonmultiplying bacteria. For multiplying bacteria, cultures in logarithmic growth ($\approx 10^9$ bacteria/ml) were centrifuged at 14,000 rpm (Eppendorf 5415 C centrifuge; Gerätebau Eppendorf GmbH, Engelsdorf, Germany) for 1 min at 4°C. The supernatant was then removed, and the pelleted bacteria were resuspended at a density of 10^6 CFU/ml in TSB. Antibiotics were then added at a concentration of 1 to 10 times their MIC, and the number of viable bacteria (CFU) was determined by plate assay after appropriate dilution. For nonmultiplying bacteria, *Listeria* strains were collected as described above but resuspended in PBS to prevent further growth as reported for other bacterial species (3, 53). We checked that the number of CFU remained effectively close to the original value ($\approx 10^6$ CFU/ml) for up to 5 h in the absence of antibiotics.

Cells. THP-1 cells, a myelomonocytic cell line derived from the blood of a 1-year-old boy with acute monocytic leukemia (49), were maintained in RPMI 1640 medium supplemented with 10% decomplemented FCS and 2 mM glutamine in an atmosphere of 95% air–5% CO_2 at 37°C. Cells, which grow spontaneously in loose suspension under these conditions, were subcultured every third day by gentle shaking followed by pelleting and reseeding at a density of 2×10^5 cells per ml.

Interferon and antireceptor antibodies. Human recombinant IFN-γ, with a specific activity of 2×10^7 U/mg of protein, was purchased from Roche Diagnostics (formerly Boehringer Mannheim GmbH, Mannheim, Germany) and stored at −20°C. Aliquots were thawed immediately before use. Anti-human IFN-γ receptor (CD119) was purchased from Genzyme Diagnostics (Cambridge, United Kingdom).

IFN-γ cell binding experiments. Binding assays were performed by incubating cells at 4°C for 2 h with increasing concentrations of ^{125}I-IFN-γ (specific radioactivity of 82.7 μCi/μg; Du Pont, NEN Research, Boston, Mass.) in U-bottomed microtiter plates at a density of 10^7 cells/ml (200 μl/well) in RPMI 1640 medium containing 2% FCS (binding medium). After incubation, cells were washed four times by centrifugation in the cold with PBS supplemented with 2% FCS, and the cell-associated radioactivity was thereafter determined by gamma scintillation counting. Nonspecific binding was determined in parallel in the presence of a 50-fold excess of unlabeled IFN-γ. Nonspecific binding, which never exceeded 15% of the total amounts of radioactivity detected at saturation, was subtracted for determining the specific binding. Binding parameters were determined by graphic interpolation by the Scatchard plot approach.

Assay for IFN-γ receptor expression by flow cytometry. Cells were seeded in U-bottomed microtiter plates at density of 2×10^6 cells/ml (100 μl per well) in Hanks' balanced salt solution supplemented with 3% FCS and 10 mM sodium azide and incubated with a mouse monoclonal antibody raised against human IFN-γ receptor at a final concentration of 5 μg/ml for 1 h at 4°C. Cells were then washed with ice-cold incubation medium without antibody and thereafter exposed for 45 min at 4°C to a fluorescein isothiocyanate (FITC)-labeled goat polyclonal antibody raised against mouse immunoglobulin G1 (IgG1). Cells were then washed again in Hanks' balanced salt solution–3% FCS, fixed in 1.25% paraformaldehyde, and kept at 4°C in the dark until analysis by flow cytometry with a FACScan (Becton Dickinson, San Jose, Calif.). In parallel, cells were incubated either in the incubation medium alone or with FITC-labeled goat anti-mouse antibody alone to assess autofluorescence and nonspecific binding of the secondary antibody, respectively.

Cell activation. THP-1 cells (5×10^5 cells/ml) were activated by exposure to IFN-γ (100 U/ml) for 24 h at 37°C. This activation did not cause adhesion, and cells kept growing as a loose suspension.

Cell infection and assessment of intracellular activity of antibiotics. All experiments were conducted in six-well multidishes (4-cm-diameter wells; 2 ml of medium per well) at an initial density of approximately 5×10^5 cells per ml. Cells were collected by gentle shaking and centrifugation at $600 \times g$ for 10 min (Damon/IEC CRU-5000 centrifuge; Damon, Needham Heights, Mass.), resuspended in fresh medium inoculated with bacteria (2.5×10^6 CFU/ml for *L. monocytogenes* Hly$^+$ strain and 10^7 CFU/ml for *L. monocytogenes* Hly$^-$ strain), and then incubated at 37°C for 1 h to allow phagocytosis. Cells were then again centrifuged, the medium was decanted, and the infected cells were washed with prewarmed PBS by four successive centrifugations. At this time, the ratio of viable bacteria (CFU counting) to macrophages was approximately 1:1. Cells were then incubated with a control medium or with a medium containing the antibiotics (at an extracellular concentration of 10 times their MICs). At selected intervals, this medium was decanted and the cells were washed with ice-cold PBS. Cells were pelleted and lysed in distilled water (in this process, the cell sample was diluted at least 2,000-fold on a volume basis, so that carried-over antibiotic could not interfere with the CFU determination). No detergent was used to avoid interference with bacterial survival and/or subsequent antibiotic assay. The resulting suspension was used for determination of the number of viable bacteria by colony counting after plating on tryptic soy agar (CFU) and for assay of total cell protein (27). All results are expressed as CFU per milligram of cell protein.

Determination of cellular antibiotic accumulation. The uptake of sparfloxacin by THP-1 cells was determined by means of a radiochemical assay with ^{14}C-labeled drug. Cells were exposed to antibiotic at a final concentration of 10 mg/liter, and cell-associated radioactivity was measured on cell lysates, obtained as described above, by liquid scintillation counting. In preliminary experiments, we checked by thin-layer chromatography that the bulk of the ^{14}C collected from cells under these conditions was associated with genuine sparfloxacin. For azithromycin and ampicillin, cells were incubated with 10 and 30 mg/liter, respectively, and the cell antibiotic content was determined by radial diffusion assay in agar with *Bacillus subtilis* as the test organism with lower limits of detection set at 0.25 and 0.125 μg/ml, respectively. Standard curves were prepared in water, as described previously (50), after it was found that the low amounts of protein found in cell samples did not interfere with the assays. The cell antibiotic content was expressed by reference to the protein content of the samples. This protein content was used to estimate the cell volume, with a conversion factor of 5 μl of cell volume per mg of cell protein, a value close to that found experimentally for cultured fibroblasts (50), mouse peritoneal macrophages (44), and several other types of cultured cells, and the level of accumulation of each antibiotic was then expressed as the ratio of its apparent cellular concentration to its extracellular concentration.

Inhibition of the production of reactive nitrogen intermediates (RNI) and hydrogen peroxide. Cells were incubated with 400 μM L-monomethyl arginine (L-MMA; Calbiochem-Novabiochem International Inc., San Diego, Calif.) and 1,500 U of catalase (Sigma Chemical Co., St. Louis, Mo.) per ml, separately or in combination, during 24 h before infection with *L. monocytogenes* and during the 5-h postinfection period. We checked that the increase in H_2O_2 production stimulated in THP-1 cells by preincubation with IFN-γ was entirely suppressed by catalase alone under these conditions (horseradish peroxidase-dependent oxidation of phenol red by H_2O_2 [35]). Similarly, we checked that L-MMA completely suppressed the production of NO by THP-1 cells (Greiss reaction [10]).

Subcellular localization of phagocytosed bacteria. (i) Confocal microscopy. To distinguish between phagosomal and cytosolic *L. monocytogenes*, we used the double-fluorescence technique of labeling the bacteria with fluorescein prior to phagocytosis and the cell actin with rhodamine-phalloidin after cell fixation. In this system, naked bacteria will fluoresce in green whereas bacteria surrounded with actin will fluoresce in red or yellow because of the superimposition of a thick layer of rhodamine over the fluorescein. Viable *L. monocytogenes* cells were labeled with FITC [5-(((2(carbohydrazino)methyl)-thio)acetyl)amino-fluorescein; Molecular Probes, Eugene, Oreg.] by an overnight incubation with 0.5 mg of FITC per ml in TSB followed by sedimentation at 14,000 rpm (Eppendorf 5415 C centrifuge) for 1 min at 4°C and washing with PBS. This treatment did not alter the phagocytosis and intracellular survival of *Listeria* compared to those of controls. Infection was carried out at a bacterium-to-macrophage ratio of approximately 50 for the *L. monocytogenes* Hly$^+$ strain and of 200 for the *L. monocytogenes* Hly$^-$ strain (these higher ratios, compared to other experiments, were chosen to facilitate the observation of a large number of intracellular bacteria soon after phagocytosis). At appropriate times after infection, cells were washed three times with cold PBS. They were fixed as a suspension in 3.7% (vol/vol) formaldehyde in PBS for 15 min at room temperature and permeabilized and stained for actin by exposure to 1.7×10^{-7} M rhodamine-phalloidin (Molecular Probes) in 0.2% Triton X-100 and as described by Dabiri et al. (8). After washing, specimens were dried and mounted in 2.5% 1,4-diacylbicyclo-(2,2,2)octane (Dabco; Sigma Chemical Co.) in Mowiol (Calbiochem-Novabiochem International Inc.). Observations were made under oil immersion with a 63× objective with an MRC1024 (Bio-Rad Laboratories, Richmond, Calif.) confocal microscope. Images were digitally recorded with a Focus Graphics image recorder and used for direct computer-assisted reproduction with an ink-jet photo printer.

(ii) Electron microscopy observations. Infection of macrophages was carried out as described for the confocal microscopy studies, but cells were thereafter washed four times with PBS containing 3.6 mM Ca^{2+} and 3 mM Mg^{2+}, pelleted at 1,000 rpm in conical centrifuge tubes, and fixed for 30 min at 4°C with a freshly prepared solution of 2% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH

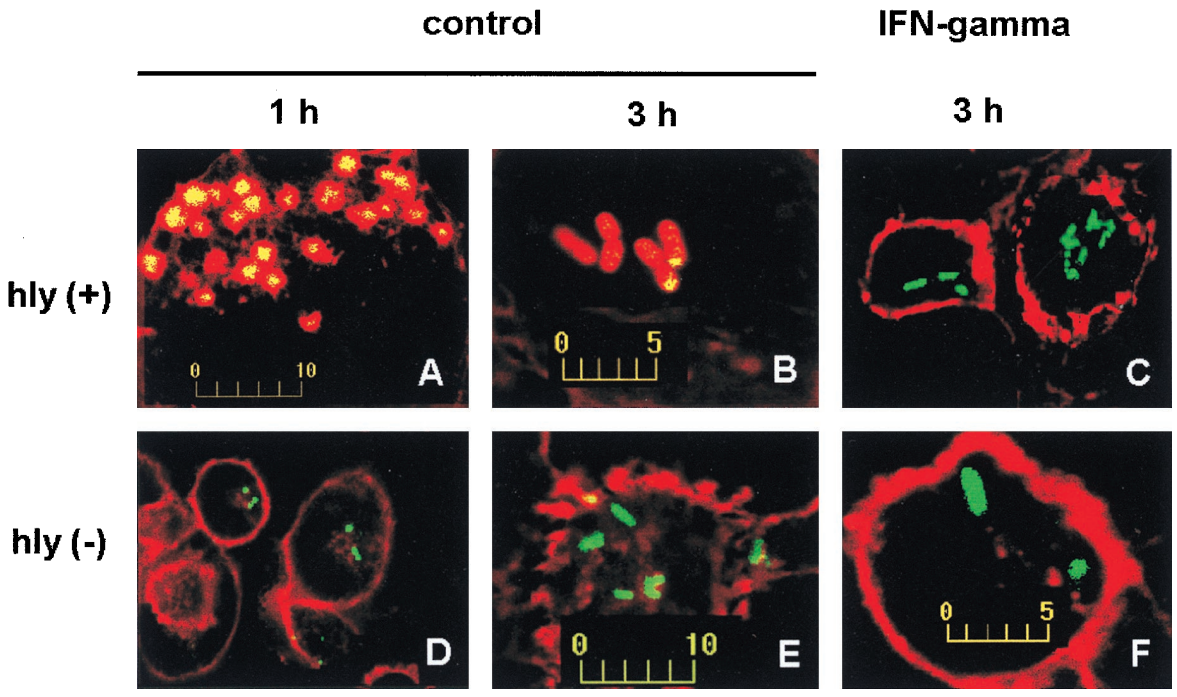

FIG. 1. Confocal microscopy of THP-1 macrophages after phagocytosis of *L. monocytogenes*. Bacteria were labeled with fluorescein, and cell actin was labeled with phalloidin-rhodamine. Upper row, *L. monocytogenes* Hly^+ strain; lower row, *L. monocytogenes* Hly^- strain. Columns marked "control" refer to THP-1 cells with no previous contact with IFN-γ. "IFN-gamma" refers to THP-1 cells preexposed for 24 h to 100 U of IFN-γ per ml prior to phagocytosis. Photographs were taken 1 h (A and D) and 3 h (B, C, E, and F) after phagocytosis. Scales are in micrometers.

7.4). Cells were then washed four times with the same buffer and postfixed for 1 h with 1% osmium tetroxide in cacodylate buffer in the dark. The samples were then washed three times with cacodylate buffer, once with distilled water, and once with Veronal acetate buffer (pH 7) and then stained en bloc in 0.5% uranyl acetate for 2 h at room temperature in the dark. Samples were then washed four times with Veronal acetate buffer (pH 7), immersed in melted 2% agar, dehydrated in alcohol, and then embedded in Spur resin. Thin sections were cut with a diamond knife, picked up on uncoated grids (300 mesh), stained with lead citrate, and examined in a Philips EM 301 microscope at 80 kV.

Materials. ^{14}C-labeled sparfloxacin was obtained from the French Commissariat à l'Energie Atomique, Saclay, France, on behalf of Rhône-Poulenc Rorer, Anthony, France, at a specific radioactivity of 26.8 mCi/mmol. Unlabeled sparfloxacin and azithromycin were obtained as laboratory samples for microbiological evaluation from Rhône-Poulenc Rorer and Pfizer s.a., Brussels, Belgium, respectively. Ampicillin was purchased from Sigma Chemical Co. Gentamicin was procured as Geomycin (the commercial brand distributed for clinical use in Belgium) from Schering-Plough s.a., Brussels, Belgium. Cell culture media and sera were from Gibco Biocult, Paisley, Scotland, and unless stated otherwise, all other reagents were purchased from E. Merck AG, Darmstadt, Germany.

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

RESULTS

Influence of IFN-γ on the intracellular trafficking of *L. monocytogenes* (confocal and electron microscopy). In the first step, we examined by confocal microscopy the association of *L. monocytogenes* (labeled with fluorescein) with the cell actin (stained with rhodamine-phalloidin). Figure 1 shows that 1 h after phagocytosis, the *L. monocytogenes* Hly^+ strain is already associated with actin since all labeled bacteria within cells display an orange staining surrounded by a thick red rim (Fig. 1A). Upon higher magnification (Fig. 1B), bacteria, many of which were in the process of division, appeared as red, rod-shaped bodies with spotty yellow patches. In contrast, all intracellular bacteria in IFN-γ-treated cells were brillantly stained in green even after 3 h, whereas actin was mainly detected on the pericellular edges of the cells (Fig. 1C). When the same experiments were performed with the *L. monocytogenes* Hly^- strain, all intracellular bacteria were consistently stained in green at 1 and 3 h in control cells (Fig. 1D and E) as well as in IFN-γ-treated cells (Fig. 1F).

This striking effect of IFN-γ on the subcellular environment of the *L. monocytogenes* Hly^+ strain was then further characterized by electron microscopy. Figure 2 shows the various stages of the intracellular trafficking of these bacteria in comparison with their nonvirulent variant (Hly^-). In control cells, bacteria were first seen associated with microvilli of the pericellular membrane of THP-1 macrophages and entering cells through long membrane invaginations while already multiplying (Fig. 2A). They were thereafter observed in phagosomes from which, however, they quickly escaped (Fig. 2B) to appear in the cytosol surrounded by a thick rim of filamentous material (Fig. 2C).

When these studies were repeated with cells pretreated with IFN-γ, bacteria remained confined in phagosomal vacuoles and were never seen in the cytosol for the whole duration of our observations (Fig. 2D and E). A similar confinement in phagosomal vacuoles was observed for the *L. monocytogenes* Hly^- strain in control cells (Fig. 2F and G) as well as in IFN-γ-treated cells (Fig. 2H). Moreover, the *L. monocytogenes* Hly^- strain cells often appeared as multiple organisms inside one vacuole, suggesting a fusion between several vacuoles containing single bacteria or an active multiplication of bacteria within a given vacuole.

Influence of IFN-γ on the intracellular *Listeria* growth pattern and roles of RNI and H_2O_2. We demonstrated earlier that preincubation of THP-1 with 100 U of IFN-γ per ml makes these cells nonpermissive for the intracellular growth of the *L. monocytogenes* Hly^+ strain (41). In the present study, we first

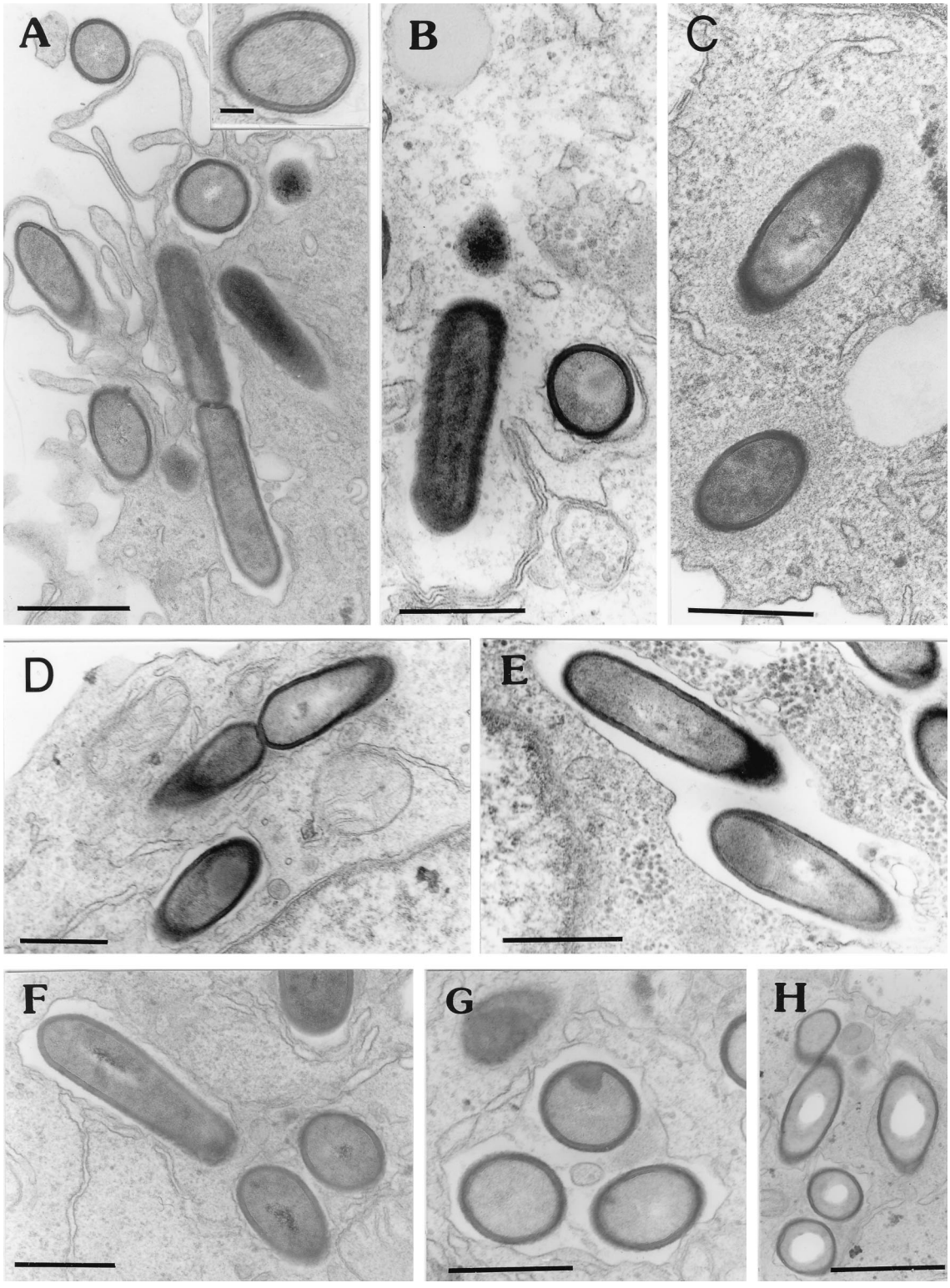

FIG. 2. Electron microscopy of THP-1 macrophages after phagocytosis of *L. monocytogenes*. Upper row, *L. monocytogenes* Hly^+ strain in control cells 1 h after phagocytosis (A [including inset] and B) and 3 h after phagocytosis (C). Middle row, *L. monocytogenes* Hly^+ strain in IFN-γ-pretreated cells 3 h after phagocytosis (D) and 5 h after phagocytosis (E). Lower row, *L. monocytogenes* Hly^- strain in control cells 3 h after phagocytosis (F and G) and in IFN-γ-pretreated cells 5 h after phagocytosis (H). Bars = 0.5 μm, except for inset of panel A (0.1 μm).

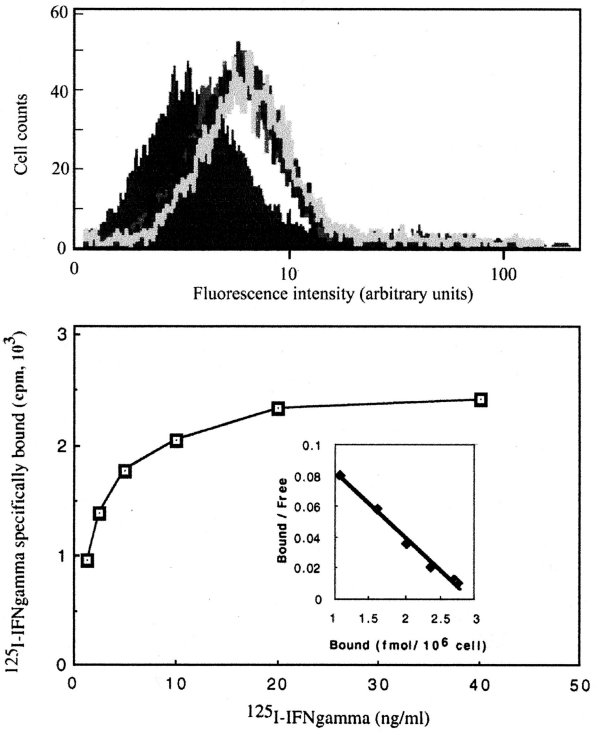

FIG. 3. (Upper panel) Expression of the IFN-γ receptor as determined by fluorescence-activated cell sorting of THP-1 macrophages (open histograms, cells treated with mouse IFN-γ receptor antibody and goat anti-mouse fluorescein-labeled IgG [the two histograms correspond to two independent sets of measurements]; solid histogram, cells stained with goat anti-mouse fluorescein-labeled IgG only). (Lower panel) Binding of IFN-γ to THP-1 macrophages; the specific binding of ^{125}I-IFN-γ is expressed as a function of the ligand concentration in the incubation medium (inset, Scatchard plot of the same data).

documented by fluorescence-activated cell sorting that THP-1 cells display receptors for IFN-γ. Figure 3 (upper panel) shows that a clear-cut signal was obtained for the whole population of cells exposed to a monoclonal antibody raised against the IFN-γ receptor (revealed with a secondary fluorescein-labeled antibody). In parallel, we directly measured and characterized the binding of ^{125}I-labeled IFN-γ to THP-1 cells. As shown in Fig. 3 (lower panel), the binding of IFN-γ was saturable, with an estimated maximum of 1,750 receptors per cell and a dissociation constant of 3×10^{-10} M (105 U/ml). Next, we examined whether the influence of IFN-γ on intracellular bacterial growth was dose dependent at concentrations with

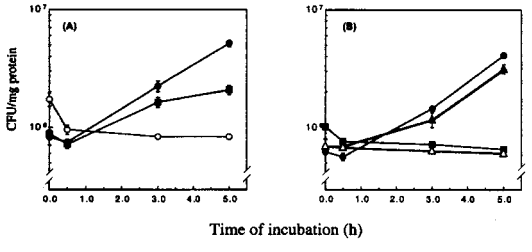

FIG. 4. Characterization of the effect of IFN-γ on the intracellular growth of the *L. monocytogenes* Hly^+ strain. (A) Dose dependency. ●, control (no IFN-γ); ■, IFN-γ (50 U/ml); ○, IFN-γ (100 U/ml). (B) Specificity. ■, IFN-γ alone (100 U/ml); ▲, IFN-γ plus monoclonal anti-human IFN-γ receptor mouse antibody; △, IFN-γ plus control isotype IgG; ●, no IFN-γ. Results are shown as means ± SD ($n = 3$).

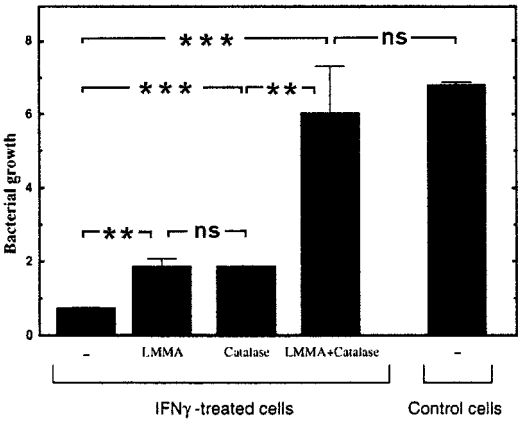

FIG. 5. Influence of IFN-γ alone and in combination with L-MMA, catalase, and their combination on the intracellular growth of the *L. monocytogenes* Hly^+ strain. Bacterial growth is defined as the ratio of the CFU observed in cell samples 5 h after phagocytosis to the number of CFU observed immediately after phagocytosis and washing. Data are shown as means ± SD ($n = 3$). Differences between paired sets of data were analyzed by the Student t test. **, $P < 0.005$; ***, $P < 0.001$; ns, not significant.

suboptimal receptor occupancy. Figure 4A shows that IFN-γ exerts at 50 U/ml an effect which, at 5 h, is about half of that observed at 100 U/ml. Figure 4B shows also that the addition of anti-IFN-γ receptor antibodies completely suppresses the effect of IFN-γ, demonstrating the role of its specific recognition by THP-1 cells (no effect was seen with an isotype control IgG). The growth of *L. monocytogenes* in control cells was unaffected by the presence of gentamicin (at an extracellular concentration of 10× its MIC), demonstrating its intracellular character.

Because IFN-γ is known to induce the production of bactericidal RNI and reactive oxygen intermediates (ROI) (10), we tested whether the addition of L-MMA, used to inhibit nitric oxide synthesis, and of catalase, to destroy hydrogen peroxide, would prevent IFN-γ from exerting its effects on intracellular *Listeria*. As shown in Fig. 5, L-MMA and catalase given alone made the cells partly permissive for *Listeria* growth in the presence of IFN-γ. When the two agents were given together, IFN-γ-treated cells became more permissive and the *L. monocytogenes* Hly^+ strain grew in these cells as well as it grew in controls (L-MMA and catalase had by themselves no significant effect on the intracellular growth of the *L. monocytogenes* Hly^+ strain in control cells [data not shown]). L-MMA, catalase, or their combination, however, did not suppress the ability of IFN-γ to constrain the *L. monocytogenes* Hly^+ bacteria within vacuoles. Electron microscopic studies, indeed, failed to disclose cytosolic, actin-surrounded bacteria in these cells. To the contrary, and as illustrated in Fig. 6, *L. monocytogenes* Hly^+ bacteria phagocytosed by IFN-γ-treated cells exposed to L-MMA and catalase remained consistently in vacuoles, many of which contained multiple bacterial profiles (Fig. 6A to C). Bacteria were also often seen in the process of division within these vacuoles (Fig. 6D to F), strongly suggesting that the growth seen in Fig. 5 was due to a multiplication of phagosomal bacteria.

Effect of IFN-γ and L-MMA combined with catalase on the intracellular activities of antibiotics toward internalized *L. monocytogenes*. We showed earlier that ampicillin, azithromycin, and sparfloxacin, at extracellular concentrations of 10× their MICs, exerted a slowly developing bactericidal effect on the intracellular growth of *L. monocytogenes* Hly^+ bacteria in

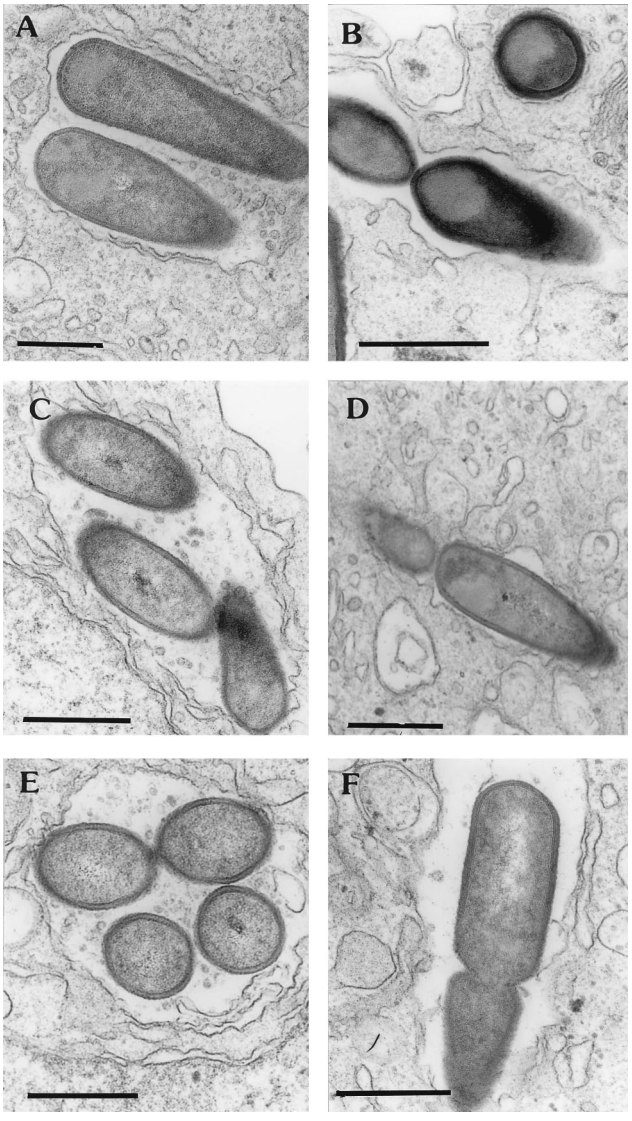

FIG. 6. Electron microscopy of IFN-γ-treated THP-1 macrophages exposed to L-MMA and catalase 5 h after phagocytosis of the *L. monocytogenes* Hly^+ strain. Catalase (A and B), L-MMA (C and D), or both (E and F) were further added immediately after phagocytosis. Bars = 0.5 μm.

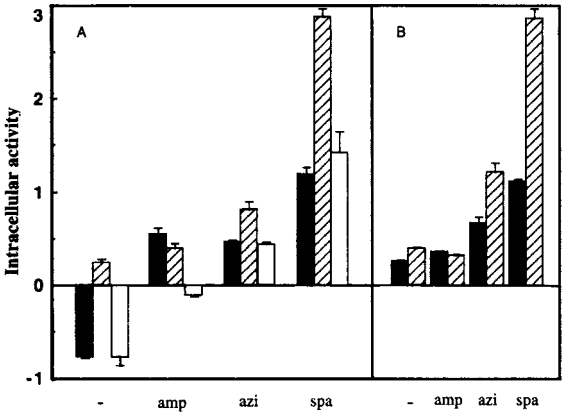

FIG. 7. Influence of the exposure of THP-1 macrophages to IFN-γ (100 U/ml), catalase, and L-MMA on the intrinsic activity of antibiotics towards intracellular *L. monocytogenes*. −, no antibiotic; amp, ampicillin; azi, azithromycin; spa, sparfloxacin. (A) Infection performed with the virulent variant Hly^+ in control (closed bars), in IFN-γ-treated cells (hatched bars), or in IFN-γ-treated THP-1 cells exposed to L-MMA and catalase (open bars). (B) Infection performed with the nonvirulent variant Hly^- in control (closed bars) and in IFN-γ-treated cells (hatched bars). Activity is defined as the $\log_{10}$ of the ratio of the number of CFU observed immediately after phagocytosis and washing to that after 5 h of incubation with the antibiotics (a negative value therefore means bacterial growth). Data are shown as means ± SD ($n = 3$). A statistical analysis of the differences seen between pertinent experimental groups of panel A is presented in Tables 1 and 2. For panel B, the difference between the data obtained for cells incubated with azithromycin or sparfloxacin alone and cells incubated with the same antibiotics but preexposed to IFN-γ is significant ($P < 0.005$ for azithromycin; $P < 0.001$ for sparfloxacin).

THP-1 macrophages (≈1 log reduction of CFU after 5 h). The changes in bacterial growth patterns caused by exposure of macrophages to IFN-γ caused a complete loss of intrinsic activity for ampicillin (i.e., the addition of ampicillin did not change the slight bactericidal effect obtained in cells by exposure to IFN-γ). In contrast, the effect of azithromycin was additive to that of IFN-γ, while synergy was demonstrable for sparfloxacin. These data are presented again here (Fig. 7) for the sake of comparison with the next set of data. We indeed show now (Fig. 7A; see Tables 1 and 2 for statistical analysis) that the addition of L-MMA and catalase, which caused IFN-γ-treated cells to become again permissive for *Listeria* growth, also allowed ampicillin to regain some intrinsic antibacterial effect even though only a static effect was seen under these conditions. Interestingly enough also, the activities of azithromycin and sparfloxacin in the simultaneous presence of IFN-γ, L-MMA, and catalase were not different from those observed in control cells (i.e., cells unexposed to IFN-γ).

To examine whether the modulation of the antibiotic action brought about by IFN-γ was related to changes in the bacterial growth patterns only, we examined the behavior of the *L. monocytogenes* Hly^- strain in this system. These nonvirulent bacteria do not multiply in THP-1 macrophages (41) and remain confined in phagosomes (see above). Figure 7B shows that intracellular *L. monocytogenes* Hly^- bacteria are insensitive to ampicillin in control as well as in IFN-γ-treated cells. Yet, IFN-γ increased the activity of azithromycin and sparfloxacin to an extent similar to that seen with the Hly^+ virulent variant.

Modulation of antibiotic cellular accumulation by IFN-γ and by L-MMA and catalase. Figure 8A, B, and C show the kinetics of the uptake and the accumulation levels recorded for ampicillin, azithromycin, and sparfloxacin in control and IFN-γ-treated macrophages. As observed for many other cell types, the ampicillin cell content remained lower than the extracellular one, while sparfloxacin achieved a fair degree of accumulation (approximately 12-fold) and azithromycin accumulated to a very great extent (up to 70- to 90-fold). Pretreatment of

TABLE 1. Influence of IFN-γ and IFN-γ plus L-MMA–catalase on antibiotic activity[a]

Antibiotic	IFN-γ vs control (*P*)	IFN-γ plus L-MMA–catalase vs control (*P*)
Ampicillin	− (<0.01)	− (<0.001)
Azithromycin	++ (<0.005)	NS
Sparfloxacin	+++ (<0.001)	NS

[a] The table shows a synopsis and statistical analysis of the differences observed between experimental groups as described for Fig. 7A. Statistical analysis was performed by the Student *t* test with a corresponding level of significance. +, ++, and +++, lowest to highest increase, respectively; −, decrease; NS, no significant change.

TABLE 2. Influence of antibiotics on *L. monocytogenes* growth[a]

Condition	AMP vs none (*P*)	AMP vs AZI (*P*)	AMP vs SPA (*P*)	AZI vs SPA (*P*)
Control	++ (<0.001)	NS	++ (<0.001)	++ (<0.001)
With IFN-γ	NS	+ (<0.01)	+++ (<0.001)	++ (<0.001)
With IFN-γ and L-MMA–catalase	++ (<0.001)	++ (<0.001)	+++ (<0.001)	+++ (<0.005)

[a] The table shows a synopsis and statistical analysis of the differences observed between experimental groups as described for Fig. 7A. Statistical analysis was performed by the Student *t* test with a corresponding level of significance. None, no antibiotics; AMP, ampicillin; AZI, azithromycin; SPA, sparfloxacin; +, ++, and +++, lowest to highest increase, respectively; NS, no significant change.

THP-1 cells with IFN-γ did not significantly modify the cellular concentration of ampicillin. In contrast, the accumulation of sparfloxacin and that of azithromycin were increased 1.5- and 1.7-fold, respectively. This effect was noted already after 2 h of incubation with the drugs and was maintained for up to at least 24 h. In parallel, we tested whether the combination of L-MMA and catalase influenced the accumulation of sparfloxacin in IFN-γ-treated cells, but as shown in Fig. 8D, no significant effect was observed.

Influence of antibiotic concentration, pH of incubation medium, and bacterial growth on the activity of antibiotics towards the *L. monocytogenes* Hly$^+$ strain in broth. Because IFN-γ not only prevents intracellular growth of *Listeria* but also increases the cellular concentrations of azithromycin and sparfloxacin and affects the pH to which intracellular *Listeria* is exposed (i.e., preventing it from reaching the neutral environment of the cytosol and restricting it to the slightly acidic medium of the phagosomes), we systematically tested the influence of the drug concentration and of the acidity on the intrinsic activities of the antibiotics used toward both actively multiplying and nonmultiplying *Listeria* organisms. Kill curves were obtained by exposing bacteria for up to 5 h to drug concentrations ranging from 1 to 10× their MICs at pH 7.3 and 6.8, with cultures in logarithmic growth (typical increase of 2 log CFU in the absence of antibiotic), as well as in a nongrowing stage (by maintaining bacteria in PBS rather than in TSB,

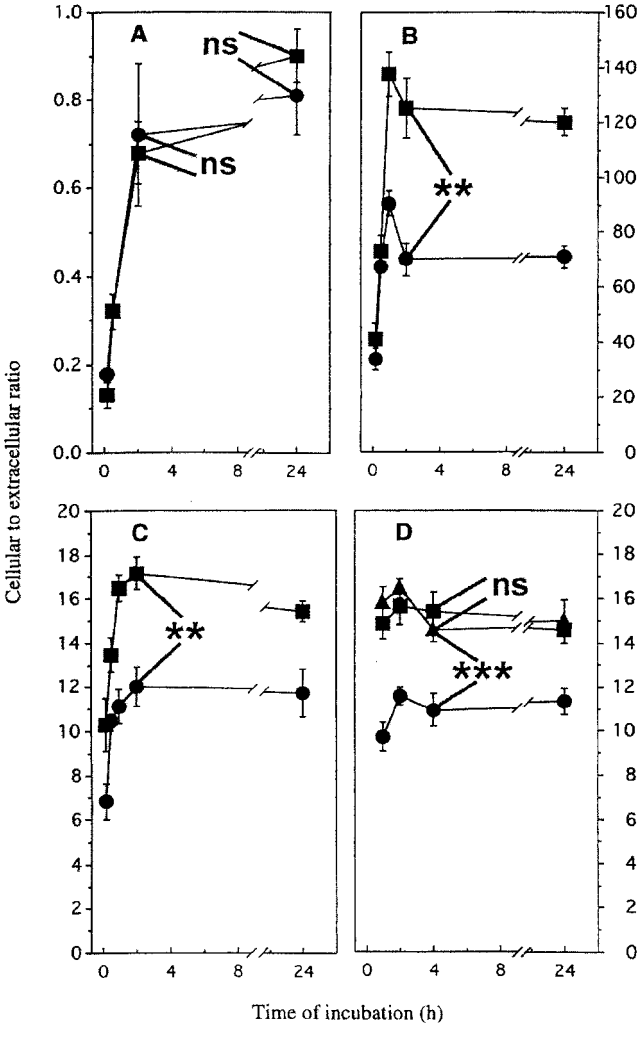

FIG. 8. Accumulation of antibiotics in THP-1 macrophages. The ordinate shows the apparent cellular-to-extracellular-drug-concentration ratio (see Materials and Methods). (A) Ampicillin; (B) azithromycin; (C and D) sparfloxacin. ●, control cells; ■, cells pretreated with IFN-γ (100 U/ml); ▲ (D), cells pretreated with IFN-γ and incubated with catalase and L-MMA. Data are means ± SD (n = 3). The results of the statistical analysis of the differences seen at 2 h (and at 24 h for ampicillin) are shown in the graph (**, $P < 0.005$; ***, $P < 0.001$; ns, nonsignificant).

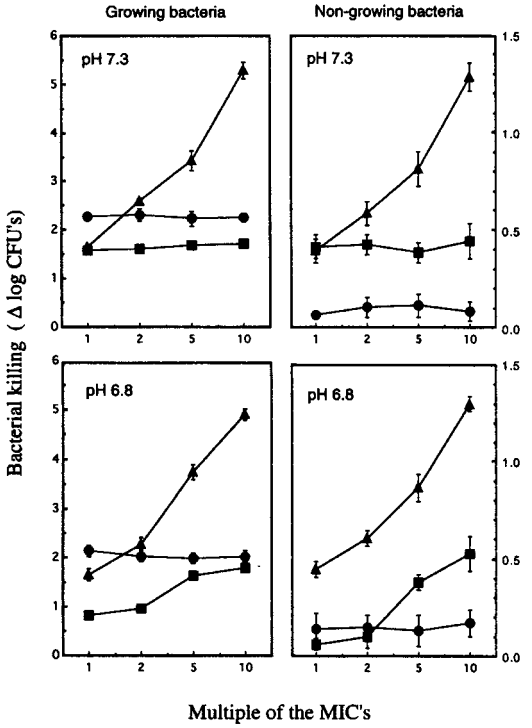

FIG. 9. Killing patterns of growing and nongrowing *L. monocytogenes* Hly$^+$ bacteria upon exposure to antibiotics at multiples of their MICs in broth (growing bacteria) and PBS (nongrowing bacteria), respectively (●, ampicillin; ■, azithromycin; ▲, sparfloxacin). Bacterial killing is defined as the decrease of the $\log_{10}$ of the number of bacteria over a 5-h period (positive numbers therefore mean a reduction of the number of viable bacteria). Data are means ± SD (n = 3).

which completely prevents their division, as observed earlier with *Escherichia coli* [53] and *Staphylococcus aureus* [3]).

Figure 9 shows the data obtained at 5 h. Considering growing bacteria first, it clearly appears that ampicillin exerts a significant bactericidal effect, which, however, is not dose dependent or influenced by the decrease of pH in the limits of our studies. The effect of azithromycin was also largely dose independent, but its activity was severely impaired by the decrease of pH. In striking contrast, the activity of sparfloxacin was consistently dose dependent and not affected by the pH change. With nongrowing bacteria, ampicillin lost almost all its bactericidal activity. Azithromycin was very modestly active at neutral pH and lost all activity at acid pH at low multiples of its MIC. Sparfloxacin remained bactericidal in a dose-dependent fashion at both pH 7.4 and pH 6.8, but its overall activity was markedly reduced toward nongrowing compared to growing bacteria.

DISCUSSION

L. monocytogenes is an invasive organism which causes prolonged, recurrent infections because of its ability to enter cells, thrive intracellularly, and spread from cell to cell (12, 48). Eradication of the intracellular forms of *Listeria* appears therefore critical for effective therapy. The control of listeriosis is very dependent on an efficient T-cell immune response (2), suggesting a key role for activated macrophages. The importance of IFN-γ, the secretion of which is triggered and maintained by the persistent production of interleukin 12 (IL-12) (52), has been clearly recognized in this context (7, 23, 29). Yet, few studies have so far examined directly the potential cooperation of IFN-γ with antibiotics. Somewhat surprisingly, also, convential antibiotic therapy of listeriosis rests mostly on the use of ampicillin and gentamicin (2), i.e., two classes of antimicrobials classically which do not rapidly nor extensively accumulate in phagocytes (51) and which are not therefore expected to actively act against the intracellular forms of *Listeria*. We, accordingly, have attempted to set up a model in which the influence of IFN-γ and its cooperation with antibiotics could be examined in a systematic fashion. We have used THP-1 macrophages since these cells share many specific markers with human phagocytes, including the expression of receptors for cytokines and IFN-γ in particular (38). Our data on IFN-γ binding kinetics unambigously confirm this for the cells that we used. We also showed earlier that THP-1 cells provide a suitable environment for *Listeria* growth and that this growth is effectively prevented by preexposure of these cells to IFN-γ (41). In the same study, we showed that *Listeria* phagocytosed by THP-1 cells is sensitive to ampicillin, azithromycin, and sparfloxacin but not to gentamicin at equipotent, microbiologically meaningful concentrations (10× the MIC). IFN-γ was also shown to cooperate with azithromycin and sparfloxacin to achieve more significant killing than that observed with these antibiotics alone but to suppress the intrinsic activity of ampicillin.

A first critical observation made in the present study is that the effect of IFN-γ on macrophage permissiveness toward *Listeria* is clearly dependent on its specific binding and is probably mediated by nitrogen- and oxygen-derived reactive species. First, the control that IFN-γ exerts on intracellular bacterial growth is abolished by exposing the cells to antibodies raised against the IFN-γ receptor and is concentration dependent at suboptimal IFN-γ receptor occupancy. These data are consistent with a recent report indicating that an interferon consensus sequence binding protein (ICSPB-IRF2 complex) is essential for IFN-γ-mediated protection against *Listeria* (15). Next, we show that the effect of IFN-γ on bacterial growth is entirely suppressed in cells exposed to L-MMA and catalase. IFN-γ has been shown to trigger the production of H_2O_2 and to induce substantial NO secretion by macrophages (10, 19, 25). The fact that L-MMA and catalase must be used together to obtain complete suppression of the effect of IFN-γ suggests that both oxygen-derived and nitrogen-derived reactive species must be released and/or act synergistically to control *Listeria* growth. This is also consistent with the finding that an absence of the production of RNI, without a concomitant effect on oxygen-derived reactive intermediates, fails to always decrease bacterial density (15, 25). We ourselves found that THP-1 cells transfected with the gene coding for inducible NO synthase, to overexpress this protein and enhance NO production in the absence of IFN-γ, are still partly permissive for bacterial growth (34a). In parallel, we confirm for THP-1 cells the fact that IFN-γ completely prevents the escape of *L. monocytogenes* Hly^+ bacteria from phagosomes to the cytosol (37). The present data rule out a direct role of oxygen- and nitrogen-derived reactive species in this confinement, e.g., through an inactivation of listeriolysin O, since L-MMA and catalase are unable to reverse this effect. Yet, it is likely that such a confinement of *Listeria* in phagosomes is important to ensure an optimal contact of the oxygen-derived reactive species with the bacteria since the latter are produced at the time of phagocytosis or within the phagocytic vacuoles, i.e., in close contact with the bacteria.

A second critical observation is that the antagonism that IFN-γ exerts on the activity of ampicillin, already evidenced in our earlier study (41), is partly suppressed when cells are also treated with L-MMA and catalase (ampicillin becoming now able to exert a static effect under these conditions). Yet, in these cells *Listeria* remains located in phagosomes, which demonstrates that, contrary to what we proposed earlier, ampicillin must have access to this subcellular compartment. Since ampicillin shows no concentration dependence in its activity on *Listeria*, the present experiments provide, however, no clue as to the proportion of intracellular ampicillin that effectively reaches the phagosomes but merely indicate that its concentration therein must probably exceed its MIC. This point, therefore, needs to be further studied by directly determining quantitatively the ampicillin subcellular distribution.

A third important observation made in the present study is that the synergy that we observed earlier between IFN-γ and sparfloxacin appears entirely due to the capacity of the cytokine to trigger the production of H_2O_2 and NO, because this synergy is completely lost in the presence of L-MMA and catalase. Interestingly, IFN-γ shows also a synergy with sparfloxacin toward the *L. monocytogenes* Hly^- strain, an organism which is always phagosomal. Yet, the data do not allow us to assess the importance of the phagosomal confinement of *Listeria* in this synergy per se, since we have not, in the present experiments, triggered the production of nitrogen- and oxygen-reactive species without at the same time causing the sequestration of *Listeria* in phagosomes. At first glance, it would seem that the increased accumulation of sparfloxacin induced by IFN-γ, and for which we have no simple explanation, should also participate in the synergistic effect described here, since this drug shows a marked dose dependency in its antimicrobial activity against *Listeria*, at least in broth. Yet, this potential pharmacodynamic effect must be considered as unimportant since L-MMA and catalase completely suppress the synergy between sparfloxacin and IFN-γ without reducing the increase in drug accumulation caused by IFN-γ. Actually, a direct cooperation between sparfloxacin and IFN-γ through RNI-ROI appears a more plausible hypothesis when taking into account

the mode of action of fluoroquinolones. These drugs indeed are inhibitors of topoisomerase II and are highly genotoxic in procaryotes (especially the most recent generation of fluoroquinolones, of which sparfloxacin is a typical member) (1). They, thereby, induce SOS DNA repair mechanisms that can be impaired by RNI-ROI. Moreover, fluoroquinolones themselves generate oxidant species (13) and may stimulate oxidative metabolism (47). Finally, the phototoxicity of fluoroquinolones, which sparfloxacin clearly demonstrates (43), has been related to their capacity to induce the generation of ROI and singlet oxygen (30). In contrast to sparfloxacin, azithromycin, the accumulation of which is also markedly enhanced by treatment with IFN-γ but which is not known to trigger RNI-ROI production, shows only a more modest increase of activity in IFN-γ-treated cells. This could also have been explained by the lack of dose dependency of the activity of azithromycin and would have emphasized the fact that the intracellular activity of an antibiotic cannot be simplistically correlated with its level of accumulation only. Yet, because we ruled out a pharmacodynamic mechanism to explain the increased activity of sparfloxacin, we probably cannot use this argument here without caution. It is indeed possible that IFN-γ increases drug accumulation while at the same time decreasing its intracellular bioavailability, for instance, by confining the excess of drug in an organelle with low exchange capabilities. This possibility is perhaps of critical importance for azithromycin, for which a change in the lysosomal pH or composition could easily induce a marked increase in drug storage without concomitant increase of the net amount of free, active drug (51). Finally, the confinement of *Listeria* in phagosomes and the decreased activity of azithromycin that it implies because of the lower pH prevailing in these vacuoles may also play a significant role.

Beyond these mechanistic considerations of the effects of IFN-γ on *Listeria* intracellular infection, the present data may also suggest new avenues for biological and clinical research. First, they emphasize the potential roles of cytokines and of the involvement of oxygen- and nitrogen-derived reactive species for the control of *Listeria* infection. Thus, in addition to IFN-γ, other cytokines such as tumor necrosis factor alpha, IL-12, and IL-4, which play important but contrasting roles in *Listeria* eradication (19, 25, 45, 52), may well be worthwhile investigating in this context. With respect to antibiotic therapy, the data presented here and in our previous report (41) also suggest that gentamicin will always be inactive against the intracellular forms of *Listeria* whether the cells are activated or not. This result is consistent with other reports which pointed to gentamicin inactivity at least in short-term experiments (31) (long-term exposure may indeed result in a significant intracellular accumulation of aminoglycosides [50]). Actually, gentamicin was even used to unambiguously distinguish between intracellular and extracellular models of bacterial multiplication of *Listeria* in several cell culture models, including macrophages (36). Our data also suggest that ampicillin may become ineffective against the intracellular forms of *Listeria* in macrophages of patients with an adequate IFN-γ response. This raises obvious questions concerning the usefulness of this antibiotic for eradication of *Listeria* in chronically infected patients. Conversely, a macrolide or, even better, a fluoroquinolone might be more effective than usually thought in these situations. As suggested earlier (41), these issues may warrant animal and clinical studies, especially since rational explanations for the differences observed are now partially available. Yet, it must be recognized that the data presented here were obtained with cells exposed to a single, equipotent concentration for all antibiotics studied (10× the MIC), for obvious reasons of homogeneous pharmacological comparison. These concentrations do not correspond exactly to those obtained in serum and extracellular fluids during conventional therapies (thus, 2 mg/liter is probably quite low for ampicillin, while 4 and 12 mg/liter for azithromycin and sparfloxacin are quite above extracellular concentrations that can be obtained under clinically acceptable conditions of administration). Further studies will therefore need to explore the influence of the drug extracellular concentration on the effects described here. Yet, the discovery and development of new derivatives of macrolides and fluoroquinolones with enhanced activity against *Listeria* may allow successful application in the clinic of some suggestions made here. *Listeria* infection is usually limited to elderly, immunocompromised patients, neonates, and pregnant women (2) and may therefore be considered not a very important medical problem. Yet, it may constitute a general paradigm of protracted, recurrent infections, and the results obtained with this facultative intracellular pathogen could be taken into consideration for the design of improved approaches in many other situations of intracellular infection.

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