Modulation of Intracellular Growth of *Listeria monocytogenes* in Human Enterocyte Caco-2 Cells by Interferon-γ and Interleukin-6: Role of Nitric Oxide and Cooperation with Antibiotics

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The influence of interferon (IFN)–γ and interleukin (IL)–6 on the intracellular growth of *Listeria monocytogenes* phagocytosed from the apical pole was examined in polarized Caco-2 cells. IFN-γ (from the apical pole) and IL-6 (from the basolateral pole) considerably reduced the bacterial intracellular growth, an effect largely abolished by l-monomethyl arginine. Both cytokines caused overexpression of inducible nitric oxide synthase. IL-6, but not IFN-γ, caused a partial restriction of *L. monocytogenes* in phagosomes and largely prevented the cytosolic forms from being surrounded by actin. Ampicillin was bacteriostatic in unstimulated cells but modestly bactericidal in cells treated with IFN-γ and IL-6. Azithromycin (a macrolide) was fairly bactericidal and sparfloxacin (a fluoroquinolone) highly bactericidal in all situations. IFN-γ and IL-6 may therefore be important determinants in the protection of epithelial cells from intracellular multiplication of *L. monocytogenes.* Ampicillin may fail in their absence, requiring the use of other antibiotics such as the fluoroquinolones.

Listeria monocytogenes is a gram-positive pathogen that has devised strategies to locate itself intracellularly where humoral host defense mechanisms are minimal. It may infect a variety of cell types, including macrophages, Küpffer cells, hepatocytes, epithelial cells, and fibroblasts [1–7]. Yet, being a food-borne pathogen, *L. monocytogenes* enters the host first by invading the epithelial cells of the gut. Caco-2 cells derive from a human colorectal carcinoma [8] and mimic the functional behavior of normal intestinal epithelial cells. They can be cultivated so as to display clear-cut polarization [9] and to constitute an effective epithelial barrier. Caco-2 cells, have therefore been widely used since the mid-1980s to study the early steps of *L. monocytogenes* natural invasion [3, 10–12]. In this model, *L. monocytogenes* was shown to quickly escape from the phagocytic

vacuole into the cytosol through a listeriolysin O-dependent mechanism. Enveloping itself with F-actin, *L. monocytogenes* interacts with host-cell filaments to penetrate adjacent cells [13], thereby colonizing the host while avoiding contact with the extracellular natural mechanisms of defense and chemotherapeutic agents. The development of an effective cellular response and/or the use of drugs that act intracellularly and, preferably, in cooperation with this cellular response appears therefore to be of first importance for the control of *L. monocytogenes* infection. In this context, interferon (IFN)–γ has been identified as an important cytokine for its ability to induce bactericidal macrophages toward *L. monocytogenes* and therefore help control the infection after a successful invasion [14, 15]. IFN-γ, however, also exerts a series of effects on enterocytes, such as enhancing interleukin (IL)–6 production [16], controlling lipoxin A4 receptor [17], up-regulating α- and β-chemokine secretion [18], inhibiting rotavirus entry [19], or increasing amoeba binding [20]. IL-6, which is produced by enterocytes upon bacterial stimulation and infection [21], is also important in host resistance to *Listeria* infection. IL-6 is thought to act by stimulating neutrophil production [22, 23]. These neutrophils play an important role in listericidal early innate response [24–28]. Yet IL-6 is also known to influence the enterocytes in an autocrine or paracrine fashion, causing, for instance, the release of epidermal growth factor (EGF) or EGF-like molecules, thereby modulating triacylglycerol and apolipoprotein B secretion [29].

These data prompted us to directly examine the potential influence of IFN-γ and IL-6 on experimental infection of Caco-2 cells by *L. monocytogenes.* In parallel, we have examined the cooperation of these cytokines with antibiotics, selecting am-
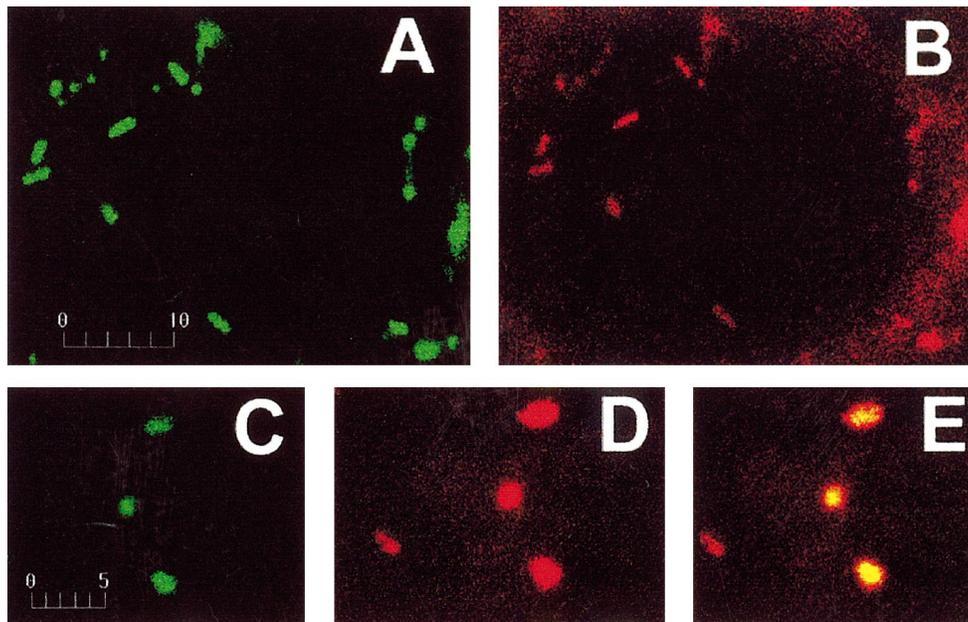
Figure 1. Confocal microscope images of Caco-2 cells 4 h after phagocytosis of fluorescein-labeled *Listeria monocytogenes*. Cell actin was revealed with Cy3-labeled anti-actin monoclonal antibody. *A, C,* cells examined for fluorescein ($\lambda_{excitation}$ = 492 nm ; $\lambda_{emission}$ = 516 nm; *B, D,* cells examined for actin $\lambda_{excitation}$ = 552 nm; $\lambda_{emission}$ = 570 nm); *E,* examination for both fluorescein and actin. Magnification: *A, B,* 10 μm; *C–E,* 5 μm.

picillin, which is an antibiotic that does not accumulate in cells but is nevertheless often considered as a first-choice therapy in listeriosis [30], and 2 other antibiotics known to accumulate in cells, namely, azithromycin (a macrolide) and sparfloxacin (a fluoroquinolone), which both have proven bactericidal in *L. monocytogenes*-infected macrophages [31].

Materials and Methods

Cells. The human carcinoma cell line Caco-2, established by J. Fogh and colleagues [32], was kindly provided by Dr Y.-J. Schneider (Biochemistry Unit, Université Catholique de Louvain, Louvain-la-Neuve, Belgium). Caco-2 cells were cultured in Dulbecco's modified Eagle medium containing glucose (2.9 g/L) and supplemented with 10% decomplemented fetal calf serum and 2 m*M* L-glutamine. Cells were seeded at a density of 1.5×10^5 cells/mL (2 mL/well) onto tissue culture inserts (Transparent PET track-etched membranes, 4.2 cm² growth area; Becton Dickinson, Erembodegem, Belgium) and maintained in an atmosphere of 95% air–5% CO_2 at 37°C. Culture was performed with 2 mL of medium in both the apical and basal chambers. Confluence of the cells and formation of an effective epithelial barrier was assessed by microscopy and determination of transepithelial electrical resistance by use of a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA).

Bacteria. *L. monocytogenes*, hemolysin-producing strain EGD (serotype 1/2a from the Trudeau Institute, Saranac Lake, NY), obtained from Dr. P. Berche (Laboratoire de Microbiologie, Faculté de Médecine Necker, Paris, France), was maintained and used exactly as described elsewhere [33]. MICs, determined in the cell

culture medium by the arithmetic dilution method (0.1 μg increment) and at a constant inoculum (10^6 bacteria / mL), were 0.3 μg/mL for ampicillin, 0.5 μg/mL for azithromycin, 1.4 μg/mL for sparfloxacin, and 0.8 μg/mL for gentamicin.

Infection of Caco-2 cells and validation of the model. Caco-2 cells, washed with PBS, were exposed from their apical membrane surface to bacterial suspensions (2 mL) prepared in fresh culture medium and adjusted to obtain the desired MOI of *L. monocytogenes*. Cells were incubated at 37°C for 1 h to allow penetration of bacteria, washed 4 times with PBS, and further incubated with culture medium containing gentamicin at a listericidal concentration (8 μg/mL 10× the MIC) for 1 h to eliminate viable extracellular bacteria. Cells were then washed with prewarmed PBS and thereafter incubated for up to 5 h to assess bacterial growth. Both electron and confocal microscopy (see [33] for methods) were used to validate the model with respect to earlier studies (3, 10–13). Thus, bacteria began to appear in the cytosol surrounded by a thick rim of finely granular material (electron microscopy) within 1 h after phagocytosis. At 4 h, confocal microscopy (figure 1) showed that almost all cell-associated bacteria (stained by exposure to fluorescein isothiocyanate prior to phagocytosis) colocalized with actin (stained with Cy3-labeled anti-actin monoclonal mouse antibody after cell fixation and permeabilization; Sigma, St Louis). Uptake efficiency was 2%–4% of the inoculum at initial bacteria to cell ratios of 5–20, and a ~10-fold increase in cell-associated cfus was seen over a period of 5 h. Gentamicin, added at a concentration of 10× the MIC, did not influence this growth, showing its intracellular character. All subsequent infection experiments (except for morphologic studies in which the bacteria to cell ratio was 50) were made at a bacteria-to-cell ratio of 5.

Treatment with cytokines. Recombinant human IFN-γ with a

specific activity of 2×10^7 U/mg protein and recombinant human IL-6 with a specific activity of 1×10^8 U/mg protein were purchased from Boehringer (Mannheim, Germany), distributed as aliquots stored at $-20°C$, and thawed immediately before use. Monolayers of Caco-2 cells cultured onto tissue culture inserts to confluency were exposed to IFN-γ or IL-6 for 24 h at 37°C through their apical and/or basolateral membrane surfaces before phagocytosis and/or exposure to antibiotics.

Quantitative morphologic evaluation of the influence of cytokines on bacteria distribution. This was done by both confocal and electron microscopy. For confocal microscopy, bacteria were stained with fluorescein (see previous section) and actin after cell fixation with rhodamine-phalloidin. Samples were examined at random and recognizable bacteria assessed as being naked (i.e., emitting a green fluorescence) or surrounded by actin (i.e., emitting a red or yellow fluorescence). For electron microscopy, samples were examined at random and recognizable bacterial profiles were assessed as being (1) in membrane-bound electron lucent profiles (phagosomes), (2) free and naked in the cytosol, or (3) free in the cytosol but surrounded by a finely granular material.

Inhibition of the production of reactive nitrogen intermediates. Cells were incubated with 400 μM l-monomethyl arginine (L-MMA, Calbiochem Novabiochem International Inc, San Diego, CA) for 24 h before infection with *L. monocytogenes,* and during the 5-h postinfection period. We checked that L-MMA effectively suppressed all detectable increase of nitric oxide (NO) production caused by the preincubation with cytokines (Greiss reaction [34]).

Cell transfection. The plasmid inducible nitric oxide synthase (piNOS) containing rat iNOS cDNA inserted into *Not*I and *Hind*III sites of the pcDNA3 vector (Invitrogen, Carlsbad, CA) was kindly provided by Dr. A. E. Karlsen (Steno Diabetes Center, Gentofte, Denmark). This expression vector was transfected into Caco-2 cells by means of calcium phosphate coprecipitation. Briefly, 60-mm dishes were seeded with 3×10^5 cells in 5 mL of culture medium. After 24 h, the cells were exposed to CaPO$_4$/DNA (2 μg DNA per dish) and incubated for 5 h. Cells were then washed with PBS and covered with medium without CaPO$_4$/DNA for 2 days before use. Expression of iNOS protein in transfectant cells was checked by Western blot analysis. Experiments made in parallel with the β-galactosidase gene (followed by histochemical staining) revealed a transfection efficiency of 25%–30% of cells.

Western blot analysis. Lysates from washed cells were prepared in ice-cold radioimmunoprecipitation assay (NP-40 1%, sodium deoxycholate 0.5%, and SDS 0.1% in PBS) lysis buffer supplemented with protease inhibitors (0.1 mg/mL phenylmethylsulfoxylfluoride, 30 μL/mL aprotinin, and 1 mM sodium orthovanadate) and subjected immunoblot analysis after SDS-PAGE with a rabbit anti-iNOS polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), by use of an anti-rabbit IgG/alkaline phosphatase conjugate for detection. Standards of known relative mobility (M$_r$) were run in parallel and used to calculate the apparent M$_r$ of the bands detected in the samples by graphic interpolation. The anti-iNOS antibody used does not cross-react with the neuronal and endothelial constitutive isoforms of NOS.

Assessment of intracellular antibiotic activity. After phagocytosis, infected cells were exposed to the antibiotics from both their apical and basolateral poles at an extracellular concentration of 10× their MIC. At selected time intervals up to 5 h, cells were washed with ice-cold PBS, removed from the membrane with a rubber policeman, and transferred to ice-cold distilled water. The resulting cell lysate was used after thorough dispersion for determination of the number of viable intracellular bacteria (by colony counting after plating on tryptic soy agar) and for assay of total cell protein [35]). All results are expressed as cfu/mg of cell protein.

Determination of cellular antibiotic accumulation. Cells were exposed to antibiotics at a final concentration of 10 mg/L in both chambers. Uptake of sparfloxacin was determined by using [^{14}C]-labeled drug, and uptake of ampicillin and azithromycin was measured by a microbiologic assay (radial diffusion assay in agar) with *Bacillus stearothermophilus* for ampicillin and *B. subtilis* for azithromycin as test organisms as described by Ouderkirk [36] and Grove and Randall [37], respectively. The cell antibiotic content was expressed by reference to the protein content of the samples, and the level of accumulation of each antibiotic was then expressed as the ratio of the apparent cellular concentration of the drug to its extracellular concentration, by use of a conversion factor of 5 μL of cell volume per mg of cell protein (a value close to that found experimentally for cultured fibroblasts [38], mouse peritoneal macrophages [39], and several other types of cultured cells).

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

Statistical analysis. These were made by the Student *t* test (comparison between groups) by using Graphpad Instat version 3.01. for windows 95 (Graphpad software, San Diego, CA).

Results

Effect of IFN-γ and IL-6 on intracellular L. monocytogenes *growth.* Figure 2 shows that both IFN-γ or IL-6 reduced the extent of growth of intracellular *L. monocytogenes* in Caco-2 cells. Yet, interestingly enough, the effect was largely dependent both on the type of cytokines and the side of the cell to which these cytokines were presented, being maximal for an exposure from the apical side for IFN-γ (50% reduction) but from the basolateral side for IL-6 (almost 80% reduction). Simultaneous treatment of cells by both sides led to a similar result to that obtained with a treatment from the side giving the largest effect.

Role of NO in IFN-γ and IL-6 effects. Figure 3 shows that L-MMA almost completely defeated the effect of IFN-γ on bacterial growth and partly suppressed that of IL-6. Yet L-MMA itself had no significant influence on the intracellular growth of *L. monocytogenes* in control cells. L-MMA did not affect the ability of *L. monocytogenes* to enter cells (no reduction in phagocytosis index; data not shown). To further char-
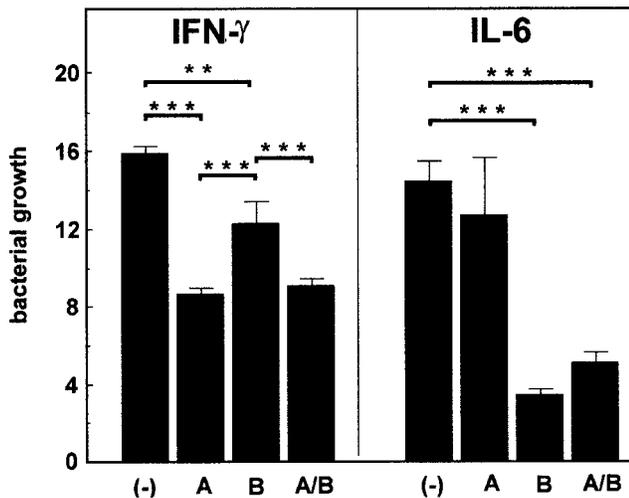
Figure 2. Influence of interferon (IFN)–γ (100 U/mL; left) and interleukin (IL)–6 (200 U/mL; right) on the intracellular growth of *Listeria monocytogenes* in Caco-2 cells. —, no treatment (control); A, cells treated through their apical membrane surfaces; B, cells treated through their basolateral membrane surfaces; A/B, cells treated simultaneously through their apical and basolateral membrane surfaces. Bacterial growth is defined as the ratio of the cfus observed in cell samples 5 h after phagocytosis to the number observed immediately after phagocytosis. Data are mean ± SD (bars, *n* = 3). The horizontal square brackets show the statistical significance of the differences used in our analysis of the data (** *P* < .01; *** *P* < .001).

acterize how NO production was involved in the Caco-2 cells response to IFN-γ or IL-6, extracts of cells treated apically with IFN-γ or basolaterally with IL-6 were fractionated on SDS-PAGE and proteins analyzed by immunoblotting by use of an anti-iNOS polyclonal IgG. Figure 4 shows that iNOS protein, which is absent in control cells, was moderately expressed in apically IFN-γ–treated cells, and prominently expressed in basolaterally IL-6–treated cells (gels showed an additional band at $M_r \approx 180,000$ but with no marked difference between samples). In parallel, we examined the expression of iNOS in cells transfected with the corresponding gene (in a construction including the SV40 promoter). Figure 4 shows that the transfected cells expressed large amounts of iNOS protein, and figure 5 shows that these transfected cells were no longer permissive for *L. monocytogenes* growth. The latter effect was largely dependent of NO production because the same cells again supported *L. monocytogenes* growth almost to the same value as control cells if exposed to L-MMA.

Influence of IFN-γ and IL-6 on the intracellular trafficking of L. monocytogenes. Both confocal and electron microscopy were then used to assess the potential changes in subcellular localization of *L. monocytogenes* in cells treated apically with IFN-γ or basolaterally with IL-6 and in control cells. A typical illustration of the electron microscopic observation made with IFN-γ and IL-6 is shown in figure 6. Most visible bacteria in

IFN-γ–treated cells were found in cytosol, surrounded by a thick rim of granular material (figure 6*A*, 6*B*). In contrast, the localization of *L. monocytogenes* in IL-6–treated cells was more diverse, with some bacteria appearing in vacuoles (figure 6*C*), whereas others appeared in the cytosol most often naked (figure 6*E*, 6*F*), but occasionally surrounded by a clearly recognizable organized granular material (figure 6*D*). Table 1 gives the results of a quantification of these observations together with those made by confocal microscopy. The data show that IFN-γ does not markedly change the fate of bacteria. In contrast, IL-6 clearly confines about half the bacteria in phagosomes and makes the largest proportion of the cytosolic bacteria unable to become surrounded by actin. The latter conclusion is confirmed by the confocal microscopy studies that show most of the bacteria to be free from actin in IL-6–treated cells (note that confocal microscopy cannot distinguish between phagosomal and cytosolic bacteria if the latter are not surrounded with actin).

Cooperation between cytokines and antibiotics against intracellular L. monocytogenes. To test whether natural host-mediators (cytokines) and drugs could cooperate for the elimination of intraepithelial *L. monocytogenes,* we tested the influence of 3 antibiotics of different classes, alone and in combination with IFN-γ or IL-6. Figure 7 shows that intracellular *L. monocytogenes* responded very differently to these antibiotics in control cells. Thus, compared with cells incubated without antibiotics in which *L. monocytogenes* showed an almost 15-
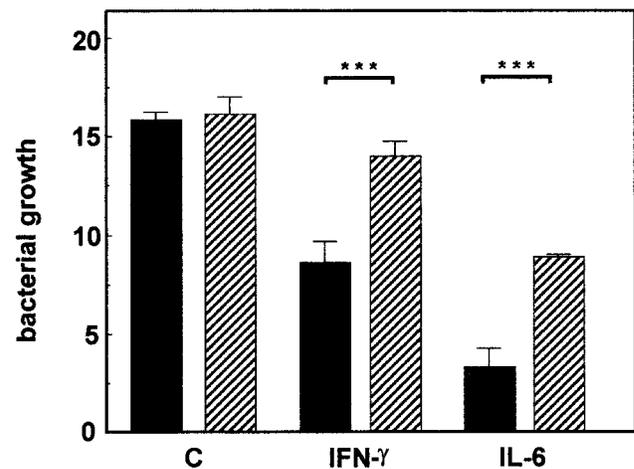

Figure 3. Influence of l-monomethyl arginine on the reduction of bacterial growth caused by interferon (INF)–γ and interleukin (IL)–6 compared with untreated cells. C, untreated cells; IFN-γ, cells treated apically with IFN-γ (100 U/mL); IL-6, cells treated basolaterally with IL-6 (200 U/mL). Bacterial growth is defined as the ratio of the cfus observed in cell samples 5 h after phagocytosis to the number observed immediately after phagocytosis. Data are mean ± SD (bars, *n* = 3). The horizontal square brackets show the statistical significance of the differences used in our analysis of the data (***, *P* < .001).
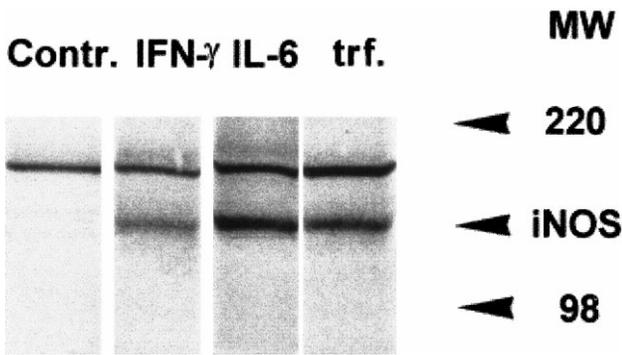
Figure 4. Western blot analysis of Caco-2 cell extract with anti-inducible nitric oxide synthase (iNOS) antibody. Contr., untreated cells; IFN-γ, cells treated apically with interferon-γ (100 U/mL); IL-6, cells treated basolaterally with interleukin-6 (200 U/mL); trf, cells transfected with the iNOS gene in a construction including the SV40 promotor. On the right are indicated the positions of the molecular mass of 2 markers in the calibration lane (not shown).

fold increase in cfu in 5 h, ampicillin (a β-lactam) at a concentration of 10× the MIC afforded only a static effect. In contrast, sparfloxacin (a fluoroquinolone) at an equipotent extracellular concentration was highly and rapidly bactericidal. Azithromycin (a macrolide), again at an equipotent extracellular concentration, was modestly bactericidal and only after 2–3 h of contact. These contrasting effects did not simply reflect differences in drug accumulation because the apparent intracellular to extracellular antibiotic concentration ratios at 5 h (i.e., at near equilibrium; table 2) were 0.50 ± 0.04 for ampicillin, 27.4 ± 4.6 for azithromycin, and 8.71 ± 0.14 for sparfloxacin. As shown by the data presented in figure 8, IFN-γ and IL-6 showed a positive cooperation with ampicillin bringing their respective effects from an almost static to a bactericidal one. In parallel, the activity of sparfloxacin was somewhat reduced by IL-6, whereas the activity of azithromycin was only very modestly affected by the cytokines. But in all cases, these antibiotics remained highly bactericidal.

Modulation of the antibiotic cellular accumulation by IFN-γ and IL-6 in Caco-2 cells infected and uninfected with L. monocytogenes. Table 2 shows the accumulation levels recorded at 5 h (i.e., at near equilibrium) for ampicillin, azithromycin, and sparfloxacin in control, IFN-γ–, and IL-6–treated Caco-2 cells. As in all other cell types studied so far, ampicillin did not accumulate in control Caco-2 cells, with an intracellular apparent concentration remaining consistently lower than the extracellular one. In contrast, and again as observed in most other cell types, sparfloxacin and azithromycin achieved a most significant accumulation in Caco-2 cells. Infection did not markedly alter this behavior. IFN-γ did not exert a biologically significant effect on drug uptake, except for ampicillin in infected cells. IL-6 most significantly increased the accumulation

of ampicillin and azithromycin in both uninfected and infected cells. Sparfloxacin accumulation was virtually unchanged in uninfected cells and only modestly increased in infected cells preexposed to IL-6.

Discussion

Caco-2 cells exhibit many of the structural and functional differentiation patterns of mature intestinal enterocytes, including a typical brush border and clear-cut polarization [40–42], and can provide signals that are important for the initiation and the amplification of an acute mucosal inflammatory immune response [43]. They have been successfully used to study the intracellular growth of *L. monocytogenes* [3, 11]. In our experimental system (cells cultivated as monolayers of high resistivity on inserts), most of the bacteria uptake must occur by the apical pole, and uptake efficiency was accordingly higher than that observed in more conventional systems, where uptake can also occur by the basolateral pole [44]. Otherwise, the model behaved as described elsewhere [3, 11, 13, 44–46]. A first conclusion of our study could therefore be that apical
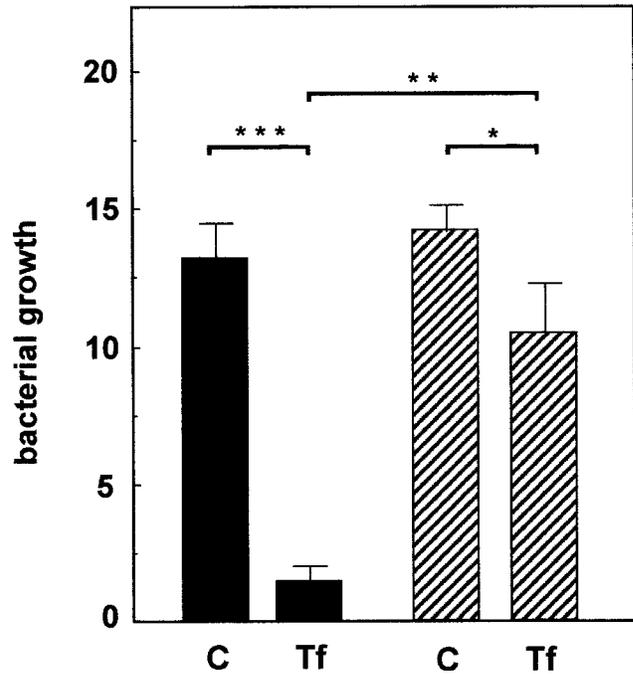

Figure 5. Influence of l-monomethyl arginine on the intracellular growth of *Listeria monocytogenes* in control Caco-2 cells (C) and in cells transfected with the inducible nitric oxide synthase gene (Tf). Bacterial growth is defined as the ratio of the colony forming units observed in cell samples 5 h after phagocytosis to the number observed immediately after phagocytosis. Data are mean $\pm$ SD (bars, $n = 3$). The horizontal square brackets show the statistical significance of the differences used in our analysis of the data ($*P < .05$; $** \ P < .01$; $*** \ P < .001$).
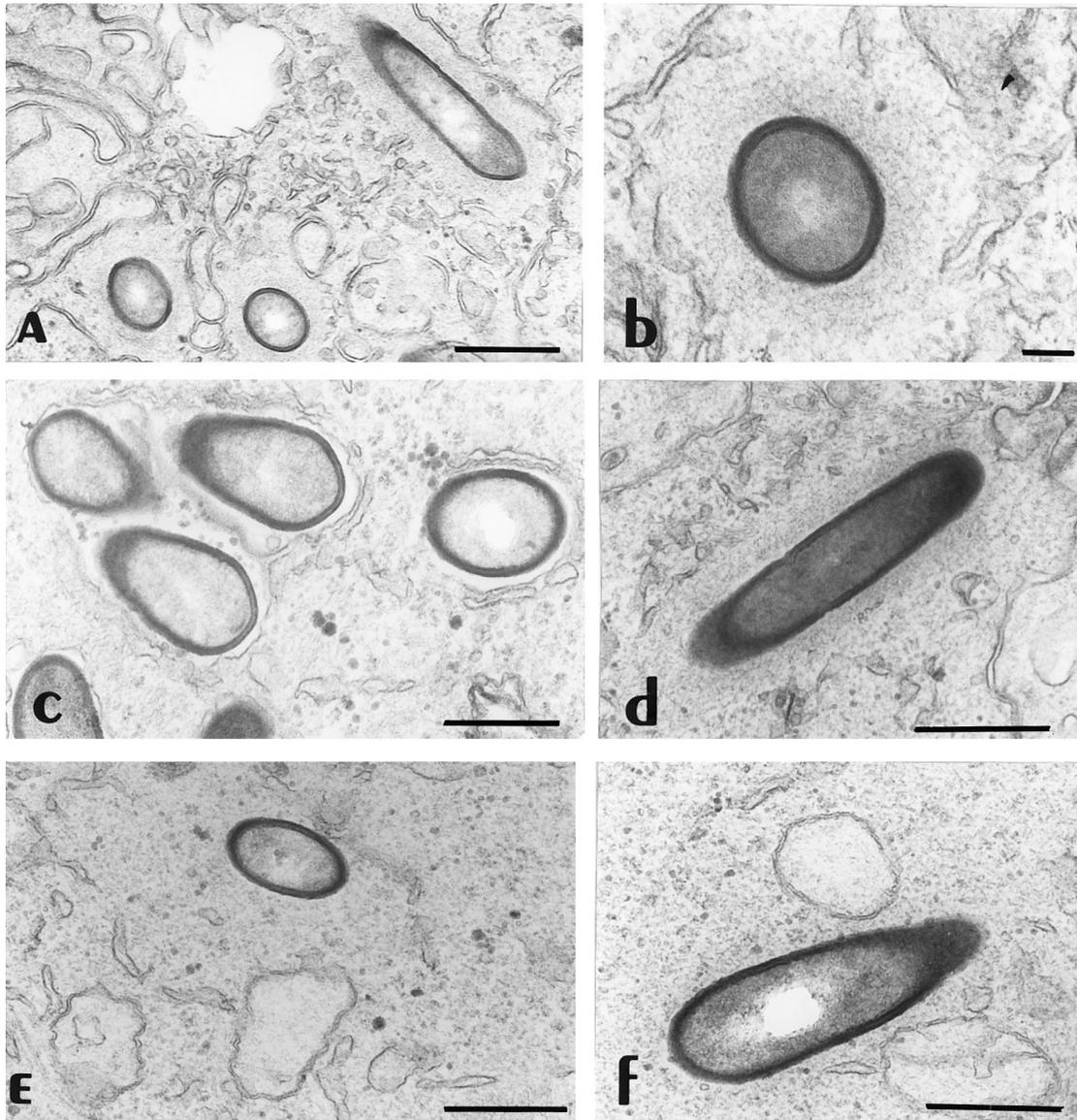
Figure 6.   Electron microscope images of Caco-2 cells infected with *Listeria monocytogenes* after treatment with cytokines. *A, B,* apical treatment with interferon-γ (100 U/mL); *C–F,* basolateral treatment with interleukin-6 (200 U/mL). Cells were fixed 3 h after phagocytosis. Bars: *A, C–F,* 0.5 μm; *B,* 0.1μm.

invasion of cells forming tight layers is a significant and important phenomenon, a point of clear pathophysiologic significance if it can be extended to intestinal cells in vivo [45] (this contention, however, does not preclude that basolateral invasion could be important for cell to cell spreading of *L. monocytogenes*).

IFN-γ, which is known to prevent *L. monocytogenes* escape from the phagosomal vacuole in macrophages [47] and to make these cells nonpermissive for intracellular *L. monocytogenes* growth [31], fails to exert marked effects on bacterial growth within Caco-2 cells when presented from the basolateral pole.

This is in contrast with other effects of IFN-γ on other polarized enterocyte-like cells, such as the HT29-D4 or T84 cell lines, in which the influence of this cytokine is maximal or even only observed when added to the basolateral pole (release of carcinoembryonic antigen from the HT29-D4 [48] or decrease of epithelial resistivity in T84 cells [49]). This raises the questions of the distribution of IFN-γ receptors between the apical and basolateral domains of Caco-2 cells, in comparison with other polarized cells, and of the coupling of these receptors with potentially domain-restricted effectors. IFN-γ, however, exerts a significant effect when administered from the apical pole. The

significance of this observation is difficult to establish, because much of the IFN-γ is probably produced at the submucosal level. Yet there is evidence for the presence of IFN-γ–producing T lymphocytes in intestinal epithelium [50], suggesting the presence of this cytokine in the lumen. Because IFN-γ does not prevent escape of *Listeria* from the phagosomal vacuoles, even when administered from the side from which it causes its maximal effect (apical side), we may reasonably assume that it will exert only a minor role in vivo.

In contrast to IFN-γ, IL-6 makes Caco-2 cells completely nonpermissive for *L. monocytogenes* growth when the cytokine is administered basolaterally. IL-6 receptor, however, is expressed at both poles of Caco-2 cells [51], again raising the question of the potentially restricted distribution of the effector protein(s). A privileged action through the basolateral pole may, however, be understandable if IL-6, which is expressed in human colon epithelial cells in response to bacterial invasion including *L. monocytogenes* [43], would act on a paracrine or autocrine fashion. We do not know, however, whether the basal production of IL-6 will be large enough in vivo to effectively protect enterocytes against invasion at an early stage.

Results obtained in the presence of L-MMA suggest that almost all of the effect of IFN-γ, and part of the effect of IL-6, on *Listeria* growth appears to be mediated by nitrogen-reactive species. The role of NO production in eliciting a stage of nonpermissiveness in Caco-2 cells toward *L. monocytogenes* is further shown by the results of the iNOS transfection experiments, because cells overexpressing this enzyme are fully capable of blocking *L. monocytogenes* net growth in a way that is totally suppressible by L-MMA. This control directly demonstrates that the transfected cells act through an increased production of NO and not through a nonspecific effect of the transfection itself; NO diffusibility would also explain why all cells need not be transfected to obtain a marked effect. Interestingly, up-regulation of iNOS has recently been shown to contribute to mucosal defense in bacterial (but not nematode) invasion [52].

The fact that not all of the of effect of IL-6 in wild cells is suppressed by L-MMA, however, suggests the intervention of
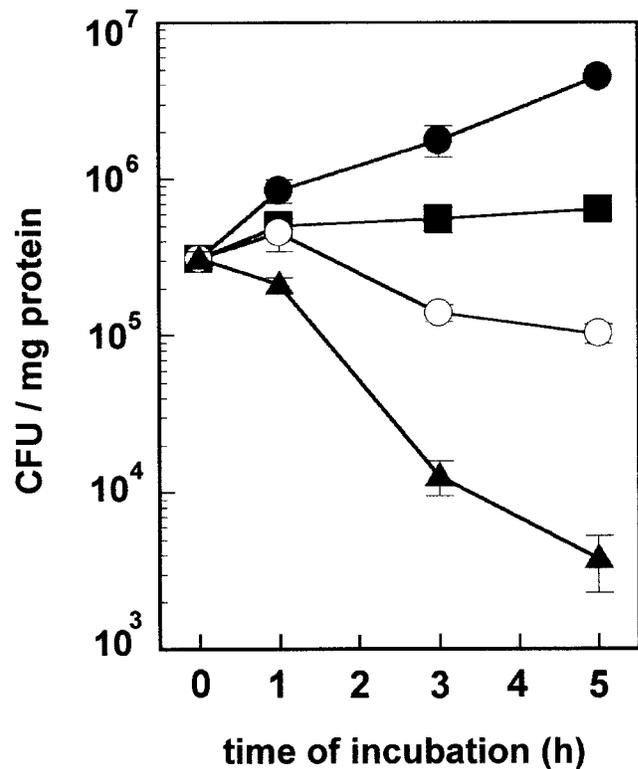

Figure 7. Influence of antibiotics on the intracellular growth of *Listeria monocytogenes* in control untreated Caco-2 cells. Antibiotics were added after phagocytosis of *L. monocytogenes* and cell washing at an extracellular concentration of 10× their MICs in both the apical and the basolateral medium. ●, no antibiotic; ■, ampicillin; ○, azithromycin; ▲, sparfloxacin. Data are mean ± SD (bars, $n = 3$). All differences at 5 h are significant with $P < .001$.

other factors than NO. The morphologic data may provide some clues in this context because we see that cytosolic *L. monocytogenes* often fail to be surrounded by a granular material, which most observers would recognize as actin. Actin is thought to play a crucial role in the intracellular movement of *L. monocytogenes* and in its spread from cell to cell [3, 13,

Table 1. Intracellular fate and appearance of *Listeria monocytogenes* in untreated and treated Caco-2 cells 3 h after infection with interferon (IFN)–γ (100 U/mL) or interleukin (IL)–6 (200 U/mL).

	Cells appearing, %						
	Electron microscopy				Confocal microscopy		
Treatment	Bacteria[a], *n*	Phagosomal[b]	Cytosolic with actin[c]	Cytosolic without actin[d]	Bacteria[a]	With actin[e]	Without actin[f]
None	104	13	75	12	319	75	25
IFN-γ	101	20	80	0	139	81	19
IL-6	114	51	9	40	106	18	82

[a] Number of profiles (electron microscopy; non adjacent sections) or bacterial bodies (confocal microscopy) counted.
[b] Bacterial profile enclosed in an electron-lucent membrane-bounded profile.
[c] Bacterial profile free in the cytosol and surrounded by a finely granular material.
[d] Bacterial profile naked and free in the cytosol.
[e] Bacteria showing a red/yellow fluorescence.
[f] Bacteria showing a green fluorescence.

53–56]. It is therefore possible that IL-6 affects the expression and/or the activity of the protein's complex participating in the nucleation of actin around *L. monocytogenes.*

The present study may also provide new insights for the optimization of antibiotic usage during *L. monocytogenes* infection in relation to the protection of the intestinal mucosa against invasion. In most cases, *Listeria* will have left the enterocytes by the time of diagnosis of the infection and when antibiotic treatment is initiated. Yet, continuous reinfection may need to be considered, as well as a potential prophylactic use of antibiotics. In this context, our data show that IFN-γ and IL-6 definitely improve the activity of ampicillin, which otherwise appears to be rather ineffective. The data also strongly suggest that these cytokines act, at least partially, by increasing drug accumulation, through a mechanism that remains, however, to be determined (increase in drug intake, decrease of drug efflux?). Thus, an active cellular immune response appears to be critical for the success of ampicillin therapy to protect against *Listeria.* Because, as discussed above, such immune response is unlikely to be present at an initial stage of the infection, prophylactic use of ampicillin appears to be largely unjustified on a pharmacologic basis. Similarly, ampicillin will appear pharmacologically ineffective against reinfection (or continuous infection) in immunodeficient patients. Azithromycin and still more sparfloxacin would appear to be considerably more effective in both situations even though their cooperation with IFN-γ and IL-6 is minimal (or even slightly negative for sparfloxacin). Azithromycin, however, remains only very modestly bactericidal, even in IL-6–treated cells, where its accumulation is enhanced. This finding is consistent with what is observed for other macrolides in *L. monocytogenes*–infected polymorphonuclear leucocytes [57–59] and suggest therefore that macrolides are likely to fail to eradicate the infection. Besides these therapeutic considerations, it would nevertheless be of interest to determine whether azithromycin acts truly on intracellular
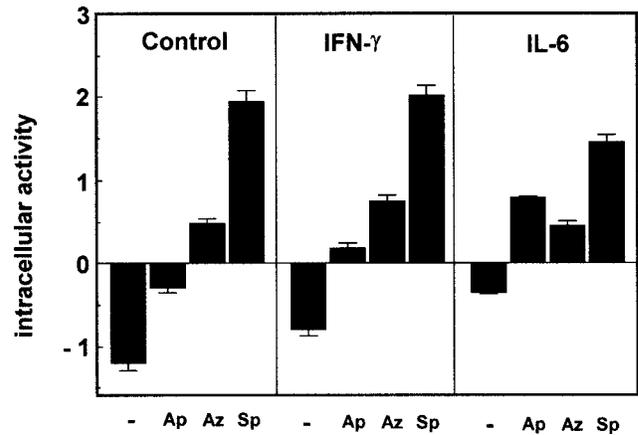

Figure 8. Modulation of the antibiotic activity against intracellular *Listeria monocytogenes* by cytokines. Control, no cytokine added; IFN-γ, cells treated apically with interferon-γ (100 U/mL); IL-6, cells treated basolaterally with interleukin-6 (200 U/mL). (-), no antibiotic; Ap, ampicillin; Az, Azithromycin; Sp, sparfloxacin. All antibiotics were added at an extracellular concentration of 10× their MICs in both the apical and the basolateral medium. Intracellular activity is defined as the $\log_{10}$ of the ratio of the number of cfus observed immediately after phagocytosis and washing after 5 h to that of incubation with the antibiotics (therefore, a negative value indicates bacterial growth). Data are mean ± SD (bars, $n = 3$). All differences between each antibiotic and the corresponding controls and between antibiotics in each series are significant at $P < .001$. All other differences between control and IFN-γ– and IL-6–treated cells are significant at $P \leq .01$ except for azithromycin when comparing control and IL-6–treated cells and for sparfloxacin when comparing control and IFN-γ–treated cells.

L. monocytogenes by inhibiting the bacterial protein synthesis, that is, by the general mode of action of macrolides toward sensitive bacteria, or whether like bafilomycin A1, it acts by neutralizing the phagosomal pH and thereby causing a containment of *L. monocytogenes* in phagosomes [see 46, 60]. It

Table 2. Accumulation of antibiotics in Caco-2 cells after 5 h of incubation at an extracellular concentration of 10 mg/L.

Antibiotic	Cell treatment	Intracellular : extracellular concentration ratio				
		Uninfected cells	P^a	Infected cells	P^b	P^c
Ampicillin	None	0.50 ± 0.04		0.73 ± 0.17		NS
	IFN-γ	0.53 ± 0.04	NS	1.40 ± 0.13	<.05 (+)	<.001 (+)
	IL-6	1.60 ± 0.36	<.05 (+)	1.70 ± 0.10	<.01 (+)	NS
Azithromycin	None	27.40 ± 4.60		26.77 ± 0.39		NS
	IFN-γ	30.06 ± 2.05	NS	26.79 ± 1.84	NS	NS
	IL-6	42.59 ± 0.89	<.05 (+)	37.22 ± 0.67	<.001 (+)	<.01 (−)
Sparfloxacin	None	8.71 ± 0.14		6.10 ± 0.56		<.01 (−)
	IFN-γ	7.50 ± 0.04	<.01 (−)	6.04 ± 0.03	NS	<.001 (−)
	IL-6	7.67 ± 0.57	<.05 (−)	9.02 ± 0.84	<.01 (+)	NS

NOTE. NS, not significant; −, decreased effect; +, increased effect.

[a] Statistical analysis of the differences between IFN-γ and IL-6 vs. control (no cytokine) in uninfected cells.

[b] Statistical analysis of the differences between IFN-γ and IL-6 vs. control (no cytokine) in infected cells.

[c] Statistical analysis of the differences between infected vs. uninfected cells.

would also be of interest to unravel the mechanism by which IL-6 increases the cellular uptake of azithromycin (by decreasing the pH of the lysosomes, causing more storage in this organelles; see discussion in [31]). In contrast to azithromycin, sparfloxacin was intensively bactericidal toward *L. monocytogenes* in Caco-2 cells (as also observed in *L. monocytogenes*–infected macrophages [31] or toward *L. monocytogenes* in acellular systems [33]). Several other fluoroquinolones have also shown activity against intracellular *L. monocytogenes* [61–63], which, together with the present data and those reported earlier for *L. monocytogenes*–infected macrophages [31], should, in our opinion, definitely justify animal and, perhaps, clinical trials.

Finally, in a context of pharmacodynamic evaluation of intracellular antibiotics, the present data would also reinforce our earlier conclusion that accumulation and intracellular activity of antibiotics are not directly and simply correlated, whereas the ranking of intracellular drug contents (azithromycin > sparfloxacin > ampicillin) was different from that of the activities recorded (sparfloxacin > azithromycin > ampicillin). Clearly, parameters such as drug subcellular disposition, bioavailability, and expression of activity [64] must exert a major influence on drug effectiveness beyond the mere intracellular drug presence within the cell.

Acknowledgments

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