Drug Targeting by Polyalkylcyanoacrylate Nanoparticles Is Not Efficient Against Persistent *Salmonella*

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Received October 29, 1997; accepted December 19, 1997

Purpose. We have investigated the efficacy of colistin and ciprofloxacin, free or bound to polyalkylcyanoacrylate nanoparticles, for the targeting and eradication of *Salmonella* persisting in the organs of the mononuclear phagocyte system.

Methods. A model of persistent *S. typhimurium* infection was developed in C57BL/6 mice using IV inoculation of the plasmid-cured strain C53.

Results. *In vivo* and *ex vivo* experiments showed that the persisting bacteria seem to evolve to a nongrowing state during experimental salmonellosis. *In vivo* treatment with free or nanoparticle-bound colistin did not significantly reduce the number of viable *Salmonella* C53, either in the liver or the spleen of infected mice. In contrast, *in vivo* treatment with ciprofloxacin led to a significant decrease of bacterial counts in the liver whatever the stage of infection and the form used. However, none of the treatments were able to sterilize the spleen or the liver. In *ex vivo* experiments, colistin was only active against bacteria recovered during the early phase of infection, whereas ciprofloxacin exerted its activity at all times postinfection.

Conclusions. We suggest that the micro-environment in which the bacterial cells persist *in vivo* probably causes dramatic changes in their susceptibility to antimicrobial agents.

KEY WORDS: colistin; ciprofloxacin; polyalkylcyanoacrylate; nanoparticles; *Salmonella typhimurium*; bacterial persistence.

INTRODUCTION

Salmonella infections remain a world-wide health problem and a major cause of morbidity. Numerous studies have dealt with the use of liposomes or polymeric nanoparticles as delivery systems for antibiotics in treating experimental infections caused by intracellular bacteria *in vivo* (for a review, see reference 1). Thus, the binding of ampicillin to polyisohexylcyanoacrylate nanoparticles has previously been shown to enhance dramatically the therapeutic efficiency of this antibiotic by increasing its concentration in the major infection sites such as the liver and the spleen, in a murine model of acute salmonellosis (2). However, a small but consistent number of persistent live, presumably nongrowing bacteria, was still present in the liver and the spleen of the mice as late as 6 weeks after the acute infection had been cured and the treatment stopped. This was also the case when ampicillin entrapped in liposomes or high doses of free ampicillin were used to treat the animals (3). These observations prompted us to determine whether antibiotics which would be active against nondividing bacteria and could be entrapped within polyalkylcyanoacrylate nanoparticles could be useful in that setting. To test this hypothesis, two antibiotics, colistin (polymyxin E) and ciprofloxacin, were chosen.

Colistin is a small cationic amphipathic peptide that exerts its antibacterial-antiseptic effect by disorganizing the cytoplasmic and outer membranes, thus inducing a disruption in the permeability barrier of most Gram-negative bacteria (4) whatever their growth rate (5,6). However, the therapeutic potential of colistin is limited *in vivo* by its very poor intratissular diffusion (5). In a previous study (7), colistin-loaded nanoparticles were shown to concentrate the antibiotic in the organs of the mononuclear phagocyte system, especially in the liver and in the spleen, in mice. Ciprofloxacin is a fluoroquinolone antibiotic which has been shown to exert a bactericidal activity against nongrowing Gram-negative bacteria both *in vitro* (8,9,10) and in a murine stationary *E. coli* or *P. aeruginosa* granuloma pouch model (11,12).

In order to obtain a model of infection with persistent *Salmonella*, C57BL/6 mice were infected by intravenous inoculation of *Salmonella typhimurium* strain C53 (13). This variant of *S. typhimurium* strain C5, cured of its large virulence plasmid pIP 1350, is able to move through tissues at rates equal to those of the wild-type strain, but grows less rapidly within the host cells in mice, and does not lead to the death of the animals (14). This model was used to assess the effectiveness of colistin and ciprofloxacin (free or entrapped within nanoparticles) in the eradication of persistent *Salmonella.*

MATERIALS AND METHODS

Antibiotics and Reagent Sources

Colistin sodium methanesulfonate was purchased from Roger Bellon (Neuilly-sur-Seine, France), ciprofloxacin (CIP) chlorhydrate from Armos (Spain), ciprofloxacin (Ciflox®, injectable solution) from Bayer Pharma (Germany), and ampicillin trihydrate from Sigma (Paris, France). Hexylcyanoacrylate (HCA) was obtained from Henkel (Boulogne-Billancourt, France) and isohexylcyanoacrylate (IHCA) from Loctite (Dublin, Ireland).

Bacterial Strain

The strain C53 (kindly provided by F. Norel, Institut Pasteur, Paris, France) of *Salmonella enterica* serovar *typhimurium* was obtained from strain C5 after curing of the plasmid pIP1350 (13). A stock suspension of strain C53 was prepared in brain heart infusion broth (Difco Laboratories, Detroit, USA) with 16% glycerol, divided into aliquots (1 ml), and stored at −70°C.

Susceptibility Testing

Minimal Inhibitory Concentrations (MICs) were measured in Mueller-Hinton broth (Diagnostics Pasteur, Marnes-La-

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0724-8741/98/0400-0544$15.00/0

Coquette, France) by a standard microdilution method (15). The MIC was defined as the lowest concentration inhibiting visible growth after incubation for 18 h at 37°C and the Minimal Bactericidal Concentration (MBC), as the lowest concentration of antibiotic which decreased the number of viable bacteria by 3 $\log_{10}$.

Mice

Female C57BL/6 mice of 7–8 weeks (18g) (IFFA-CREDO, Les Oncins, L'Arbresle, France) were housed in the central animal facilities of our institution under class II security conditions. Each group received food and water *ad libitum*.

Nanoparticle Preparation

Ciprofloxacin-loaded nanoparticles were prepared according to the emulsion polymerization method (16) with the following modifications: 0.1 ml of IHCA was added under magnetic stirring to 10 ml of an aqueous polymerization medium (1% w/v dextran 70, 5% w/v D-glucose, pH 2.5) containing 10% v/v acetone and ciprofloxacin at a previously optimized concentration of 0.5 mg/ml. After 6 h of polymerization, the organic solvent was removed under vacuum (Rotavapor®, Bioblock, France) and nanoparticles were freeze-dried (at −30°C/+30°C for 24 h under a 0.05 mbar vacuum, Alpha I-5, CHRISS, Germany) in order to stabilize the preparation during storage. Colistin-loaded nanoparticles (7) and ampicillin-loaded nanoparticles (17) were prepared as described elsewhere. Unloaded PIHCA nanoparticles were prepared under the same conditions, but no antibiotic was added to the polymerization medium.

Nanoparticle Characteristics

Particle size was estimated before and after freeze-drying of nanoparticles by quasi-elastic laser light scattering (Nanosizer Coulter N4MD, Coultronics, Paris, France) and the entrapment efficiency was estimated by measuring the residual antibiotic concentration in the supernatant obtained after ultracentrifugation of the nanoparticle suspension (100,000 × *g* for 1 h at +4°C; L7-55 ultracentrifuge, Beckman). Ampicillin and ciprofloxacin concentrations were assayed by reversed-phase high-performance liquid chromatography (HPLC) (18, adapted from 19) and by a diffusion agar method (2, adapted from 20). Colistin concentrations were measured by a diffusion assay in antibiotic medium 10 (Difco) by using the growth of *Bordetella bronchiseptica* (CIP 53.157) as an indicator (21). The entrapment efficiency was calculated as the total quantity of antibiotic minus the free antibiotic divided by the total antibiotic.

Distribution of Antibiotic Activity in Mice

The biodistribution of colistin (7) and ampicillin (2) were previously studied. To compare the biodistribution of free and nanoparticle-bound ciprofloxacin, a single dose (4 mg/kg) was administered intravenously to five groups of five uninfected C57BL/6 mice. Urine was collected during 18 h, 24 h and 48 h in metabolism cages and stored at −20°C. The mice were killed in a CO_2 chamber at different times (1 h, 6 h, 18 h, 24 h, and 48h). The total blood was collected by cardiac puncture without pooling, immediately centrifuged and sera were stored at −20°C. The spleen, liver, kidneys and lungs were excised, washed in phosphate-buffered saline (PBS) to remove any trace of non tissue-associated drug, dried on a filter paper (65g/m^2) (OSI, France) and weighed. Then, the samples were frozen at −20°C and subsequently homogenized in PBS with an Ultraturrax mixer (Bioblock, Vanves, France). Antibiotic concentrations in sera, urine and organ homogenates were determined by a diffusion agar method (adapted from 20) in antibiotic medium 5 (Difco), using the growth of *Escherichia coli* (IPP 7624) as an indicator.

Experimental Infection

An aliquot of the stock suspension of *S. typhimurium* C53 was grown overnight in 10 ml of broth medium. The suspension was adjusted by dilution in physiological sterile saline (0.9% NaCl) to obtain a bacterial concentration of 2×10^3 CFU/ml. The inoculum concentration was verified for each experiment by counting on Drigalski agar. Fifty six C57BL/6 mice were experimentally infected by injecting 100 µl of *S. typhimurium* strain C53 (2×10^2 CFU) into the tail vein. Eight mice were sacrificed on days 2, 7, 14, 21, 28, 35 and 40 postinfection, the liver and the spleen were removed aseptically, homogenized in 2 ml of sterile PBS, and viable bacteria counted on tryptic soy agar.

Effectiveness of Antibiotic Treatment at Various Stages of Infection with *S. typhimurium*

In vivo antimicrobial activity was assayed both in the early phase of the infection during which the bacteria were actively dividing and in the late phase during which their number was stable. Thus, antibiotics, free or bound to nanoparticles, were injected intravenously daily for 5 days beginning either on day 1 or on day 21 postinfection. Three days after the last injection, mice were sacrificed and viable bacteria were counted in the spleen and the liver on tryptic soy agar as described above.

Ex vivo Assay of Antimicrobial Activity

In order to study antibiotic activity without taking into account problems related to the crossing of biological barriers, direct contact between the bacteria and the free form of the antibiotics was made. For this test, the spleen was preferred to the liver because the cells could be separated by gentle mechanical means without having to resort to enzymatic treatment. Thus, on days 2, 7, 14, and 21 postinfection, five animals were sacrificed and their spleens were aseptically isolated. Each spleen was homogenized in 2 ml of sterile PBS with an Ultraturrax mixer (Bioblock, France) and centrifuged at 3000*g* for 10 min. The cell pellet was then resuspended in 2 ml of PBS and sonicated at 35 kHz (Transsonic Digital Elma®, France) for 4 min in order to liberate viable bacteria from their intracellular location (22). Aliquots (0.4 ml) of sonicates were incubated for 30 min at 37°C with the MBC of ampicillin, colistin or ciprofloxacin (free form) and viable bacteria were counted by spreading 0.1 ml of 10-fold dilutions onto tryptic soy agar plates (Diagnostics Pasteur, Marnes-la-Coquette, France). The control did not contain any antibiotic. For all fractions, the minimal sensitivity level was defined as the 10^{-1} dilution (>100 CFU/ml) in order to avoid carryover effects.

Statistical Analysis

Bacterial counts, expressed in decimal logarithms, were compared by the nonparametric Mann and Whitney test. For all data, the criterion of significance was $P < 0.05$.

RESULTS

Susceptibility Testing

The MIC for *S.* serovar *typhimurium* strain C53 was 1 μg/ml for ampicillin, 1 μg/ml for colistin, and 0.062 μg/ml for ciprofloxacin. The MBC was 4 μg/ml for ampicillin, 16 μg/ml for colistin, and 0.5 μg/ml for ciprofloxacin.

Determination of Entrapment Efficiency and Nanoparticle Size

Drug content, nanoparticle size and entrapment efficiency for ampicillin, colistin and ciprofloxacin are shown in Table I. The entrapment efficiency was greater than 80% for all three compounds. However, the drug amount per mg of polymer was lower for ciprofloxacin than for colistin. Moreover, ciprofloxacin nanoparticles were larger than unloaded nanoparticles, whereas drug encapsulation only slightly affected the size of ampicillin- and colistin-loaded nanoparticles.

Distribution of Ciprofloxacin in Mice

After IV injection, the concentrations of free and nanoparticle-bound ciprofloxacin (Table II) decreased rapidly in serum. Comparison of tissue concentrations showed that the formulation did not affect the antibiotic level in the kidneys, and in the lungs. In contrast, the concentrations of ciprofloxacin were significantly higher in liver and spleen, 1 h after injection of the nanoparticle form, than after injection of the free form (5.75-fold and 3.2-fold, respectively). Thereafter, concentrations of ciprofloxacin decreased very slowly from 6 to 24 h in these organs. Moreover, the urine excretion was greatly reduced (3-fold) when the drug was associated with nanoparticles.

Kinetics of Experimental Infection

After intravenous administration, the strain C53 was able to colonize the liver and the spleen of C57 BL/6 mice (Fig. 1). The bacterial counts reached a maximum level after 14 days, then decreased slowly before reaching a plateau at week 5. The number of bacteria was more often higher in the spleen than in the liver. Moreover, no animal died during the course of infection.

In vivo Antibacterial Activity at Various Stages of Infection with *S. typhimurium*

Free ampicillin and free ciprofloxacin displayed a bactericidal activity against the strain C53 during the early phase of infection, whereas free colistin did not (Table III). However, during the late phase of infection (Table IV), neither free nor nanoparticle-bound ampicillin or colistin caused a significant decrease in the number of viable C53, either in the liver or the spleen. The treatment with 30 mg of free ciprofloxacin per kg or 26 mg of free ciprofloxacin plus 4 mg of nanoparticle-bound ciprofloxacin per kg significantly reduced the number of CFU in the liver only (0.8 and 0.6 $\log_{10}$ CFU, respectively) compared with that of control mice ($P < 0.05$). Whatever the antibiotic, bacterial counts were not different between mice treated with the free form and those treated with the nanoparticle form.

Ex vivo Assay of Antimicrobial Activity

Salmonella recovered from spleen one day postinfection were highly sensitive to ampicillin, colistin and ciprofloxacin (Fig. 2). In contrast, on day 7 postinfection, only ciprofloxacin sterilized the homogenized spleen. Incubation with ampicillin or colistin induced a reduction of 1.8 $\log_{10}$ and 0.8 $\log_{10}$ respectively in the number of CFU per organ. On days 14 and 21 postinfection, there was no significant difference between bacterial counts obtained after *ex vivo* treatment with ampicillin or colistin and those obtained in the control. However, ciprofloxacin maintained its *ex vivo* bactericidal effect upon splenic *Salmonella,* with a significant drop of 2.56 $\log_{10}$ on day 14 and 2.90 $\log_{10}$ on day 21, in comparison with the control.

DISCUSSION

In agreement with previous findings (14), the virulence plasmid-cured strain C53 was able to cause a nonlethal, systemic infection in C57BL/6 mice after IV inoculation. The number of bacteria in spleen and liver increased progressively over 14 days, whereas it increased rapidly for the parental strain (6×10^6 CFU/organ on day 5 postinfection, data not shown). The virulence plasmid is not required for *Salmonella* to move through deep tissues and to resist host defense mechanisms (14). Indeed, it was recently shown that the *rpoS* chromosomal locus which encodes an alternative sigma factor (σ^s) is highly involved in *Salmonella* virulence in mice (23–25).

The characterization of loaded-nanoparticles revealed that the antimicrobial activity of ampicillin, colistin and ciprofloxacin was not altered by the process of polymerization used for nanoparticle preparation. In the *in vivo* antimicrobial activity assay, ampicillin and ciprofloxacin (but not colistin) were active

Table I. Characteristics of Unloaded Nanoparticles and Nanoparticles Loaded with Ampicillin, Colistin or Ciprofloxacin ($n = 5$)

		Nanoparticle characteristics			
Antibiotics	Monomer	Size (nm) ± SD	Initial drug concentration (μg/ml)	Amount of antibiotic(μg) per mg of freeze-dried nanoparticles	Entrapment efficiency (%) ± SD
None	IHCA	180 ± 10	—	—	—
Ampicillin	IHCA	214 ± 35	2000	172	86 ± 4
Colistin	HCA	211 ± 40	500	93	93 ± 2
Ciprofloxacin	IHCA	305 ± 60	500	41	82 ± 2

Table II. Distribution of Ciprofloxacin After One Intravenous Injection to Noninfected C57BL/6 Mice (4 mg/kg of Free or Nanoparticle-bound Ciprofloxacin)

Ciprofloxacin	Organ	Mean concentration[a] (SD)[b] at the following times after administration				
		1h	6h	18h	24h	48h
Free	Serum	0.17 (0.08)	nd[c]	nd	nd	nd
	Lungs	0.83 (0.19)	nd	nd	nd	nd
	Liver	0.20 (0.05)	nd	nd	nd	nd
	Spleen	1.09 (0.26)	nd	nd	nd	nd
	Kidneys	0.62 (0.22)	nd	nd	nd	nd
	Urine[d]	—	—	9.10	16.0	10.6
Nanoparticle-bound	Serum	0.74 (0.06)	nd	nd	nd	nd
	Lungs	1.25 (0.70)	nd	nd	nd	nd
	Liver	1.15 (0.14)	1.93 (0.52)	0.32 (0.09)	0.12 (0.017)	nd
	Spleen	3.50 (0.22)	2.45 (1.14)	1.14 (0.33)	0.71 (0.30)	nd
	Kidneys	1.10 (0.71)	nd	nd	nd	nd
	Urine	—	—	3.80	5.50	3.30

Note: The detection limit was 0.031 μg ciprofloxacin/ml in sera and urine, 0.32 μg/g in lungs, 0.05 μg/g in liver, 0.48 μg/g in spleen and 0.17 μg/g in kidneys.
[a] Values are in micrograms per milliliter for serum and urine, and in micrograms per gram for the organs.
[b] Five mice per time point.
[c] nd, not detected.
[d] Urine collected during the above-mentioned time.

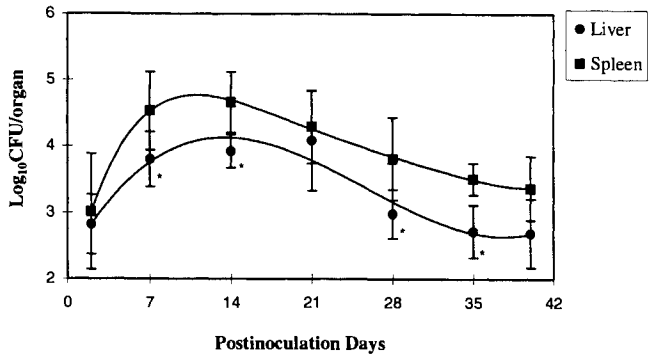

Fig. 1. Kinetics of hepatic and splenic colonization after intravenous challenge with strain C53. At various times postinfection (2, 7, 14, 21, 28, 35, 40 days), C57 BL/6 mice (8 per group) were sacrificed and the bacterial counts were determined in the spleen and the liver. Data are expressed as means ± SD. * Significant differences from the splenic counts ($P < 0.05$).

Table III. *In vivo* Activity of a 5-Day Treatment (from the 1st to the 5th Days After Inoculation with *S. typhimurium* C53) with the Free Form of Ampicillin, Colistin or Ciprofloxacin on the Bacterial Counts in the Liver and the Spleen of C57 BL/6 Mice ($n = 5$)

Treatment	Mean $\log_{10}$CFU/organ (±SD)	
	Liver	Spleen
0.9% NaCl (control)	3.81 ± 0.31	4.14 ± 0.45
Ampicillin (1 g/kg)	1.87 ± 0.5[a]	2.42 ± 0.22[a]
Colistin (5 mg/kg)	3.62 ± 0.44	4.02 ± 0.47
Ciprofloxacin (30 mg/kg)	1.79 ± 0.28[a]	2.09 ± 0.17[a]

[a] $P < 0.05$ versus control.

Table IV. *In vivo* Activity of a 5-Day Treatment (from the 21st to the 25th Days After Inoculation with *S. typhimuriurn* C53) with the Free or Nanoparticle-bound Forms of Antibiotics. The Results Are Expressed as Bacterial Counts in the Liver and the Spleen of C57 BL/6 Mice

Treatment	Mean $\log_{10}$CFU/organ (±SD)	
	Liver	Spleen
0.9% NaCl (control) ($n = 10$)	2.98 ± 0.37	3.81 ± 0.62
Unloaded nanoparticles (50 mg/kg) ($n = 6$)	2.79 ± 0.22	3.74 ± 0.27
Free ampicillin (1 g/kg) ($n = 18$)	3.01 ± 0.77	3.79 ± 0.36
Nanoparticle-bound ampicillin (40 mg/kg) ($n = 10$)	3.02 ± 0.30	3.81 ± 0.33
Free colistin (5 mg/kg) ($n = 10$)	2.93 ± 0.43	3.77 ± 0.35
Nanoparticle-bound colistin (5 mg/kg) ($n = 18$)	2.88 ± 0.25	3.74 ± 0.26
Free ciprofloxacin (30 mg/kg) ($n = 14$)	2.18 ± 0.39[a]	3.41 ± 0.31
Free ciprofloxacin (26 mg/kg) + nanoparticle-bound ciprofloxacin (4 mg/kg) ($n=10$)	2.38 ± 0.26[a]	3.48 ± 0.24

[a] $P < 0.05$ versus control.

assay, ampicillin and ciprofloxacin (but not colistin) were active in the early phase of infection. However, in the later phase of infection, ciprofloxacin was the only drug which showed antibacterial activity. This is in sharp contrast with the previous finding (2) that the binding of ampicillin to PIHCA nanoparticles dramatically increased its efficacy against *S. typhimurium* in a model of acute infection. This apparent discrepancy is probably due to a different growth state of the bacteria during the acute and the persistent infection.

In order to analyze the sensitivity of the bacteria during the infection, an *ex vivo* assay was applied to isolated, infected

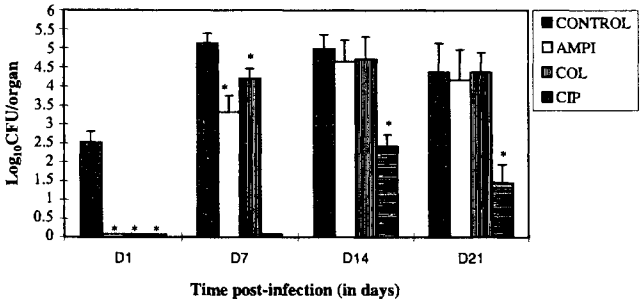

Fig. 2. *Ex vivo* susceptibility of the strain C53 in splenic homogenates after 30 min exposure to MBC of ampicillin, colistin or ciprofloxacin (free form). For each time, control denotes the bacterial counts of C53 in absence of antibiotics. Data are the means ± SD of five experiments. * Significant differences from the control values ($P < 0.05$).

spleen. Interestingly, bacteria acquired a gradual resistance to ampicillin during the experimental salmonellosis. Since this antibiotic is only active against dividing bacteria, these results could be related to the low rate of bacterial division in the late stage of infection. It should be noted that, under these conditions, colistin was active against bacteria recovered during the early phase of infection only, whereas ciprofloxacin exerted its *ex vivo* bactericidal activity at all times after infection.

In our model of persistent *Salmonella* infection, the data obtained with ampicillin can be explained by its mechanism of action. On the contrary, the lack of activity observed with colistin and the low activity of ciprofloxacin were unexpected phenomena. Indeed, both antibiotics are recognized as being active against some nongrowing bacteria (4,8–12,26). However, a recent study (27) has shown that stationary-phase *S. typhimurium* exhibited a several-fold-higher resistance to polymyxins than log-phase bacteria. Moreover, during infection, *Salmonella* has to adopt survival mechanisms directly implicating the bacterial wall. The flexibility of the cell envelope composition, regulated by the growth rate of the bacteria, may cause considerable alterations in the ability of antibacterial molecules such as colistin to interact with bacterial membrane (28). These observations could explain the difference of activity observed in the *ex vivo* experiments during the early phase and the late phase of infection, after treatment with colistin. On the other hand, the fact that dividing *Salmonella* were not sensitive to colistin *in vivo* whereas they were *ex vivo* (one day postinfection) suggests that the concentration of this antibiotic in the free form was not sufficient in the infected organs. Its *in vivo* distribution profile, which has been reported elsewhere (7), confirms this assumption.

In order to explain why ciprofloxacin was not more active *in vivo* against persistent *Salmonella,* whereas it exerted a marked bactericidal activity *ex vivo,* during the late stage of infection, different hypothesis can be put forward. The first is an insufficient concentration of ciprofloxacin in the major foci of infection. As shown by our tissue distribution studies, the binding of ciprofloxacin to nanoparticles led to higher drug accumulation in the liver and the spleen. The average concentrations reached in these organs exceeded the MICs by several-fold (1- to 20-fold in the liver and 1- to 4-fold in the spleen, according to the time after administration). Nevertheless, in terms of bactericidal activity (MBC), the concentration of ciprofloxacin was probably not sufficient to eradicate the persistent bacteria. An alternative hypothesis is that the deep lymphoid system, such as the spleen (22) and the lymphatic nodes, or vascular endothelia could constitute real « safe-sites » for *S. typhimurium.* In this respect, we cannot exclude the possibility that the persistent bacterial population could form biofilms on the surface of these tissue (29,30). Indeed, it is now well documented that many chronic infections involve consortia of bacteria growing as an adherent biofilm within an extended polysaccharide glycocalyx (31) conferring on the bacteria an increased resistance to the host defenses and antimicrobial agents (32,33). In our model, this assumption could account for the fact that the bactericidal activity of ciprofloxacin was retained *ex vivo* on direct contact with the homogenized, infected organs, but not *in vivo* after IV administration. In addition, this could explain the finding that *Salmonella* were more sensitive in the liver than in the spleen, and more sensitive to ciprofloxacin in the early phase of infection, a time at which they were not yet protected into "safe- sites". On the other hand, in order to understand why the entrapment of ciprofloxacin within nanoparticles did not improve its *in vivo* activity against persistent *Salmonella* compared to the free agent, it must be pointed out that the action of a targeted antibiotic is observed *in vivo* either after direct contact with bacteria, requiring co-location in the same compartment, or after intracellular redistribution of drug released from nanoparticles. In the present case, the partial inhibition of macrophage phagosome-lysosome fusion due to *S. typhimurium* (34) could prevent the contact between the bacteria mainly present in phagosomes, and the antibiotic-loaded nanoparticles, present in secondary lysosomes (35,36). In addition, because it is in its cationic form within lysosomes, ciprofloxacin is not able to diffuse through the cell to *Salmonella* located at other intracellular sites. Finally, all these mechanisms could be supposed to limit the intracellular targeting of these bacteria by nanoparticles.

In conclusion, it seems likely that the accessibility of bacteria to the drug and the host cell barriers to drug transport are responsible for the differences of activity observed. The eradication of persistent bacteria *in vivo* appears thus to be far more complicated than originally thought.

ACKNOWLEDGMENTS

We thank Loctite and Armos for the gift of the IHCA monomer and ciprofloxacin chlorhydrate, respectively. We are grateful to F. Norel for fruitful discussions. This study was supported by the "réseau vectorisation des médicaments" (DRED, Ministère de l' Education Nationale, France).

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