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Treatment of Experimental Salmonellosis in Mice with Ampicillin-Bound Nanoparticles

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We tested the effectiveness of ampicillin bound to nanoparticles of polyisohexylcyanoacrylate (PIHCA) in treating C57BL/6 mice experimentally infected with *Salmonella typhimurium* C5. The diameter of the nanoparticles was 187 ± 13 nm, and the ampicillin/PIHCA ratio was 0.2/1. The proportion of ampicillin bound was 90 ± 3%. All control mice and all those treated with nonloaded nanoparticles died within 10 days of infection. By contrast, all mice treated with a single injection of 0.8 mg of nanoparticle-bound ampicillin survived. With free ampicillin, a similar curative effect required three doses of 32 mg each. Lower doses delayed but did not reduce mortality. The sharp increase in the therapeutic index of ampicillin after linkage to PIHCA nanoparticles was explained by studies of the distribution of ampicillin, which showed that when bound to nanoparticles, the ampicillin was concentrated mainly in the liver and spleen, the primary foci of infection in the experimental model that we used. These findings warrant further development of intracellular targeting of antibiotics on biodegradable polymeric carriers such as PIHCA.

Biodegradable polymeric nanoparticles have aroused interest as potent drug carriers (for a review, see reference 6) that are capable of enhancing intracellular drug delivery. The available results regarding the toxicity of polyisohexylcyanoacrylate (PIHCA) nanoparticles suggest that this toxicity is low (6, 27).

This is why we recently defined the conditions required for the binding of ampicillin to nanoparticles of PIHCA and showed that the antimicrobial activity of the drug is not altered by linkage to this carrier (12). We found that nanoparticle-bound ampicillin is significantly more effective than free ampicillin in chronic experimental listeriosis in athymic nude mice (27). However, its effect was mostly limited to the liver, and bacterial counts in the spleen changed little (27).

Since nothing is known about the effectiveness of ampicillin-bound PIHCA nanoparticles in lethal infections, in the present study we examined the effect of this treatment on the outcome of an acute fatal infection experimentally induced in mice by a strain of *Salmonella* serotype *typhimurium*. Experimental salmonellosis is considered as a disease typical of infection by intracellular bacteria (5, 11, 17, 19) and in many respects resembles typhoid fever in humans (14, 17, 22, 26). It has been adopted as a model system for investigating the therapeutic effects of various antibiotics both in free form (1–4, 9, 25) and entrapped in liposomes (8, 24).

In mice, susceptibility to salmonella infections is genetically controlled (13, 16, 21, 23, 26). C57BL/6 mice are susceptible (*Ity*^{s}) to this infection. We therefore used this strain in the experiments described here.

MATERIALS AND METHODS

A total of 100 μl of isohexylcyanoacrylate monomer (Sopar, Saint-Darmes-Avelines, Belgium) was mechanically stirred at room temperature into 10 ml of an aqueous polymerization medium (1% dextran 70, 5% glucose, and 0.001 N HCl) containing 2 mg of ampicillin trihydrate (Negma, Buc, France) per ml. After polymerization of the monomer for 6 h, a milky suspension was obtained which was neutralized with 1 N NaOH. Unbound PIHCA nanoparticles were prepared in the same way, but no ampicillin was added to the polymerization medium.

The amount of ampicillin bound to the nanoparticles was determined after ultracentrifugation (110,000 × *g* for 90 min) by measuring the free ampicillin in the supernatant by a reversed-phase high-performance liquid chromatography assay (18). When the concentration of ampicillin in the polymerization medium was 2 mg/ml, 90 ± 3% of the drug was firmly bound to the nanoparticles (n = 4). The nanoparticle diameter, as determined by laser light scattering (Nanosizer Coulter; Coultronics, Paris, France), was 172 ± 10 nm for unbound nanoparticles (n = 4) and 187 ± 13 nm for ampicillin-bound nanoparticles (n = 4).

S. typhimurium C5 from a lyophilized vial (Institut Pasteur, Paris, France) (20) was cultured on Drigalski agar at 37°C for 18 h. A stock solution was prepared by harvesting the cells in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with 10% glycerol, and 40 portions (1 ml) of this suspension were frozen in liquid nitrogen and stored at −70°C.

C57BL/6 mice (age, 8 to 10 weeks; IFA-CREDO, Les Oncins, L'Arbresle, France) were experimentally infected. On the day before the experiment, one frozen portion of the stock solution of *S. typhimurium* C5 was thawed, inoculated into 10 ml of broth, and incubated at 37°C for 18 h. The resulting culture was diluted 10^{-4} in sterile saline, and 0.2 ml of the dilution was then injected into each mouse through the tail vein. Counts of viable bacteria in inocula ranged from 6 × 10^3 to 8 × 10^3 CFU/ml. At 3, 6, and 8 days after bacterial inoculation, groups of five mice each were injected in the retroorbital sinus with 0.4 ml of one of the following: unloaded nanoparticles, free ampicillin, unloaded nanoparticles and free ampicillin, or ampicillin-bound nanoparticles. The therapeutic effects of the different forms of drugs were evaluated according to the duration of survival.

To study the effects of the different forms of drugs on the course of infection in the liver and spleen, three or four

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TABLE 1. Effects of nanoparticle-bound ampicillin, free ampicillin, and unbound nanoparticles on the survival of C57BL/6 mice inoculated with *S. typhimurium* C5

Treatment			No. of survivors/no. tested on the following days after challenge:							
Agent	Dose of (mg/mouse) ampicillin	No. of doses	5	8	10	15	20	30	40	60
None			10/10	4/10	0/10					
Unbound nanoparticles[a]	0	3	10/10	4/10	0/10					
Free ampicillin	0.8	3[b]	10/10	10/10	6/10	4/10	0/10			
	16	3	10/10	10/10	10/10	4/10	0/10			
	32	3	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
	32	1[c]	10/10	10/10	4/10	2/10	0/10			
	64	1	0/5							
Ampicillin-bound nanoparticles	0.8	3	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
	0.8	1	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
	0.4	1	10/10	10/10	7/10	5/10	0/10			
Unbound nanoparticles and free ampicillin	0.8	3	10/10	10/10	6/10	2/10	0/10			

[a] The total dosage was 12 mg of PIHCA in three doses of 4 mg each.
[b] At days 3, 6, and 8 after bacterial inoculation.
[c] At day 3 after bacterial inoculation.

surviving mice were killed by chloroform anesthesia at days 5, 10, 15, 20, 30, 40, and 60 after inoculation. The spleens and livers were immediately removed under aseptic conditions and were individually homogenized with an electric mixer (Ultraturrax; Bioblock, Paris, France) in 5 ml of sterile saline. Homogenates were serially diluted 10-fold in saline. To count viable bacteria, 0.1 ml of the appropriate dilutions was plated on Drigalski agar and incubated at 37°C for 48 h. Residual materials from undiluted homogenates were tested for viable bacteria in Mueller-Hinton agar (Diagnostic Pasteur) by the pour-plate technique (15).

To compare the distribution of free and nanoparticle-bound ampicillin in tissue, we gave a single retroorbital injection of 0.8 mg of one of the two preparations to noninfected C57BL/6 mice. At 1, 6, 24, and 72 h thereafter, four mice from each group were killed and blood was collected by cardiac puncture. The spleens, livers, kidneys, and lungs were then excised, washed three times in phosphate-buffered saline (pH 6.0), and dried on Whatman paper to remove as much non-tissue-associated ampicillin as possible. The tissues were then homogenized in phosphate-buffered saline with an electric mixer, and the ampicillin concentration was measured in a diffusion assay in antibiotic medium 2 (Difco) by using the growth of *Bacillus subtilis* ATCC 6633 as an indicator.

RESULTS

All untreated mice, as well as those given three injections of unloaded nanoparticles, died within 10 days of bacterial inoculation (Table 1). By contrast, all mice injected with three doses of 0.8 mg each of nanoparticle-bound ampicillin were alive 60 days after bacterial inoculation. With free ampicillin, three doses of 32 mg each were necessary to obtain a similar result. Lower doses of free ampicillin, and also of free ampicillin mixed with unloaded nanoparticles, delayed but did not eliminate mortality. Furthermore, all the mice that were only given a single injection of 0.8 mg of ampicillin bound to nanoparticles survived, whereas all those treated with a single injection of 32 mg of free ampicillin ultimately died. When twice this dose of ampicillin was tested, all the mice died rapidly of seizures. An attempt to have the minimum therapeutic dose of nanoparticle-bound ampicillin reduced by administering a single injection of 0.4 mg resulted in the resurgence of mouse mortality.

The bacterial counts in the livers and spleens of the mice treated with these different regimens (Table 2) were greater than 10^7 CFU per organ in all control mice and were not significantly lower in the organs of the mice given unloaded nanoparticles. They were 10 to 10,000 times lower in the mice given ampicillin free from, mixed with, or bound to nanoparticles. None of the treatments tested was, however, able to sterilize the organs of the mice, as we found living bacteria in the livers and spleens of all those mice that were still alive 60 days after bacterial inoculation.

Study of the distribution in tissue of ampicillin injected free or bound to nanoparticles to noninfected mice showed that antibiotic levels in sera, kidneys, and lungs were not significantly different, whatever the form of antibiotic used (Table 3). However, in the livers and spleens, these levels at 6 h after injection were, respectively, 96 and 11 times higher for nanoparticle-bound ampicillin than for free ampicillin. Furthermore, when nanoparticle-bound ampicillin was used, antibiotic concentrations were the same in the livers and spleens, whereas with the free ampicillin they were 10 to 20 times higher in the spleens than in the livers.

DISCUSSION

Our results show that the linkage of ampicillin to PIHCA nanoparticles dramatically increases its efficacy in treating *S. typhimurium* infections in C57BL/6 mice, because a total dose of 0.8 mg of ampicillin bound to nanoparticles suppressed all mortality, whereas three doses of 32 mg each of the free drug were required to suppress mortality. The therapeutic index of ampicillin, calculated on the basis of mouse mortality, was therefore increased by 120-fold when it was bound to nanoparticles. Furthermore, a single injection of 0.8 mg of nanoparticle-bound ampicillin was just as efficient as three in suppressing all mortality, whereas a

TABLE 2. Effects of ampicillin-bound nanoparticles, free ampicillin, and unbound nanoparticles on counts of *S. typhimurium* C5 in the livers and spleens of C57BL/6 mice at various times after bacterial inoculation

Organ	Agent	Dose of (mg/mouse) ampicillin	No. of doses	Mean $\log_{10}$ CFU/organ (SD)[a] on the following days after challenge:								
				2	5	8	10	15	20	30	40	60
Liver	None			3.40 (0.36)	5.70 (0.27)	8.03 (0.20)						
	Unbound nanoparticles[b]	0	3[c]	ND[d]	5.57 (0.29)	7.36 (0.50)						
	Free ampicillin	0.8	3	ND	5.64 (0.53)	ND	6.90 (0.43)	6.11 (0.55)				
		16	3	ND	4.15 (0.30)	ND	6.52 (0.32)	6.37 (0.29)				
		32	1[e]	ND	4.49 (0.42)	ND	7.99 (0.71)	4.47 (0.30)				
		32	3	ND	2.69 (0.56)	ND	2.85 (0.66)	3.41 (0.29)	2.79 (0.58)	2.02 (0.35)	2.24 (0.42)	1.56 (0.97)
	Ampicillin-bound nanoparticles	0.8	3	ND	2.69 (0.36)	ND	1.95 (0.24)	2.02 (0.44)	1.94 (0.33)	2.78 (0.15)	2.28 (0.52)	2.21 (0.50)
		0.8	1	ND	1.63 (1.16)	ND	2.51 (0.38)	4.88 (0.89)	4.78 (0.39)	3.68 (0.15)	3.49 (0.33)	2.41 (0.38)
		0.4	1	ND	4.93 (1.50)	ND	4.93 (0.45)	5.23 (0.77)				
	Unbound nanoparticles and free ampicillin			ND	4.53 (0.64)	ND	6.78 (0.76)	7.24 (0.48)				
Spleen	None			3.12 (0.48)	5.85 (0.31)	7.71 (0.58)						
	Unbound nanoparticles	0	3	ND	5.96 (0.52)	7.59 (0.12)						
	Free ampicillin	0.8	3	ND	5.83 (0.66)	ND	5.67 (0.35)	5.38 (0.45)				
		16	3	ND	4.87 (0.28)	ND	6.48 (0.36)	6.22 (0.21)				
		32	1	ND	5.57 (0.42)	ND	6.51 (1.43)	4.67 (0.03)				
		32	3	ND	2.55 (0.87)	ND	3.15 (0.86)	3.77 (0.31)	2.90 (0.27)	2.37 (0.37)	2.24 (0.42)	1.56 (0.97)
	Ampicillin-bound nanoparticles	0.8	3	ND	3.23 (0.18)	ND	2.92 (0.21)	3.26 (0.22)	2.92 (0.36)	2.73 (0.18)	2.11 (0.35)	2.52 (0.36)
		0.8	1	ND	1.46 (0.99)	ND	3.31 (0.76)	5.04 (0.57)	4.78 (0.27)	3.99 (0.17)	3.73 (0.23)	2.53 (0.31)
		0.4	1	ND	5.09 (1.60)	ND	5.40 (0.33)	5.56 (1.23)				
	Unbound nanoparticles and free ampicillin			ND	4.45 (0.55)	ND	6.44 (0.53)	6.84 (0.39)				

[a] Three or four mice per group.
[b] The total dosage was 12 mg of PIHCA in three doses of 4 mg each.
[c] At days 3, 6, and 8 after bacterial inoculation.
[d] ND, Not done.
[e] At day 3 after bacterial inoculation.

single injection of the maximum nonlethal dose of free ampicillin (32 mg) delayed mortality, but did not reduce it.

It must be pointed out, however, that neither the liver nor the spleen was sterilized in any of the surviving mice, even 60 days after injection. It is known that T-cell-mediated immunity induces resolution of disease in the late stage of murine thyphoid even in the carrier state (5, 11, 17, 19).

The high efficacy of nanoparticle-bound ampicillin observed in the treatment of acute murine experimental salmonellosis confirms our previous results for chronic *Listeria monocytogenes* infection in nude mice (27). This efficacy is probably attributable to the combined effect of two types of targeting. First, as shown by our tissue distribution studies, the linkage of ampicillin to nanoparticles led to the concentration of the drug in the livers and spleens; this is important, since these organs are the major foci of infection in the experimental model that was used (20). Second, as discussed elsewhere (27), the cellular uptake of ampicillin by macro-

TABLE 3. Distribution of activity after intravenous injection of noninfected C57BL/6 mice with 0.8 mg of free or nanoparticle-bound ampicillin

Ampicillin	Organ	Mean concn (μg/g[a] [SD[b]]) at the following times after administration:			
		1 h	6 h	24 h	72 h
Free	Serum[c]	2.0	<0.1	<0.1	<0.1
	Spleen	3.2 (0.7)	1.6 (0.7)	1.9 (0.4)	0.9 (0.2)
	Liver	0.3 (0.3)	0.2 (0.2)	<0.1	<0.1
	Kidneys	1.1 (0.3)	0.8 (0.5)	0.6 (0.2)	0.14 (0.1)
	Lungs	1.9 (0.3)	0.6 (0.2)	1.6 (0.2)	0.4 (0.1)
Nanoparticle-bound	Serum	1	0.15	0.14	<0.1
	Spleen	12.4 (2.3)	18.3 (9)	2 (0.7)	1.2 (0.2)
	Liver	12.8 (1.1)	19.2 (3.7)	1.8 (0.3)	<0.1
	Kidneys	3.0 (1.6)	0.3 (0.1)	0.8 (0.2)	0.25 (0.1)
	Lungs	0.9 (0.5)	1.4 (1.1)	1.2 (0.1)	0.5 (0.1)

[a] Values are in micrograms per milliliter for serum.
[b] Five mice per time point per treatment.
[c] The sera from five mice were pooled.

phages is probably increased more when the drug is bound to nanoparticles than when it is in the free form. In this connection, previous studies have shown that the uptake of nanoparticles by an endocytotic mechanism allows intralysosomal localization of the carrier (7) and a subsequent increase in the intracellular concentration of the targeted drug (10). Taken together, these results suggest that ampicillin-bound nanoparticles should be further explored for the treatment of intracellular bacterial infections in animals and humans.

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