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Effectiveness of Nanoparticle-Bound Ampicillin in the Treatment of *Listeria monocytogenes* Infection in Athymic Nude Mice

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The effectiveness of nanoparticle-bound ampicillin was tested in the treatment of experimental *Listeria monocytogenes* infection in congenitally athymic nude mice. Nanoparticles of polyisohexylcyanoacrylate (PIHCA) 187 ± 13 nm in diameter were bound to ampicillin at an ampicillin/PIHCA ratio of 0.2:1. The proportion of ampicillin bound was 90% ± 3%. After adsorption onto nanoparticles, the therapeutic activity of ampicillin increased dramatically over that in the free state. Thus, 2.4 mg of nanoparticle-bound ampicillin (three doses of 0.8 mg each) had a greater therapeutic effect than 48 mg of free ampicillin (three doses of 16 mg each). These results might provide an incentive for further development of intracellular targeting of antibiotics on biodegradable polymeric carriers.

Infections caused by obligate or facultative intracellular microorganisms such as *Listeria monocytogenes* are difficult to treat, especially in immunocompromised patients (15). The need for intracellular chemotherapy of these infections has been recognized for many years (19). It was recently observed that the use of particulate carriers able to undergo endocytosis increased intracellular delivery of antibiotics (18, 20). For instance, when ampicillin was entrapped in liposomes it had an enhanced therapeutic effect on listeriosis in normal mice (1). This was probably because the liposomal encapsulation of ampicillin made it more readily available to intracellular bacteria and allowed lysosomal uptake of the drug (2).

Although these results are interesting, large-scale manufacture of liposomes is still difficult. The disruptive effects of human serum on liposomes may lead to some extrusion of trapped drugs (11), and the limited stability of liposomes in circulating blood has been stressed (17). For these reasons, we investigated the possibility of using biodegradable polymeric nanoparticles as an alternative carrier to enhance intracellular drug delivery (4). Owing to their polymeric nature, polyalkylcyanoacrylate nanoparticles may be more stable than liposomes in biological fluids and during storage, and they can generally entrap molecules in a stable and reproducible way (5). This is why we recently described the conditions required for the binding of ampicillin to nanoparticles of polyisohexylcyanoacrylate (PIHCA) and showed that the antimicrobial activity of the drug remained unaltered after linkage to this carrier (8). Desorption of ampicillin from PIHCA nanoparticles is only 10% of the loaded dose after 2 h of incubation in phosphate buffer at 37°C (8).

In the present study of experimental murine listeriosis, we found that such nanoparticle-bound ampicillin was significantly more effective than free ampicillin.

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MATERIALS AND METHODS

Isohexylcyanoacrylate monomer (Sopar, Saint-Darmes-Avelines, Belgium) (100 μl) was mechanically stirred at room temperature into 10 ml of an aqueous polymerization medium (1% dextran 70 [Sigma], 5% glucose, 0.001 N HCl) containing 2 mg of ampicillin trihydrate (Negma, Buc, France) per ml. After a 6-h polymerization of the monomer, a milky suspension was obtained, which was then 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 reverse-phase high-performance liquid chromatography assay (14).

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 diameter of nanoparticles as determined by laser light scattering (Nanosizer Coulter; Coultronics, France) was 172 ± 10 nm for unbound nanoparticles ($n = 4$), and 187 ± 3 nm for ampicillin-bound nanoparticles ($n = 4$). The stability of nanoparticles as estimated by the release of ampicillin in the presence of plasma was measured as follows. Ampicillin-loaded PIHCA nanoparticles (0.2 mg of ampicillin per mg of polymer) were mixed with 90% newborn calf serum (GIBCO Laboratories, Grand Island, N.Y.). The final concentration of ampicillin bound to nanoparticles was 0.2 mg/ml of serum. This preparation was aliquoted before and after a 1-h incubation at 37°C. The aliquots were centrifuged at 10,000 × *g* for 90 min, and proteins in the supernatant were precipitated with 0.5 M perchloric acid (Prolabo, Paris, France). Ampicillin was assayed as described above, and the results showed that 2.9% ± 1.3% ($n = 3$) of the total amount of ampicillin bound to the nanoparticles was released.

L. monocytogenes EGD (NTCC 7973) serovar 1/2a was cultured on blood agar at 37°C for 18 h from a lyophilized vial

TABLE 1. Effects of nanoparticle-bound ampicillin, free ampicillin, and unbound nanoparticles on counts of *L. monocytogenes* in the livers and spleens of nude mice at various times after bacterial inoculation

Organ	Organ and treatment[a]	Mean $\log_{10}$ CFU/organ (standard deviation)[b] on:					
		Day 2	Day 4	Day 7	Day 12	Day 16	Day 19
Liver	None	4.7 (1.0)	4.8 (0.8)[c]	5.0 (0.9)	5.5 (0.8)[c]	5.4 (1.6)	5.0 (0.5)
	Unbound nanoparticles[d]	ND[e]	4.8 (0.6)	4.2 (0.4)	3.4 (0.7)[c]	4.3 (0.7)	4.1 (0.2)
	Free ampicillin						
	2.4 mg[f]	ND	3.2 (0.3)[c]	3.6 (0.3)	3.5 (0.3)	3.7 (0.2)	4.0 (0.2)
	24 mg[g]	ND	2.3 (0.4)	1.6 (0.9)	2.1 (0.3)	2.8 (0.5)	3.0 (0.3)
	48 mg[h]	ND	1.5 (0.8)	1.0 (1.4)	2.2 (0.25)	2.7 (0.5)[c]	3.1 (0.5)
	Unbound nanoparticles + free ampicillin[i]	ND	2.2 (1.0)	2.3 (1.0)	2.4 (0.6)	3.2 (0.5)	3.6 (1.1)
	Ampicillin-bound nanoparticles[i]	ND	1.6 (1.2)	0	0.3 (0.7)	1.7 (1.4)[c]	3.2 (0.9)
Spleen	None	6.0 (0.4)	5.6 (0.9)	5.5 (0.7)[c]	5.3 (0.2)	5.0 (0.5)	5.1 (0.3)
	Unbound nanoparticles	ND	5.4 (0.3)	4.8 (0.5)	4.7 (0.5)	5.1 (0.3)	4.9 (0.4)
	Free ampicillin						
	2.4 mg	ND	5.0 (0.5)	4.1 (0.3)	5.0 (0.5)	4.7 (0.6)	4.6 (0.7)
	24 mg	ND	4.4 (0.6)	2.9 (0.1)	4.0 (0.7)	3.9 (0.5)	4.2 (0.3)
	48 mg	ND	3.8 (0.3)	3.2 (0.4)	4.4 (0.4)	4.1 (0.6)	4.8 (0.6)
	Unbound nanoparticles + free ampicillin	ND	4.4 (0.7)	4.3 (0.6)	5.2 (0.3)	5.4 (0.5)	5.0 (0.6)
	Ampicillin-bound nanoparticles	ND	4.2 (0.3)	3.4 (0.3)[c]	4.9 (0.6)	4.7 (0.7)	4.6 (0.9)

[a] All products were injected intravenously in three separate doses of 0.4 ml each on days 2, 5, and 8 after bacterial inoculation.
[b] Five mice per group.
[c] $P < 0.05$ (see text).
[d] Total dosage of 12 mg divided into three doses of 4 mg each.
[e] ND, Not done.
[f] Total dosage divided into three doses of 0.8 mg each.
[g] Total dosage divided into three doses of 8 mg each.
[h] Total dosage divided into three doses of 16 mg each.
[i] Total dosage of 12 and 2.4 mg for nanoparticles and ampicillin, respectively, divided into three doses of 4 and 0.8 mg.

(Institut Pasteur, Paris, France). A stock solution was prepared by harvesting the cells in brain heart infusion broth with 10% glycerol, and 40 1-ml portions of this suspension were frozen in liquid nitrogen and stored at −70°C. Locally bred 8- to 10-week-old C57BL/Ka mice, homozygous for the nude mutation (*nu/nu*), were used.

On the day before the experiment, each frozen aliquot of the stock solution 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 1×10^3 to 3×10^3 CFU/ml. At 2, 5, and 8 days after bacterial inoculation, the mice were divided into groups of five, and all members of each group were injected in the tail vein with one of the following (at 0.4 ml per mouse): unbound nanoparticles, free ampicillin, unbound nanoparticles plus free ampicillin, or ampicillin-bound nanoparticles.

The mice were killed by chloroform anesthesia on days 2, 4, 7, 12, 16, and 19 after bacterial inoculation. The spleen and liver were immediately removed aseptically from each mouse and homogenized separately with an electric mixer (Ultra-turrax; Bioblock, Paris, France) in 5 ml of sterile saline. For ampicillin-treated mice, 50 U of penicillinase (Institut Pasteur Production) was added to the homogenates, which were serially diluted 10-fold in saline. To count viable bacteria, we plated 0.1 ml each of the appropriate dilutions on blood agar and incubated them at 37°C for 48 h. Residual materials from undiluted homogenates were tested for viable bacteria by the pour-plate technique (12) in Mueller-Hinton agar. Mean bacterial counts were compared by Student's *t* test.

RESULTS

The results of the present experiment (Table 1) show that when chronic infection was established in untreated mice, mean bacterial counts in the livers and spleens exhibited no significant variation during the 19-day follow-up period. Treatment with unbound PIHCA nanoparticles was followed by a slight drop in bacterial counts in the liver, which reached a significant level only on day 12 after infection, after the three injections had been completed ($\log_{10}$ CFU per liver, 5.5 ± 0.8 versus 3.4 ± 0.7; $P < 0.05$). Treatment with 0.8-mg doses of free ampicillin produced the same reduction in bacterial counts, but it appeared earlier, reaching a significant level on day 4 after bacterial inoculation ($\log_{10}$ CFU per liver, 4.8 ± 0.8 versus 3.2 ± 0.3; $P < 0.05$). Doses of 0.8 mg of free ampicillin mixed with unbound PIHCA nanoparticles induced a significantly larger drop in bacterial counts in the liver than that observed after administration of either free ampicillin alone or unbound PIHCA nanoparticles alone ($\log_{10}$ CFU per liver, 2.3 ± 1.0 versus 3.6 ± 0.3 and 4.2 ± 0.4, respectively). This drop was equivalent to that observed after the administration of three 8-mg doses of free ampicillin. In addition, 4 days after bacterial inoculation, a single injection of 0.8 mg of nanoparticle-bound ampicillin had the same effect as a single injection of 16 mg of free ampicillin on bacterial counts in the liver ($\log_{10}$ CFU per liver, 1.6 ± 1.2 versus 1.5 ± 0.8). Liver sterilization was observed by day 7 postinfection in the mice treated with nanoparticle-bound ampicillin, but never in those treated with free ampicillin, even at a total dose of 48 mg. Furthermore, for 8 days after the last therapeutic injection, nanoparticle-bound ampicillin in a total dose of 2.4 mg continued

to act more effectively than a total dose of 48 mg of free drug ($\log_{10}$ CFU per liver, 1.7 ± 1.4 versus 2.7 ± 0.5; $P < 0.05$).

Bacterial counts in spleens were less affected by the four treatments than counts in livers were. Neither unbound PIHCA nanoparticles nor a total dose of 2.4 mg of free ampicillin (or a combination of both) had any significant effect on bacterial counts in spleens when compared with those for untreated mice. However, in mice treated with three 0.8-mg injections of nanoparticle-bound ampicillin, significant drops in bacterial counts in spleens were observed 7 days after bacterial inoculation ($\log_{10}$ CFU per spleen in control mice, 3.4 ± 0.3 versus 5.5 ± 0.7; $P < 0.05$). With free ampicillin alone, a similar reduction in bacterial counts was obtained only after three injections of 8 or 16 mg each.

DISCUSSION

We previously showed that the binding of ampicillin to PIHCA nanoparticles was stable over time in phosphate buffer (8) and that the antimicrobial activity of the drug remained unchanged after linkage (8). In the present study we have shown that the complex is also stable in plasma.

We demonstrated the antimicrobial efficiency of PIHCA nanoparticle-bound ampicillin in vivo against experimental listeriosis in athymic nude mice, a model involving a chronic infection of both liver and spleen macrophages (1, 16). The therapeutic index of ampicillin for bacterial counts in the liver rose at least 20-fold after its linkage to PIHCA nanoparticles.

In addition, this form of ampicillin was capable of ensuring liver sterilization after two injections of 0.8 mg of nanoparticle-bound drug, whereas no such sterilization was ever observed with any of the other regimens tested. It is noteworthy that liposome-entrapped ampicillin showed no activity in the same experimental model (1). However, the dose of ampicillin used in that model was only 23% of the dose bound to nanoparticles. In the present study, the reappearance of living bacteria in the liver after the end of the treatment was probably due to a secondary infection derived from other organs such as the spleen, which were not sterilized by the treatment. A moderate antilisteria activity was noted in vivo with unbound nanoparticles in both livers and spleens of athymic nude mice. We previously found that unbound nanoparticles exhibited some antibacterial activity in an in vitro test by using the growth of *Bacillus subtilis* as indicator (8). However, the mechanism of this activity is unknown, and it remains to be determined whether it is responsible for the in vivo effect of nanoparticles on bacterial counts.

The higher efficiency of ampicillin when it was combined with nanoparticles of PIHCA was consistent with the in vivo distribution profile of this cyanoacrylic drug carrier after intravenous administration (4), since cyanoacrylic nanoparticles were found to be rapidly eliminated from the bloodstream by reticuloendothelial cells, which are found mostly in the liver and spleen (13). In addition, autoradiographic studies performed with polyisobutylcyanoacrylate and polyisohexylcyanoacrylate nanoparticles have shown that most of these particles were present in the liver 5 min after intravenous administration. Later, they also concentrated in the spleen, but to a lesser extent (7). The profile distribution of [^{14}C]PIHCA nanoparticles in nude mice showed that 60 to 80% of the polymer concentrated in the liver, whereas only 2 to 6% was in the spleen (6).

Our results can be explained by the fact that the cellular uptake of ampicillin was probably much better when the drug was bound to nanoparticles than when it was free. Previous findings have suggested that nanoparticles are internalized via endocytosis, leading to intralysosomal localization of the carrier and the drug (5). Free acidic beta-lactam antibiotics, like ampicillin, do not diffuse through the lysosomal membrane, because of their ionic character at neutral extracellular pH (P. Tulkens, personal communcation). Therefore, in intracellular infections, bacteria entrapped inside lysosomes are difficult to kill with free ampicillin (9). As the present findings suggest, nanoparticles might overcome these difficulties by increasing the intracellular delivery of antibiotics. Results available to date show that polyalkylcyanoacrylate nanoparticles induce cellular damage to macrophages in culture only at relatively high concentrations in the cell culture medium. No mutagenicity has been shown for polyalkylcyanoacrylate nanoparticles. The 50% lethal dose for mice of polyisobutylcyanoacrylate is 196 mg/kg of body weight (10), and that of PIHCA is greater than 500 mg/kg (P. Couvreur, unpublished results).

Chronic toxicity in rats injected with 16 doses (over 4 months) or 8 doses (over 1 month) of 20 mg of PIHCA nanoparticles per kg has been studied. No toxicity was observed in terms of mortality, weight loss, or histopathological or biological parameters (3).

Taken as a whole, these results open up exciting prospects for the targeting of antibiotics with nonliposomal carriers.

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LITERATURE CITED

1. **Bakker-Wondenberg, I. A. J. M., A. F. Lokerse, F. H. Roerdink, D. Regts, and M. F. Michel.** 1985. Free versus liposome-entrapped ampicillin in treatment of infection due to *Listeria monocytogenes* in normal and athymic (nude) mice. J. Infect. Dis. **151**:917–924.
2. **Bakker-Wondenberg, I. A. J. M., A. Lokerse, J. C. V. van den Berg, F. H. Roerdink, and M. F. Michel.** 1986. Effect of liposome-entrapped ampicillin on survival of *Listeria monocytogenes* in murine peritoneal macrophages. Antimicrob. Agents Chemother. **30**:295–300.
3. **Brasseur, F., A. Biernacki, V. Lenaerts, L. Galanti, P. Couvreur, C. Deckers, and M. Roland.** 1983. Etude de la toxicité des nanoparticles de polycyanoacrylate d'alkyl, p. 194–202. *In* Proceedings 3rd International Conference on Pharmaceutical Technology. Association de Pharmacie Galénique et Industrielle, Paris.
4. **Couvreur, P., L. Grislain, V. Lenaerts, F. Brasseur, P. Guiot, and A. Biernacki.** 1986. Biodegradable polymeric nanoparticles as drug carrier for antitumor agents, p. 28–94. *In* P. Guiot and P. Couvreur (ed.), Polymeric nanoparticles and microspheres. CRC Press, Inc., Boca Raton, Fla.
5. **Couvreur, P., B. Kante, M. Roland, and P. Speiser.** 1979. Adsorption of antineoplastic drugs to polyalkylcyanoacrylate nanoparticles and their related characteristics in a calf serum medium. J. Pharm. Sci. **68**:1521–1523.
6. **Gipps, E. M., R. Arshady, J. Krenter, P. Groscurth, and P. P. Speiser.** 1986. Distribution of polyhexylcyanoacrylate nanoparticles in nude mice bearing human osteosarcoma. J. Pharm. Sci. **25**:256–258.
7. **Grislain, L., P. Couvreur, V. Lenaerts, M. Roland, D. Deprez-Decampenere, and P. Speiser.** 1983. Pharmacokinetics and distribution of a biodegradable drug carrier. Int. J. Pharm. **15**:335–345.
8. **Henry-Michelland, S., M. J. Alonso, A. Andremont, P. Main-**

cent, J. Sauzières, and P. Couvreur. 1987. Attachment of antibiotics to nanoparticles: preparation, drug-release and antimicrobial activity in vitro. Int. J. Pharm. **35**:121–127.
9. **Horwitz, M. A.** 1982. Phagocytosis of microorganisms. Rev. Infect. Dis. **4**:104–123.
10. **Kante, B., P. Couvreur, G. Dubois-Krack, C. de Meester, P. Guiot, M. Roland, M. Mercier, and P. Speiser.** 1982. Toxicity of polyalkylcyanoacrylate nanoparticles. I. Free nanoparticles. J. Pharm. Sci. **71**:786–790.
11. **Kimelberg, H. K., E. Mayhewe, and P. Papahajopoulos.** 1975. Distribution of liposome-entrapped cations in tumor-bearing mice. Life Sci. **17**:715–724.
12. **Koch, A. L.** 1981. Growth measurement, p. 179–207. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
13. **Lenaerts, V., J. F. Nagelkerke, T. J. C. van Berkel, P. Couvreur, L. Grislain, M. Roland, and P. Speiser.** 1984. In vivo uptake of polyisobutylcyanoacrylate nanoparticles by rat liver Kupffer, endothelial, and parenchymal cells. J. Pharm. Sci. **73**:980–983.
14. **Margosis, M.** 1982. Quantitative reversed-phase high-performance liquid chromatographic analysis of ampicillin. J. Chromatogr. **236**:469–480.
15. **Nieman, R. E., and B. Lorber.** 1980. Listeriosis in adults: a changing pattern. Report of eight cases and review of the literature, 1968–1978. Rev. Infect. Dis. **2**:207–227.
16. **North, R. J.** 1981. Immunity of *Listeria monocytogenes*, p. 201–219. *In* A. J. Nahimas and R. J. O'Reilly (ed.), Immunology of human infection. Part I. Bacteria, mycoplasma, chlamydiae, and fungi. Plenum Publishing Corp., New York.
17. **Scherpoff, G., F. Roerdink, D. Hoekstra, J. Zborowski, and E. Wisse.** 1980. Stability of liposomes in presence of blood constitutants: consequences for uptake of liposomal lipid and entrapped compounds by rat liver cells, p. 179–209. *In* G. Gregoriadis and A. C. Allison (ed.), Liposomes in biological systems. John Wiley & Sons, Inc., New York.
18. **Stevenson, M., A. J. Baillie, and R. M. E. Richards.** 1983. Enhanced activity of streptomycin and chloramphenicol against intracellular *Escherichia coli* in the J774 macrophage cell line mediated by liposome delivery. Antimicrob. Agents Chemother. **24**:742–749.
19. **Tulkens, P.** 1985. The design of antibiotics capable of an intracellular action, p. 179–194. *In* R. Buri and R. Gumma (ed.), Aims, potentialities and problems in drug targeting. Elsevier Biomedical Press, Amsterdam.
20. **Vladimrsky, M. A., and G. A. Ladigina.** 1982. Antibacterial activity of liposome-entrapped streptomycin in mice infected with *Mycobacterium tuberculosis*. Biomed. Pharmacother. **36**: 375–377.