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Effect of Liposome-Entrapped Ampicillin on Survival of *Listeria monocytogenes* in Murine Peritoneal Macrophages

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The effect of liposomal encapsulation of ampicillin on the antibacterial activity against intracellular *Listeria monocytogenes* was studied by comparing survival of *L. monocytogenes* within peritoneal mouse macrophages in the presence of free ampicillin alone or in combination with liposome-entrapped ampicillin. In the presence of 50 μg of free ampicillin per ml of the incubation medium, intracellular growth of *L. monocytogenes* was still observed, although less as compared with intracellular growth in the absence of ampicillin. At a concentration of 50 μg of free ampicillin plus 100 μg of liposome-entrapped ampicillin per ml, 99% of the intracellular bacteria were killed. On the other hand, a concentration of 150 μg of free ampicillin per ml plus empty liposomes only inhibited intracellular bacterial growth, and the bacteria were not killed. In addition, empty liposomes at a concentration of 1 μmol of lipid per ml had no effect on intracellular bacterial growth. In broth, liposome-entrapped ampicillin at a concentration of 100 μg/ml was not bactericidal for *L. monocytogenes*, indicating that significant leakage of ampicillin from the liposomes with subsequent killing of the bacteria by the free drug did not occur. Therefore, we concluded that liposomal encapsulation of ampicillin results in an increased availability of the antibiotic for the intracellular bacteria.

Clinical experience has indicated that infections caused by intracellular bacteria are difficult to treat, especially in patients with underlying malignant disease or in patients receiving immunosuppressive drugs (18). While the bacteria are susceptible to antibiotics in vitro, they seem to be protected from the action of antibiotics by their intracellular localization. The relative inefficiency of antibiotics in dealing with intracellular bacteria may be due to their poor penetration into cells. In a previous report it was shown that ampicillin in relatively high doses cured immunologically normal mice with infections due to the facultative intracellular bacterium *Listeria monocytogenes* (2). In a recent study we observed a considerable enhancement (80-fold) in the therapeutic activity of ampicillin resulting from encapsulation of the antibiotic in liposomes (3). We speculated that the mechanism by which the liposomes improved the therapeutic index of ampicillin was an increased delivery of the antibiotic to the liver and spleen, the organs infected with *L. monocytogenes*. Seventy-nine percent of the injected amount of liposome-entrapped ampicillin could be recovered from the liver and spleen. After isolating the Kupffer cells by pronase perfusion of the liver and subsequent elutriation centrifugation of the nonparenchymal cells, substantial amounts of radioactive liposomal ampicillin could be recovered from this cell type, the target cells of *L. monocytogenes*, after intravenous infusion (data not shown). In this study we investigated whether the enhancement of the therapeutic activity of liposome-entrapped ampicillin is also due to an increased availability of the drug inside the macrophages, the target cells of *L. monocytogenes*. We examined the effect of free versus liposome-entrapped ampicillin on the survival of *L. monocytogenes* in murine peritoneal macrophages in vitro.

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MATERIALS AND METHODS

Animals. Female C57Bl/Ka mice were used in all experiments. Mice were 11 to 13 weeks old and had been bred at REPGO-TNO Rijswijk, The Netherlands.

Bacteria. A serum-resistant strain of *L. monocytogenes* type 4b was used. The MIC of ampicillin (Beecham Research Laboratories, Amstelveen, The Netherlands) for this strain was 0.16 μg/ml by the tube dilution test. Bacteria were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd., London, England). This stationary-phase culture contained 2×10^9 CFU/ml (range, 1.8×10^9 to 2.2×10^9). Bacteria were washed twice in phosphate-buffered saline by centrifugation for 10 min at 3,500 × *g*. *L. monocytogenes* organisms (2×10^8 CFU/ml) were opsonized by incubation with 10% newborn calf serum (NCS) under continuous rotation at 8 rpm (rotating rack; Breda Scientific, Breda, The Netherlands) for 30 min at 37°C.

Preparation of liposomes. Multilamellar vesicles consisting of cholesterol, sphingomyelin, and L-α-phosphatidyl-L-serine in a molar ratio of 5:4:1 were prepared as described previously (3). All liposome preparations were sized by extrusion through polycarbonate filters (final pore size, 0.4 μm) and freed of nonentrapped ampicillin on a Sepharose CL-2B column. For binding studies of liposomes to peritoneal cells, trace amounts of [^{14}C]ampicillin were added to the lipid mixtures. [^{14}C]ampicillin (specific activity, 1 mCi/mmol) was kindly supplied by Beecham Pharmaceuticals (Betchworth, Surrey, England). Incorporation of antimicrobially active ampicillin was found to be 100 ± 4.9 (mean of four determinations) μg of ampicillin per μmol of vesicle lipid.

Isolation of *L. monocytogenes*-infected peritoneal macrophages from mice. Isolation of *L. monocytogenes*-infected peritoneal macrophages was performed as described by van Dissel et al. (26), with some modifications. A number of 10^8

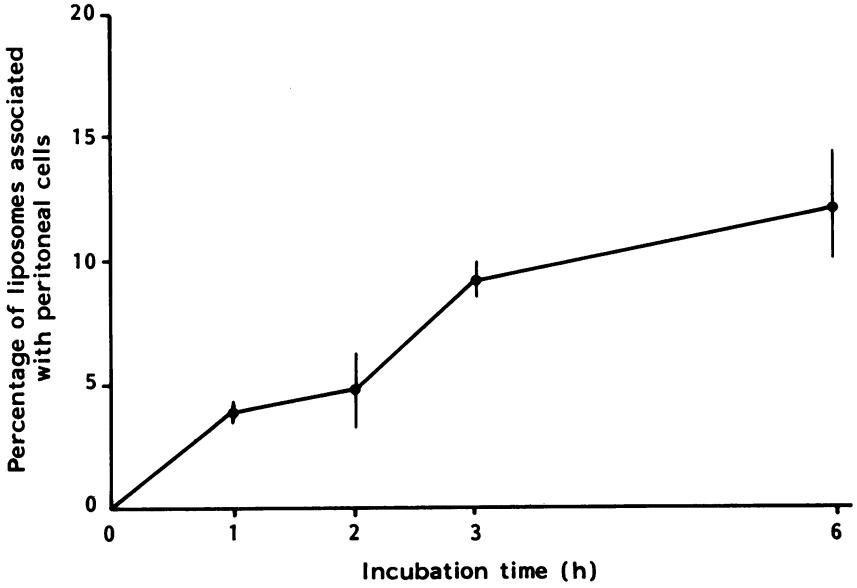

FIG. 1. Association of liposomal [^{14}C]ampicillin with peritoneal cells containing ingested *L. monocytogenes*. Suspensions of 5 × 10^6 cells per ml were incubated in HBSS-gel with 10% NCS at 37°C under rotation at 8 rpm (rotating rack; Breda) in the presence of liposomes containing 100 μg of ampicillin, to which trace amounts of [^{14}C]ampicillin were added, plus 50 μg of free ampicillin. The liposomal lipid concentration was 1 μmol per ml of culture medium. The data given are means ± standard deviations of three determinations.

CFU of opsonized *L. monocytogenes* in 1 ml of Hanks balanced salt solution (HBSS; Oxoid) containing 10% NCS was injected intraperitoneally. Mice were sacrificed after 30 min. The peritoneal cells were obtained by peritoneal washings with 5 ml of cold phosphate-buffered saline–50 U of heparin per ml, followed by centrifugation at 110 × *g* for 10 min at 4°C. To remove the noningested bacteria, cells were centrifuged at 25 × *g* for 7 min at 4°C and washed once with cold HBSS–0.1% gelatin (HBSS-gel)–0.5 U of heparin per ml, followed by two washings with cold HBSS-gel. Cell suspensions consisted of mononuclear cells and less than 3% granulocytes. Forty-six percent of the cells were macrophages containing ingested *L. monocytogenes*. Suspensions of cells were adjusted to a concentration of 5 × 10^6 cells per ml. Immediately after the last washing step the cells were incubated in HBSS-gel–10% NCS at 37°C under continuous rotation at 8 rpm (rotating rack; Breda) in the presence or absence of ampicillin alone or in combination with empty liposomes or liposomal ampicillin. The time interval between removal of macrophages from the peritoneum and the initiation of the in vitro incubation did not exceed a period of 40 min at 4°C.

Cellular uptake of [^{14}C]ampicillin-containing liposomes. To test the cell association of the liposomes, *L. monocytogenes*-infected peritoneal cells were incubated with liposomes containing 100 μg of ampicillin, to which trace amounts of [^{14}C]ampicillin (10^4 dpm) were added, plus 50 μg of free ampicillin per ml. Samples of 0.5 ml containing 2.5 × 10^6 cells were removed and chilled in ice water. Cells and liposomes not associated with cells were separated by the method of Lewis et al. (13) with some minor modifications. Each suspension was layered onto 0.5 ml of 10% Ficoll (Pharmacia, Uppsala, Sweden) in a polypropylene tube and centrifuged for 20 min at 400 × *g* at 4°C. Radioactivity was determined in the cell pellet and the supernatant. Control studies showed that this separation method was effective. After liposomes were mixed with peritoneal cells derived from uninfected mice at 4°C, 95 ± 2.6% (n = 3) of the cells were recovered from the cell pellet, whereas 95 ± 0.6% (n = 3) of the liposomes not associated with the cells were recovered from the supernatant.

Intracellular survival of *L. monocytogenes*. Cells were exposed to free ampicillin alone or in combination with either liposome-entrapped ampicillin or empty liposomes. Multilamellar vesicles were used at a concentration of 1 μmol of vesicle lipid per ml of the incubation mixture. Control suspensions were incubated without ampicillin. At different time intervals the number of surviving intracellular *L. monocytogenes* was determined. In case the incubation occurred in the absence of free ampicillin, the extracellular bacteria were removed by centrifugation as described above. Small fractions of the cell suspensions were diluted 10-fold in cold distilled water containing 0.01% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), 0.1% Triton X-100 (BDH, Poole, England), and 10 U of penicillinase SR106 (Oxoid) per ml. Triton X-100 was added to disrupt the liposomes. Penicillinase was added to inactivate the free ampicillin already present in the medium or released after disruption of cells and liposomes. Suspensions were mixed vigorously. Cells were disrupted by quickly freezing the sample in liquid nitrogen and thawing at 37°C; this was repeated three times. Freezing and thawing did not affect the viability of *L. monocytogenes*. Tenfold serial dilutions in saline containing penicillinase were prepared and tested for viable bacteria by spreading 200 μl of each dilution onto blood-agar plates. The plates were incubated for 48 h at 37°C. Intracellular survival of *L. monocytogenes* was calculated from these viable counts. Survival of leukocytes and cell viability (trypan blue exclusion) was determined throughout the incubation period.

Bacterial killing curves. To study whether intact liposomes containing ampicillin showed an inhibitory effect on the growth of *L. monocytogenes* in vitro, we incubated the bacteria with liposome-entrapped ampicillin, free ampicillin, or empty liposomes. A stationary-phase culture which had been grown for 16 h at 37°C was diluted in Todd-Hewitt

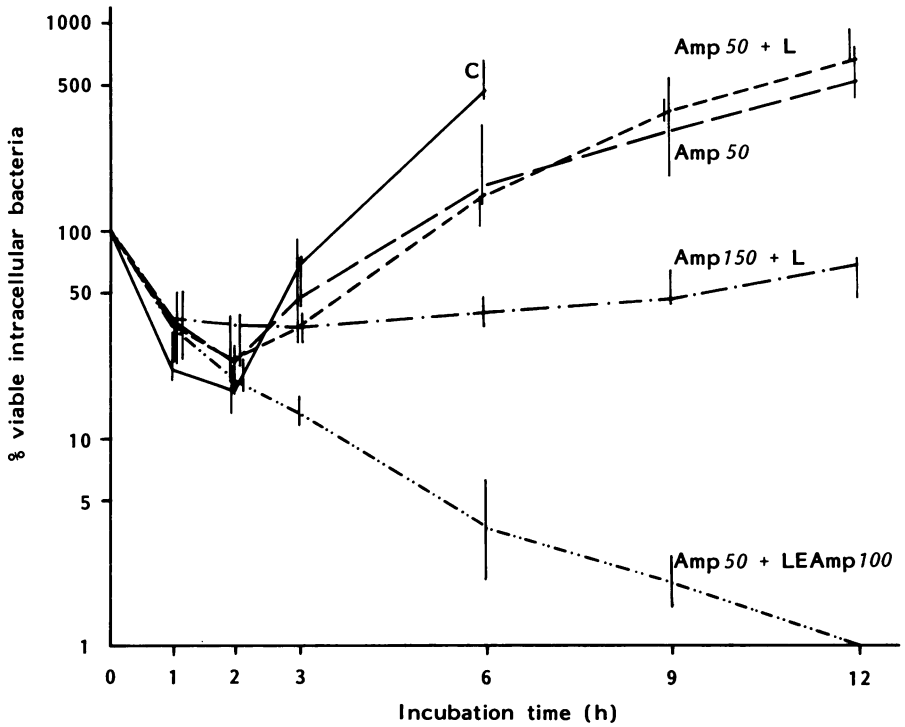

FIG. 2. Intracellular survival of *L. monocytogenes* in peritoneal macrophages of mice after in vivo phagocytosis. Suspensions of 5×10^6 cells per ml of HBSS-gel with 10% NCS were incubated at 37°C without ampicillin (C), in the presence of free ampicillin at a concentration of 50 μg/ml alone (Amp50) or in combination with empty liposomes at a concentration of 1 μmol of lipid per ml (Amp50 + L), 100 μg of liposome-entrapped ampicillin in 1 μmol of lipid per ml (Amp50 + LEAmp100), or 100 μg of free ampicillin plus empty liposomes at 1 μmol of lipid per ml (Amp150 + L). The data given are median values of three determinations, with ranges.

broth to a concentration of 5×10^4 CFU/ml. After incubation for 2 h at 37°C, ampicillin, liposome-entrapped ampicillin, or empty liposomes were added at time zero. The numbers of viable organisms were then determined at regular time intervals over a 6-h period by plating 10-fold serial dilutions in saline onto blood-agar plates containing penicillinase.

RESULTS

Uptake of liposomes by peritoneal cells. Uptake of [^{14}C]ampicillin-labeled liposomes by peritoneal macrophages infected with *L. monocytogenes* was studied during a 6-h incubation period. Infected cells were incubated with liposomes containing 100 μg of ampicillin, to which trace amounts of [^{14}C]ampicillin had been added, plus 50 μg of free ampicillin per ml. Separation of the *L. monocytogenes*-infected cells and the liposomes at time zero, as described above for uninfected cells, resulted in a recovery of 100 ± 2.9% (n = 3) of the cells from the pellet, whereas 97 ± 3.7% (n = 3) of the liposomes not associated with the cells were recovered from the supernatant. Uptake of ampicillin-containing liposomes by the cells increased up to 12% of the added dose after 6 h of incubation (Fig. 1).

Effect of liposome-entrapped ampicillin and free ampicillin on intracellular survival of *L. monocytogenes* within peritoneal macrophages. To investigate whether entrapment of ampicillin within liposomes resulted in an enhanced ability of ampicillin to kill intracellular *L. monocytogenes*, we compared intracellular survival of bacteria in the presence of 50 μg of free ampicillin per ml alone or in combination with empty liposomes at 1 μmol of lipid per ml or 100 μg of liposome-entrapped ampicillin in 1 μmol of lipid per ml, or 100 μg of free ampicillin plus empty liposomes at 1 μmol of lipid per ml. Fifty micrograms of free ampicillin was added to all suspensions to preclude multiplication of extracellular *L. monocytogenes*. Bacterial multiplication extracellularly is inhibited by nonentrapped ampicillin but not by liposome-entrapped ampicillin and thus complicates the determination of intracellular numbers of *L. monocytogenes* organisms. Extracellular bacteria may be present because the washing procedure to separate the peritoneal cells collected from the mice and the noningested *L. monocytogenes* eliminated most (more than 95%) but not all extracellular bacteria. In addition, the release of bacteria caused by intracellular bacteria growth and subsequent lysis of the macrophages during incubation may occur.

After 30 min of phagocytosis in vivo, the number of ingested *L. monocytogenes* organisms was found to be $5.9 \times 10^6 \pm 0.81 \times 10^6$ (100%) per 10^6 macrophages (n = 6). During the first 2 h of incubation of the macrophages in vitro, intracellular bacteria decreased in number to an average of 1.1×10^6 (19%) per 10^6 macrophages within 2 h (Fig. 2). From that time the number of intracellular *L. monocytogenes* increased about 27-fold within 4 h. In the presence of free ampicillin in combination with either empty liposomes or liposome-entrapped ampicillin during the first 2 h of incubation the initial mean number of intracellular *L. monocytogenes* decreased to an average of 1.5×10^6 (25%) per 10^6 macrophages, which was not significantly different from that of the controls. From that time the number of intracellular *L. monocytogenes* in the incubation mixture with free ampicillin at a concentration of 50 μg/ml alone (Amp50) or in combination with empty liposomes increased about sevenfold within 4 h. Although the number of intracellular bacteria

TABLE 1. Survival of murine macrophages with ingested *L. monocytogenes* in the presence of ampicillin in combination with either liposome-entrapped ampicillin or empty liposomes

Treatment[a]	Survival percentages of cells with ingested *L. monocytogenes* at the following times (h) after incubation[b]					
	1	2	3	6	9	12
Amp50 + L	99 (100)	99 (100)	93 (93)	97 (93)	89 (76)	81 (82)
Amp50 + LEAmp100	96 (100)	100 (96)	99 (94)	100 (91)	97 (96)	96 (97)
Amp150 + L	100 (100)	92 (100)	93 (90)	92 (93)	87 (92)	78 (84)
Controls	100 (99)	99 (97)	100 (91)	91 (89)		

[a] Suspensions of 5×10^6 cells per ml in HBSS-gel with 10% NCS were exposed to free ampicillin at concentrations of 50 μg/ml in combination with empty liposomes at a concentration of 1 μmol of lipid per ml (Amp50 + L), 100 μg of liposome-entrapped ampicillin in 1 μmol of lipid per ml (Amp50 + LEAmp100), or 100 μg of free ampicillin plus empty liposomes at 1 μmol of lipid per ml (Amp150 + L) and incubated at 37°C under rotation at 8 rpm for up to 12 h.

[b] The data given are means of two determinations. Values in parentheses are cell viability percentages.

increased in the presence of free ampicillin, the total intracellular number was significantly ($P < 0.01$) less compared with that in untreated controls. In contrast to this intracellular bacterial multiplication, the addition of 100 μg of liposome-entrapped ampicillin in 1 μmol of lipid to the concentration of 50 μg of free ampicillin per ml resulted in killing of more than 99% of intracellular *L. monocytogenes*. The lack of intracellular multiplication in the presence of liposome-entrapped ampicillin is reflected in the minimal decrease in number and viability of cells. After 12 h of incubation the number of cells was unchanged compared with the start of incubation, and there was no significant loss in cell viability (Table 1). However, the intracellular bacterial multiplication in the presence of 50 μg of free ampicillin plus empty liposomes during the 12-h incubation period resulted in a gradual decrease in the number of cells as well as loss of cell viability. The addition of 100 μg of free ampicillin in combination with empty liposomes at 1 μmol of lipid to the concentration of 50 μg of free ampicillin per ml resulted not in killing of the intracellular bacteria but in significant inhibition of intracellular growth of *L. monocytogenes*.

Effect of liposome-entrapped ampicillin, free ampicillin, or empty liposomes on the growth of *L. monocytogenes* in broth. Intact ampicillin-containing liposomes did not affect the growth of *L. monocytogenes* in broth (Fig. 3). The presence of 100 μg of ampicillin entrapped within liposomes at 1 μmol of lipid per ml resulted in a slight delay in growth during the first 2 h, followed by a normal growth rate of the bacteria. Empty liposomes had no effect on the bacterial growth rate at all. These data, in combination with the observation that a concentration of 0.16 μg of free ampicillin per ml (the MBC) resulted in bacterial killing, demonstrate only a limited leakage (less than 0.2%) of ampicillin from the liposomes during the first 2 h.

DISCUSSION

The use of liposomes as carriers of therapeutic agents has been reviewed extensively by Gregoriadis (11). Intracellular delivery of antimicrobial agents by liposomes has been demonstrated in experimental models of the intracellular parasitic infections leishmaniasis and malaria in hamsters and mice with intact host defense (1, 17, 20), and of mycotic infections such as *Candida albicans* infection both in normal mice (16) and cyclophosphamide-treated mice (15). Results of these studies demonstrated an improved therapeutic index and reduced toxicity resulting from encapsulation of the drug in liposomes. Liposomal entrapment of antibacterial drugs may also be interesting for the purpose of delivering the agents in relatively high concentrations into cells or specific tissues (22). In our model of intracellular bacterial infection caused by *L. monocytogenes*, we demonstrated in mice with intact host defenses a considerable enhancement (80-fold) in the therapeutic activity of ampicillin resulting from liposomal encapsulation (3). We speculated that the mechanism by which liposomes improve the therapeutic index of ampicillin in the treatment of listeriosis in mice is an increased delivery of the drug to macrophages of the liver and spleen. To determine whether the use of liposomes indeed resulted in an increased availability of the drug for the intracellular bacteria, we studied the effect of free versus liposome-entrapped ampicillin on the survival of *L. monocytogenes* within peritoneal mouse macrophages. We used macrophages because it is generally accepted that after intravenous inoculation in mice *L. monocytogenes* organ-

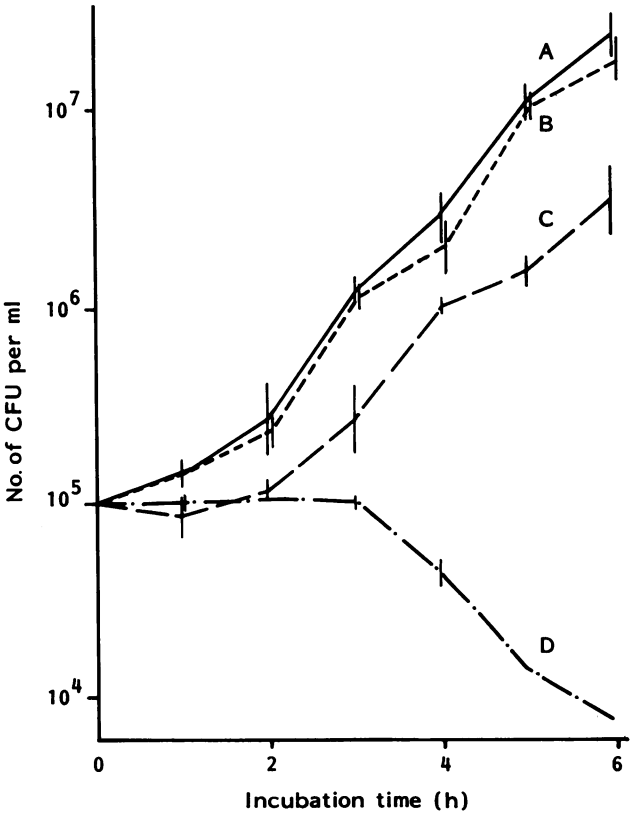

FIG. 3. Growth of *L. monocytogenes* in broth without ampicillin (A) and after the addition of empty liposomes at a concentration of 1 μmol of lipid per ml (B), liposome-entrapped ampicillin at a concentration of 100 μg of ampicillin in 1 μmol of lipid per ml (C), or free ampicillin at a concentration of 0.16 μg/ml (D). The data given are means ± standard deviations of three determinations.

isms reside within macrophages, especially of the liver and spleen (19). We observed that ampicillin-containing liposomes are taken up by *L. monocytogenes*-infected cells. It is also well known from results of other in vitro studies that macrophages can take up liposomes very avidly (8, 21, 23, 27). Uptake occurs predominantly by way of phagocytosis, followed by extensive degradation of the vesicles within the lysosomal compartment of the cells (9, 24; F. Roerdink, J. Regts, T. Daemen, I. Bakker-Woudenberg, and G. Scherphof, *in* G. Gregonodis, G. Poste, J. Senior, A. Trouet, ed., *Targeting of Drugs with Synthetic Systems*, in press).

The growth of *L. monocytogenes* in broth was not influenced by liposomes, whether empty or ampicillin containing. The very low and transient antibacterial activity of liposome-entrapped ampicillin at a concentration of 100 μg/ml is probably the result of a very limited leakage (<0.2%) of entrapped ampicillin, considering the low MBC of 0.16 μg of ampicillin per ml for the *L. monocytogenes* strain. The lack of activity of liposome-entrapped ampicillin against *L. monocytogenes* in broth is in line with the observation of Stevenson et al. (25), who showed that liposome-entrapped chloramphenicol and streptomycin were inactive against *Escherichia coli* in vitro. Our data are not in agreement with the experiments of Hodges et al. (12) and Chowdhury et al. (6), who observed bactericidal activity of liposomal neomycin and penicillins against bacteria in vitro. The discrepancies between these results are most likely explained by differences in lipid composition and charge of the liposomes used.

The decrease in number of intracellular *L. monocytogenes* during the first 2 h of in vitro incubation of the macrophages to about 19 to 25% of the initial number, irrespective of the presence of free or liposome-entrapped ampicillin, was apparently due to intracellular killing of the bacteria. From that time the number of intracellular bacteria increased, and the different effects of ampicillin or liposomal ampicillin were observed, suggesting that the timing of exposure to ampicillin or liposomal ampicillin is not an important determinant. The increase in number of intracellular *L. monocytogenes* after 2 h of incubation was slightly but significantly influenced by the presence of 50 μg of free ampicillin per ml. The presence of 150 μg of free ampicillin per ml plus empty liposomes almost inhibited intracellular bacteria multiplication; however, *L. monocytogenes* organisms were not killed. These data suggest that ampicillin at relatively high concentrations of 50 and 150 μg/ml had a moderate effect on intracellular survival of *L. monocytogenes*. This observation confirms the finding of other investigators that relatively high concentrations of ampicillin penetrate poorly into murine macrophages (5) and leads to a slight reduction in the number of intracellular *E. coli* organisms within murine peritoneal macrophages (14). In contrast to the effect of 50 or 150 μg of free ampicillin per ml plus empty liposomes, a concentration of 50 μg of free ampicillin plus 100 μg of liposome-entrapped ampicillin per ml killed 99% of the intracellular bacteria. The absence of any effect of empty liposomes on the number of intracellular bacteria, taken together with the observation that intact liposomes containing ampicillin were not bactericidal for *L. monocytogenes*, indicate that intracellular killing of bacteria is the result of cellular uptake of liposomes followed by liposomal release of ampicillin intracellularly.

Our results on the intracellular killing of *L. monocytogenes* by liposome-entrapped ampicillin in mice are in agreement with experiments of other investigators (4, 7, 10, 25). Intracellular killing of *Staphylococcus aureus* in canine monocytes or in mouse peritoneal macrophages by aminoglycoside or dihydrostreptomycin, respectively (4, 10), and of *E. coli* in a macrophage cell line by streptomycin and chloramphenicol (25) was enhanced by encapsulating the drugs within liposomes. Desiderio and Campbell (7) showed the superiority of liposomal cephalothin to free cephalothin in effecting killing of the facultative intracellular bacterium *Salmonella typhimurium*.

From results of a previous study (3) we concluded that liposomal encapsulation of ampicillin results in a considerable enhancement in the therapeutic activity in the treatment of *L. monocytogenes* infection in mice by delivering the antibiotic in relatively high concentrations to the tissues infected with *L. monocytogenes*, the liver and spleen. Results of this study show that liposomal encapsulation of ampicillin results in an increased delivery of the antibiotic to the cells infected with *L. monocytogenes*, the macrophages, and an increased availability of ampicillin for the intracellular bacteria.

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