Effect of Lipid Composition on Activity of Liposome-Entrapped Ampicillin against Intracellular Listeria monocytogenes

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Received 15 December 1987/Accepted 14 July 1988

The use of liposomes as carriers of therapeutic agents has been extensively studied. Investigations of the delivery of antitumor or antimicrobial agents by means of liposomes were performed with experimental models of tumors or infectious disease (3, 7, 10). Liposomal delivery of antimicrobial agents has been demonstrated in models of the intracellular parasitic infections leishmaniasis and malaria; of mycotic infections such as histoplasmosis, cryptococcosis, and candidiasis; and of infection caused by Rift Valley fever virus (4). The effect of liposomal encapsulation on the therapeutic activity of antimicrobial agents against experimental intracellular bacterial infections caused by Mycobacterium tuberculosis, Legionella pneumophila, Salmonella enteritidis, or Brucella abortus was studied (4). The animal studies demonstrated an improved therapeutic index and reduced toxicity resulting from encapsulation of the drugs within liposomes.

Targeting of antimicrobial agents by means of liposomes may be of great value in the treatment of intra- or extracellular infections that prove refractory to conventional forms of antimicrobial therapy. Lopez-Berestein et al. demonstrated in a clinical study that liposome-entrapped amphotericin B was beneficial in the treatment of fungal infections in a number of leukopenic cancer patients who had been unsuccessfully treated with the nonencapsulated drug (9). Selection of the appropriate liposome carrier system for drug delivery in vivo requires better understanding of the mechanisms responsible for the increase in therapeutic effect resulting from liposomal encapsulation of the drug.

Numerous studies demonstrate that liposomes are effectively phagocyted by macrophages both in vivo and in vitro (11). It has also become apparent that both the extent of uptake and the rate of intracellular degradation of liposomes by macrophages are decisive in determining the rate at which a liposome-encapsulated drug becomes available, either intracellularly or extracellularly, and hence the therapeutic efficacy (12). In previous in vivo and in vitro studies, we demonstrated that liposomal encapsulation of ampicillin resulted in an increased availability of the antibiotic for the intracellular bacterium Listeria monocytogenes in murine macrophages (1, 2). In the work described in this paper, we used different types of liposomes in an in vitro investigation of whether the lipid composition greatly influences the rate of intracellular liposomal degradation and hence the rate at which liposome-encapsulated ampicillin is released intracellularly and becomes available to exert its antibacterial effect.

MATERIALS AND METHODS

Bacteria. A strain of L. monocytogenes type 1/2 b was used. The MIC and MBC of ampicillin (Beecham Research Laboratories, Amstelveen, The Netherlands) for this strain were both 0.16 µg/ml. The MIC was defined as the lowest concentration (twofold serial dilutions) that suppressed visible growth after incubation at 5 × 10^5 CFU/ml for 18 h at 37°C in tubes containing 4 ml of Iso-Sensitest broth (Oxoid Ltd., London, England). The MBC was defined as the lowest concentration that killed 99.9% of the original inoculum. Bacteria were grown for 16 h at 37°C in Todd-Hewitt broth (Oxoid Ltd.), and radioactively labeled with N-acetyl-d-[14C]glucosamine (specific activity, 59 mCi/mmol) up to 8.10^3 dpm/10^6 CFU. Bacteria were washed twice in phosphate-buffered saline (PBS), opsonized by incubation with 50% fetal bovine serum (Flow Laboratories, Irvine, Scotland) under continuous rotation at 8 rpm for 30 min at 37°C, and washed in PBS.

Macrophages. Macrophages were obtained from the peritoneal cavities of 12- to 14-week-old specific-pathogen-free female C57Bl/Ka mice (REPGO-TNO, Rijswijk, The Netherlands). The macrophages were washed twice in Dulbecco modified Eagle medium (Flow Laboratories) with 1% glutamine. Monolayers of peritoneal macrophages were cultured at 37°C in chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) under a humidified atmosphere of 5% CO2 in air in culture medium containing Dulbecco modified Eagle medium with 1% glutamine and 15% fetal bovine serum. After the first 2 h of incubation, fresh culture medium was added to the monolayer, and the macrophages were incubated for 48 h.

Cellular uptake and intracellular survival of L. monocytogenes. After 48 h of incubation of the macrophage monolayer, opsonized radioactively labeled L. monocytogenes cells were added at a ratio of 30 bacteria per macrophage. After a 30-min uptake period, the noningested bacteria were removed by washing the monolayer three times with PBS at
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37°C. The macrophages were reincubated (zero time) for 6 h in fresh culture medium with 15% fetal bovine serum in the presence of liposome-encapsulated ampicillin or free ampicillin plus empty liposomes. The liposomal lipid concentration was 1 μmol of lipid per ml of the incubation mixture. Control suspensions were incubated without ampicillin. At the times indicated during the period of reincubation, the monolayers were washed three times with PBS at 0°C, and the macrophages were disrupted by being quickly frozen and thawed in the presence of 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 SR 106 (Oxoid) per ml. Triton X-100 was added to disrupt the ingested liposomes. Penicillinase was added for immediate inactivation of ampicillin released after disruption of cells and liposomes. Suspensions were mixed vigorously. Freezing and thawing did not affect the viability of L. monocytogenes. The intracellular numbers of viable bacteria were determined by preparing 10-fold serial dilutions and performing subcultures on blood-agar plates. In addition, the intracellular numbers of radioactively labeled bacteria were determined.

Liposomes. Cholesterol, egg phosphatidylcholine (PC) and L-α-phosphatidyl-L-serine (PS) were obtained from Sigma. L-α-distearoylphosphatidylcholine (DSPC) and L-α-dipalmitoylphosphatidylglycerol (DPPG) were obtained from Avanti, Birmingham, Ala. Liposome preparations were multimamellar vesicles consisting of either cholesterol-PC-PS in a molar ratio 5:4:1 (fluid liposomes) or cholesterol-DSPC-DPPG in a molar ratio of 10:10:1 (solid liposomes). Liposomes were sized by extrusion through polycarbonate filters (no. 94566 [final pore size, 0.4 μm]; Nuclepore Corp., Pleasanton, Calif.) and freed of nonentrapped ampicillin on a Sephadex G-100 column (Pharmacia, Uppsala, Sweden). Liposomes were prepared essentially as described previously (1), with some modifications for the cholesterol-DSPC-DPPG liposomes: because of the high transition temperature of the DSPC used (58°C), these liposomes were prepared at 62°C. For determination of the amount of entrapped ampicillin, trace amounts of [14C]ampicillin (specific activity, 1 mCi/mmol) were added to the lipid mixtures. Incorporation of antimicrobially active ampicillin was 25 ± 50 μg/μmol of liposomal lipid. The coefficient of variation of the ampicillin determination was 2%.

Cellular uptake of liposomes. At 48 h after the macrophages had been seeded, liposomes labeled with [3H]cholesterol-hexadecylether (specific activity, 51 Ci/mmol; Du Pont, NEN Research Products, Boston, Mass.) were added in fresh culture medium at a concentration of 1 μmol of lipid per ml of culture medium. Cholesterol-hexadecylether was used because this nondegradable marker remains tightly associated with the cells after it has been taken up via liposomes. The macrophages were reincubated (zero time) for 6 h. At the times indicated, the monolayers were washed three times with PBS. The macrophages were disrupted by quick freezing and thawing in the presence of distilled water followed by a vigorous mixing. The cellular uptake of liposomes was calculated from the measurements of the amounts of radioactive label and protein in the lysate. The coefficient of variation of the determination of cellular uptake was 6%.

Intracellular degradation of liposomes. To the 48-h monolayer of macrophages, liposomes labeled with cholesteryl-[14C]oleate (specific activity, 58 mCi/mmol; The Radiochemical Centre Amersham, Little Chalfont, Buckinghamshire, England) were added at a concentration of 1 μmol of lipid per ml of culture medium, and the mixture was incubated for 2 h. Cholesteryl-oleate was used as a marker to monitor intracellular liposomal degradation. NH4Cl (10 mM) was added to the medium to prevent liposomal degradation during uptake of the vesicles by the cells by raising the intralysosomal pH. Control experiments revealed that incubation of liposomes for 2 h in the presence of 10 mM NH4Cl had no effect on the integrity of the liposomes. After 2 h the liposomes were removed, and the cells were incubated for another 30 min in the presence of 10 mM NH4Cl. Then (zero time) the cells were reincubated in fresh NH4Cl-free medium for 2 h. At the times indicated, the supernatant was removed and the macrophages were disrupted as described above. Radioactivity was measured in supernatant and cellular lysate. The lysate was evaporated to dryness, suspended in distilled water, extracted with chloroform-methanol (2:1), fractionated, and chromatographed on thin-layer plates (E. Merck AG, Darmstadt, Federal Republic of Germany) with petroleum ether (40/60)-diethyl ether-formic acid (60:40:1.5) as a solvent. The amounts of radioactive label in the relevant spots were determined and expressed as the percentage of total chloroform-soluble radioactivity in the cells. The coefficient of variation of the determination of intracellular degradation was 6%.

RESULTS

Cellular uptake of liposomes. No differences in the rates of uptake between the two types of liposomes could be observed: cellular uptake (n = 3) of liposomes increased up to 11.9 ± 0.78 and 11.2 ± 0.25 nmol of liposomal lipid per 106 macrophages for cholesterol-PC-PS (5:4:1) liposomes and cholesterol-DSPC-DPPG (10:10:1) liposomes, respectively, after 6 h of incubation (Fig. 1).

Intracellular degradation of liposomes. A difference between the two types of liposomes with regard to their

![FIG. 1. Uptake of liposomes labeled with [3H]cholesterol-hexadecylether by mouse peritoneal macrophages in monolayer culture. Two types of liposome preparations were used, differing in the lipid composition of the liposomal membranes as indicated, at a concentration of 1 μmol of lipid per ml of culture medium. At the times indicated, the macrophages were disrupted and the intracellular amounts of radioactive label were determined. Data are mean values ± standard errors of the mean for three determinations. Abbreviation: Chol, cholesterol.](http://aac.asm.org/.../acsmsr1307831561fig1.jpg)
susceptibility to lysosomal degradation was observed (Fig. 2). Fluid cholesterol-PC-PS (5:4:1) liposomes appeared to be much more sensitive to intracellular degradation than solid cholesterol-DSPC-DPPG (10:10:1) liposomes were. When incorporated in cholesterol-PC-PS (5:4:1) liposomes, 84 ± 5.3% (n = 3) of the amount of radioactively labeled cholesteryl-oleate initially incorporated was degraded within 120 min of incubation, owing to lysosomal esterase activity. The amount of liberated radioactively labeled oleate increased rapidly from the start of incubation, was partly released by the cells (data not shown), and was partly reutilized for synthesis of cellular phospholipids. When incorporated in cholesterol-DSPC-DPPG (10:10:1) liposomes, only 25 ± 4.6% (n = 3) of cholesteryl-oleate was degraded within 120 min of incubation. This relatively slow degradation is reflected in the relatively slow appearance of liberated oleate, whereas incorporation of oleate into phospholipids was almost undetectable after the 120-min incubation period.

Cellular uptake and intracellular survival of *L. monocytogenes*. The cellular uptake and intracellular survival of *L. monocytogenes* at different time intervals during the periods of uptake and reincubation in the absence of liposome-entrapped ampicillin are shown in Fig. 3. From the start of incubation of the macrophages and *L. monocytogenes* (T = −30 min), a gradual uptake of bacteria was measured in terms of viable bacteria as well as radioactively labeled bacteria, resulting in $5 \times 10^6$ *L. monocytogenes* organisms per $10^6$ macrophages after the 30-min uptake period. After removal of the noningested bacteria, during the first hour of incubation the number of intracellular bacteria did not change. After that time intracellular bacterial multiplication occurred up to threefold within 4 h of incubation and up to sixfold within 6 h. Owing to the intracellular multiplication of *L. monocytogenes*, gradual lysis of the infected macrophages occurred during the last 2 h of incubation, as reflected by a slow release of radioactivity from the cells.

**Effect of liposome-entrapped ampicillin on intracellular survival of *L. monocytogenes***. After removal of the noningested bacteria at the end of the uptake period, macrophages were reincubated in the presence of 25 or 50 μg of ampicillin encapsulated in one of the two types of liposomes (Fig. 4). In the presence of 25 μg of ampicillin per ml encapsulated in cholesterol-PC-PS (5:4:1) liposomes, the number of intracellular bacteria did not change during the first 3 h, but after 6 h of incubation 90% of the intracellular bacteria were killed. The delay in intracellular killing is probably due partly to the degradation time of the liposomes and release of ampicillin and partly to binding of ampicillin to penicillin-binding proteins PBP's of the bacteria. The decrease in the number of intracellular bacteria as measured was not due to lysis of infected macrophages, since the number of intracellular radioactively labeled bacteria remained constant during the

FIG. 2. Intracellular degradation of liposomes labeled with cholesteryl[14C]oleate by mouse peritoneal macrophages in monolayer culture. Two types of liposome preparations were used, as indicated, at a concentration of 1 μmol of lipid per ml of culture medium. At the times indicated, the macrophages were extracted with chloroform-methanol, fractionated, and chromatographed on thin-layer plates. The amounts of radioactive label in the spots representing cholesteryl-oleate, oleate, and phospholipids were determined and expressed as a percentage of total chloroform-soluble radioactivity in the cells. Data are mean values ± standard errors of the mean for three determinations. Abbreviation: Chol, cholesteryl.

FIG. 3. Cellular uptake and intracellular survival of *L. monocytogenes*. Macrophages in monolayer culture were exposed to radioactively labeled *L. monocytogenes* for 30 min. Then (zero time) the noningested bacteria were removed, and the macrophages were reincubated for 6 h. At the times indicated during the periods of uptake and reincubation, the macrophages were disrupted and the number of intracellular viable bacteria, as well as the cell-associated radioactivity, was determined. Data are mean values ± standard errors of the mean for three determinations.
period of incubation (data not shown). However, when encapsulated in cholesterol-DSPC-DPPG (10:10:1) liposomes, 25 μg of ampicillin per ml did not result in intracellular bacterial killing: although multiplication of intracellular bacteria was significantly lower than in the controls, the bacteria multiplied up to threefold within 6 h. As shown for both types of liposomes, the presence of 25 μg of free ampicillin plus empty liposomes had no significant effect on the intracellular bacterial multiplication. Doubling the amount of ampicillin encapsulated in cholesterol-PC-PS liposomes to 50 μg/ml led to a rapid killing of almost all (97%) of the intracellular bacteria. In contrast, when 50 μg of ampicillin was encapsulated in cholesterol-DSPC-DPPG liposomes, intracellular killing was delayed and less effective: only 70% of the intracellular bacteria were killed after 6 h. As shown for both types of liposomes, 50 μg of free ampicillin plus empty liposomes only inhibited intracellular bacterial multiplication but did not kill the bacteria.

**DISCUSSION**

In previous studies of experimental *L. monocytogenes* infection in mice, we observed a considerable enhancement (90-fold) in the therapeutic activity of ampicillin resulting from liposomal encapsulation (1). The mechanism by which liposomes improved the therapeutic index of ampicillin in this infection appeared to be an increased delivery of the antibiotic to the site of infection, i.e., the liver and spleen. In addition, in studies of the survival of *L. monocytogenes* in vitro within murine peritoneal macrophages after in vivo phagocytosis of the bacteria, we found that liposomal encapsulation of ampicillin resulted in an increased availability of the antibiotic for the intracellular bacteria: liposomal ampicillin killed 99% of the intracellular bacteria, whereas a similar concentration of free ampicillin plus empty liposomes had no antibacterial effect (2). These results are in line with observations made by several other investigators demonstrating in vitro the superiority of liposome-entrapped antibiotics over nonentrapped antibiotics in effecting killing of intracellular bacteria. They demonstrated that intracellular killing of *Staphylococcus aureus* in canine monocytes or mouse peritoneal macrophages by aminoglycosides or dihydrostreptomycin, of *Escherichia coli* in a macrophage cell line by streptomycin and chloramphenicol, and of *Salmonella typhimurium* in peritoneal macrophages by cephalothin was enhanced by encapsulating the drugs within liposomes (5, 6, 8, 13).

In the present study we examined the effect of free versus liposome-encapsulated ampicillin on the survival of *L. monocytogenes* in murine peritoneal macrophages after phagocytosis in vitro. We used two types of liposomes, both of which were multilamellar vesicles with a mean diameter of ca. 0.4 μm but which differed in the lipid composition of the liposomal membranes, resulting in a difference in membrane fluidity. We used liposomes composed of cholesterol, DSPC, and DPPG, a solid liposome type owing to the presence of saturated phospholipids with high transition temperatures (above 37°C). The fluid liposome type was composed of cholesterol, PC, and PS, which have transition temperatures well below 37°C. The uptake of both types of liposomes by peritoneal macrophages in monolayer culture in vitro was found to be similar. However, the rate of intracellular degradation appeared to be dependent on the lipid composition: the solid liposomes were degraded relatively slowly as compared with the fluid liposomes. The differences in degradation rate of the two types of liposomes correlated with the rate at which the encapsulated antimicrobial drug was released intracellularly as measured by differences in onset of killing of *L. monocytogenes* intracellularly by ampicillin. Encapsulation of ampicillin at a concentration of 50 μg/ml in vesicles of the solid liposome type as compared with the fluid liposome type resulted in delay of intracellular killing of *L. monocytogenes*. At an ampicillin concentration of 25 μg/ml, intracellular bacterial killing was absent when ampicillin was encapsulated in the solid liposome type; however, when
encapsulated in the fluid liposomes, ampicillin was still bactericidal at that concentration. Similar results were obtained by Storm et al. (14), who found in an experimental tumor model that the expression of antitumor activity induced by doxorubicin-containing liposomes in vivo varied with the lipid composition of the liposomes. Using double-labeled liposomes, they obtained evidence that the degradation rate of the vesicles after uptake by macrophages was dependent on the lipid composition of the bilayers. Solid liposomes, by virtue of the highly packed acyl chains in the lipid membranes, are less susceptible to lysosomal enzyme activities than are fluid liposomes, causing a delay in lysosomal degradation after uptake of the vesicles by macrophages. Slow degradation of the solid lipid type resulted in retarded onset of tumor regression, apparently induced by doxorubicin released from the macrophages. In this respect, the macrophages serve as depot cells from which the drug is released over a prolonged period. These results could recently be confirmed by using liver macrophages in maintenance culture: after uptake of doxorubicin-containing solid liposomes, a delayed release of the drug was observed in the culture medium as estimated by high-performance liquid chromatography (G. Storm, Ph.D. thesis, State University of Utrecht, Utrecht, The Netherlands, 1987).

In conclusion, evidence is presented that intracellular processing of the liposomes seems to be an important determinant for the intracellular release and hence the therapeutic effect of the encapsulated drug. By varying the lipid composition of the liposomes, it is possible to manipulate the rate of intracellular degradation and hence the therapeutic availability of the encapsulated agent. More studies are needed to establish the optimal liposomal lipid composition for delivery of agents in vivo in experimental infection models.

ACKNOWLEDGMENTS

This study was supported in part by Beecham Research Laboratories (The Netherlands). We thank Paula Jansen for secretarial help.

LITERATURE CITED