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Distribution of Free and Liposome-encapsulated Cefoxitin in Experimental Intra-abdominal Sepsis in Rats

A. KRESTA, P. N. SHEK*, J. ODUMERU AND J. M. A. BOHNEN

*Departments of Clinical Biochemistry and Surgery, Faculty of Medicine, University of Toronto, Toronto, and *Operational Medicine Section, Biosciences Division, Defence and Civil Institute of Environmental Medicine, North York, Ontario, Canada*

Abstract—The distributions of radiolabelled free cefoxitin (FC) and liposome-encapsulated cefoxitin (LC) were compared in an animal model of intra-abdominal sepsis. Intraperitoneally administered LC was initially retained in the peritoneal cavity with subsequent preferential drug targeting to the liver (14% injected LC) and spleen (6% injected LC) by 3 h post-injection. Differing patterns of liposomal drug and lipid retention indicated that drug release from the liposome complex occurred within the peritoneum, liver and spleen. Intraperitoneal FC was rapidly taken up into the systemic circulation, with peak recovery in the blood (9% injected FC) and liver (5% injected FC) at 1 h post-injection. FC was also rapidly eliminated; 7% of the injected drug was recovered in the kidney 1 h post-injection. A negligible amount of FC was recovered in the spleen and very little FC or LC was found in the lungs of treated animals. Unlike FC, LC was found to provide a sustained bactericidal drug level ($>40\ \mu g\ mL^{-1}$) in the peritoneal fluid for up to 5 h post-injection. LC also achieved significantly higher drug levels, compared with FC, within the liver at 3 and 5 h post-injection. Since severe intra-abdominal sepsis is often characterized by the presence of intraphagocytic bacteria in hepatic and splenic reticuloendothelial systems, the enhanced delivery of liposome-encapsulated anti-microbial agents, such as cefoxitin, to the liver and spleen may provide a more effective treatment for the septic condition.

Gram-negative intra-abdominal sepsis is associated with relatively high mortality rates (>30%), despite the use of broad-spectrum antimicrobial agents (Wenzel 1988; Parrillo et al 1990). The intravenous administration of antimicrobial agents, such as cefoxitin, can rapidly generate therapeutic drug levels within the blood and peritoneal fluid (Bakker-Woudenberg et al 1985; Kaplan et al 1989; Cars 1991). However, because of their short half-lives (0·5–8 h for most cephalosporins) (Roschlau & Kalant 1989), sustained therapeutic drug levels within organs such as the liver and spleen may not be attained. In experimental intra-abdominal sepsis, the liver has been shown to be a major bacterial reservoir (Bohnen et al 1991) and it is quite possible that insufficient drug targeting to the liver may accentuate the high mortality observed in such cases.

Conventional antibiotics are most effective in treating abdominal sepsis if the drug is administered within 8 to 12 h post-infection. Beyond this initial period, the infecting microbes rapidly gain entry to and reside in cells, particularly phagocytic cells of the reticuloendothelial system where the microbes appear to become shielded from further drug action. It is conceivable that any drug delivery system which promotes phagocytic uptake would allow for intracellular antimicrobial action. In this regard, it has been shown that liposome-mediated drug delivery is most effective in targeting encapsulated drugs to reticuloendothelial sites (reviewed by Shek & Barber 1986).

Liposomes are vesicular structures composed of one or more phospholipid bilayers alternating with aqueous spaces. The amphipathic nature of liposomal structures enables them to entrap both hydrophilic and hydrophobic drugs (Shek & Barber 1986). Liposomal entrapment has been shown to alter both the distribution and pharmacokinetics of entrapped agents (Gregoriadis & Senior 1980; Gabizon & Papahadjopoulos 1988; Gondal et al 1989; Goren et al 1990). Recently, liposomes have been used to improve the therapeutic efficacy of antimicrobial agents. Liposomal amphotericin B has been successfully used to treat the fungal condition of candidiasis (Gondal et al 1989), while liposomal ampicillin has been used to improve the survival of animals with listeriosis (Bakker-Woudenberg et al 1988a). The superior efficacy of liposome-encapsulated cefoxitin in enhancing the survival of animals infected with Gram-negative bacteria has also been demonstrated (Bohnen et al 1991).

Despite the improved therapeutic efficacy of liposomal cefoxitin, the fate of the administered drug is not known. The objective of this study is to determine and compare the biodistribution of free and liposome-entrapped cefoxitin in septic animals. We believe a better knowledge of the uptake and distribution of the administered drug in intra-abdominal sepsis will aid in understanding the relationship between drug targeting and improved efficacy.

Materials and Methods

Materials

Dipalmitoylphosphatidylcholine (DPPC) and phosphatidyl serine (PS) were purchased from Avanti Polar Lipids (Pelham, AL, USA). Cefoxitin was a gift of Merck, Sharp & Dohme (Kirkland, Quebec, Canada) and [thienylacetate-carbonyl-^{14}C]cefoxitin ($15{\cdot}75\ \mu Ci\ mg^{-1}$) was a gift of Dr Alan Jones (Merck, Sharp & Dohme). [^{3}H]Cholesteryl hexadecyl ether ($60\ Ci\ mmol^{-1}$), tissue digestor, Solvable, and liquid scintillation fluid, Formula-989, were purchased from New England Nuclear (DuPont, Boston, MA, USA). Brain-heart

Correspondence: P. N. Shek, Biosciences Division, Defence and Civil Institute of Environmental Medicine, 1133 Sheppard Avenue West, North York, Ontario M3M 3B9, Canada.

infusion broth was obtained from Difco Laboratories (Detroit, MI, USA).

Escherichia coli (ATCC 25922), *Enterococcus fecalis* (ATCC 19433) and *Bacteroides fragilis* (ATCC 25285) were purchased from Microbiologics (St Cloud, MN, USA). *E. coli* and *B. fragilis* are cefoxitin-sensitive organisms (minimum inhibitory concentration (MIC) for both organisms is 2–8 $\mu g\ mL^{-1}$), while *E. fecalis* is resistant to cefoxitin.

All the water used in this study was filtered through a Milli-Q Plus water system (Millipore Corp., Bedford, MA, USA).

Liposome preparation and drug entrapment

Large unilamellar vesicles formed by the extrusion technique (Hope et al 1989) were prepared as follows: DPPC and PS (110 μmol in total lipid) were dissolved in 2:1 chloroform:methanol and combined in a 9·5:0·5 molar ratio, respectively. [^{3}H]Cholesteryl hexadecyl ether, dissolved in 2:1 chloroform:methanol, was added as a lipid marker at a concentration of 2 $\mu Ci\ mmol^{-1}$ lipid. The lipids were dried into a thin film under argon gas and residual solvent was removed in-vacuo. The lipid film was hydrated at 50°C (9°C above the transition temperature of DPPC, 41°C) with a sample of [^{14}C]cefoxitin (3·6 $\mu Ci\ mmol^{-1}$) and 50 mg mL^{-1} cefoxitin in saline, such that the initial drug to lipid concentration was 760 mg $mmol^{-1}$. The multilamellar liposome preparation was shell frozen in a methanol/dry-ice mixture and freeze-dried in the freezer compartment of the apparatus to preserve drug activity. Once dried, the preparation was stored at −20°C until use.

Freeze-dried lipid-drug preparations were rehydrated at 50°C for 1 h in a small volume of Milli-Q water (1·5 mL $mmol^{-1}$ lipid) to promote maximum drug entrapment. Rehydrated samples were diluted in sterile distilled water to a concentration of 0·1 mmol lipid mL^{-1}. The diluted preparation was extruded 5 times through two polycarbonate filters (Nucleopore Corp., Pleasanton, CA, USA) of 400 nm pore size using an Extruder (Lipex Biomolecules Inc., Vancouver, BC, Canada). The extruded vesicles were washed twice in excess sterile saline by ultracentrifugation in a Beckman L8-70 ultracentrifuge to remove unentrapped drug. The final pellet was resuspended in saline to pre-extrusion volume.

Cefoxitin entrapment was measured by a fluorimetric assay (Al-Rawi & Tabaqchali 1979) as follows: samples of pre-extrusion and post-wash liposome suspensions were diluted 1:500 and 1:50, respectively, in saline to a final volume of 0·5 mL. To these samples, 0·5 mL 2 M HCl was added, followed by vortexing and incubation at room temperature (21°C) for 15 min. Samples were placed in a 100°C waterbath for 3 min and then incubated in an ice-water bath for 3 min. To each sample was added 0·5 mL 6 M NaOH followed by 60 min of incubation at room temperature. Sample fluorescence was measured in a Perkin-Elmer Fluorimeter (Model 650-10M, Baxter Diagnostics Corp., Canlab, Mississauga, Ontario, Canada), using an excitation wavelength of 385 nm and an emission wavelength of 460 nm. Drug concentrations were determined from a standard curve generated from samples run in parallel with known drug concentrations in the presence of similarly diluted empty liposomes. The mean entrapment efficiency for cefoxitin in our liposome preparations was $128·0 \pm 14·4\ \mu g\ \mu mol^{-1}$ lipid (16·8% of initial drug load).

Rat model of intra-abdominal sepsis

Adult male Sprague-Dawley rats, 180–220 g, were purchased from Charles River Canada (St Constant, Quebec, Canada). The animals were allowed free access to laboratory chow and drinking water. All animals used in this study were cared for in accordance with the principles specified in the Guide to the Care and Use of Experimental Animals, prepared by the Canadian Council on Animal Care.

Rats were injected intraperitoneally with $2·5 \times 10^7$ colony forming units (CFU) *E. coli*, $2·5 \times 10^7$ CFU *E. fecalis*, and 5×10^7 CFU *B. fragilis* in 0·3 mL brain-heart infusion broth mixed with 0·3 mL sterile faeces. Sterile faeces was prepared by removing and pooling the contents of rat caeca and combining them with an equal volume of brain-heart infusion broth. This slurry was filtered twice through gauze pads to remove large particulate matter and then autoclaved twice for sterilization. Fourteen hours after intraperitoneal inoculation, rats were anaesthetized with a combination of xylazine and pentobarbitone and underwent laparotomy. The peritoneal cavity was lavaged with 100 mL warmed sterile saline and closed with 3–0 silk sutures. Rats were injected intraperitoneally with either free cefoxitin (15 mg kg^{-1}) or liposomal cefoxitin (15 mg in 117 μmol liposomal lipid kg^{-1}). The free cefoxitin dose included 0·02 μCi [^{14}C]cefoxitin added as a tracer for the drug, while the liposomal cefoxitin preparation included both [^{14}C]cefoxitin and [^{3}H]cholesteryl hexadecyl ether as drug and lipid markers, respectively. Rats were resuscitated with 10 mL sterile saline administered subcutaneously.

At 1, 3, 5, 10, and 24 h post-injection, separate groups of rats were anaesthetized and the abdomen reopened. Peritoneal fluid was collected with sterile Pasteur pipettes, after the addition of 5 mL sterile saline; the recovered volume varied from 4·5 to 5·5 mL. Approximately 1 mL blood was collected from the dorsal aorta or by cardiac puncture. Rats were catheterized via the hepatic portal vein and perfused with 60–120 mL sterile saline; liver, spleen, kidney, and lung were excised. The fluid and tissue samples were stored in sterile plastic tubes at 4°C until preparation for liquid scintillation counting. The 10 and 24 h time-points were not included for free cefoxitin-treated animals, because preliminary work had shown that a cefoxitin dose of 45 mg kg^{-1} yielded negligible tissue and fluid drug levels after the 5 h sampling time.

Preparation of biological samples

Aliquots of peritoneal fluid and blood samples were digested with equal volumes of Solvable in capped 20-mL scintillation vials at 50°C for 3–24 h until clear. Sodium EDTA (100 mM) and hydrogen peroxide (30%) were added to digested samples as anti-foaming and bleaching agents, respectively. Ten to fifteen millilitres of Formula-989 was used as the scintillation fluid and samples were incubated overnight in the dark before counting in a Beckman LSC-5801 liquid scintillation counter. The total radioactivity recovered in the peritoneal fluid and blood was determined, respectively, based on the total peritoneal fluid volume obtained at harvesting and the total blood volume estimated according to

the relationship of blood volume to rat weight (6·5 mL/100 g) proposed by Creskoff et al (1949).

Tissue samples were weighed and homogenized in saline using a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY, USA). Kidney, lung and spleen homogenates were each made to a total volume of 5 mL, while liver homogenates were made to 13 mL. Aliquots of the homogenized tissue samples were digested, bleached, and prepared for liquid scintillation counting as described above.

Conversion of % radiolabel recovery data to tissue drug levels The distribution of cefoxitin was expressed in terms of % recovery of the injected ^{14}C-labelled drug. Cefoxitin is not biotransformed in the liver and is excreted unchanged in the urine (Kucers & Bennett 1987). Using thin layer chromatography, we were able to show that [^{14}C]cefoxitin isolated from the urine of infected rats was not degraded in-vivo (data not shown); therefore, it is justifiable to convert radiolabel recovery data to tissue drug levels.

Results

Recovery of free cefoxitin, liposomal cefoxitin and liposomal lipid

Total drug recovery. Total recovery of the labelled drug was determined by the summation of radiolabel recoveries from the peritoneal fluid, blood, liver, spleen, kidney and lung of animals injected with either free or liposome-encapsulated [^{14}C]cefoxitin. A comparison of the total drug recovery between the two groups of septic animals injected with free cefoxitin and liposomal cefoxitin is shown in Table 1. It can be seen that liposomal cefoxitin-injected animals retained a significantly higher amount of cefoxitin in the whole body at the indicated time-points post-injection. The total recovery of liposomal cefoxitin was about 1·3, 3·8 and 3·1 times as much as that of free cefoxitin from injected animals at 1, 3 and 5 h post-injection, respectively. At 24 h post-injection, total drug recovery of liposomal cefoxitin-treated animals was about 10 times that of free cefoxitin-treated animals. It appears, therefore, liposome encapsulation may slow drug elimination in the body as a whole.

Recovery in body fluids and tissue. These results are summarized in Fig. 1 and Table 2.

Table 1. Total recoveries of radiolabelled free and liposomal cefoxitin.

Time post-injection (h)	Free cefoxitin	Liposomal cefoxitin
	% recovered dose	
1	45·4 ± 7·2	70·8 ± 4·8*
3	12·2 ± 1·3	40·6 ± 4·1*
5	9·2 ± 0·2	25·6 ± 5·3*
10	n.d.	15·9 ± 5·3
24	1·3[a]	13·4 ± 3·5

The total drug recovery for each time-point post-injection represents the % of injected dose recovered from the summation of ^{14}C-counts obtained from the following body fluids and tissues: blood, peritoneal fluid, liver, spleen, lung and kidney tissues. Each number is the mean value ± s.e.m. of 3–7 animals. n.d. = not determined. *$P < 0·05$ compared with corresponding value for free cefoxitin.

[a] Single observation obtained from a series of experiments where one animal was injected with a dose of free cefoxitin (45 mg kg^{-1}) which was 3 times the dose (15 mg kg^{-1}) used in this series.

Discussion

Intraperitoneal administration of liposome-entrapped cefoxitin, as compared with free cefoxitin, resulted in a substantially higher total drug recovery in the peritoneal fluid as well as in the liver and spleen of septic animals. Free cefoxitin was rapidly transferred from the peritoneal fluid to the systemic circulation and rapidly eliminated from the body, as evidenced by the low recovery of the radiolabelled drug in the liver, spleen and lung. On the other hand, liposome-entrapped cefoxitin was transferred to the systemic circulation at a slower rate than free cefoxitin and was then accumulated primarily in the liver and spleen. Similar results have been reported by other investigators who showed that the intraperitoneal injection of liposomal polyvinylpyrollidine and liposomal doxorubicin resulted in the preferential delivery of the entrapped drug to the liver and spleen, after an initial period of retention in the peritoneum (Ellens et al 1981; Rosa & Clementi 1983).

In the present study, we observed a parallel distribution and accumulation of both liposomal lipid and liposomal drug labels in the peritoneal fluid, liver and spleen up to 3 h post-injection. Kimelberg & Mayhew (1978) also demonstrated that the distribution of radiolabelled liposomal methotrexate was similar to that of the radiolabelled liposomal lipid following intraperitoneal injection in rats. These observations of an initial temporal relationship between the distribution of liposomal lipid and drug radiolabels appear to support the hypothesis that the altered biodistribution of an entrapped drug, such as cefoxitin, is mediated by its liposomal formulation. Differences between liposomal lipid and liposomal drug recovery in the peritoneal fluid, liver and spleen within 3 h post-injection may be indicative of drug release from the liposomal complex at these sites.

An effective therapeutic drug formulation is one which can deliver the drug to the target site for maximal pharmacological action. In our model of intra-abdominal sepsis, the intended targets are the sites where the offending Gram-negative bacteria reside. The estimated amount of drug released from the liposome complex in the peritoneal fluid by 3 h post-injection was well in excess of the reported MIC (2–8 μg mL^{-1}) for the *E. coli* and *B. fragilis* strains used in the faecal inoculum. In terms of drug delivery to the liver, there was a dramatic difference between the liposomal formulation and the free drug. The hepatic cefoxitin level of liposome-injected animals was substantially elevated, about 10 times higher at 3 h and 26 times higher at 5 h post-injection, compared with that of free cefoxitin-injected animals. Using the same injected drug dose for treating rats with intra-abdominal sepsis, we have shown that there was a significant reduction in bacterial counts among liver cultures obtained from rats injected with liposomal cefoxitin compared with those injected with free cefoxitin (Bohnen et al 1991). Therefore, it appears that the cefoxitin released from liposomes in the liver may well be sufficient to mediate a significantly higher bactericidal effect.

The mechanism whereby liposomes are transported to the liver is not well understood. Hirano & Hunt (1985) demonstrated that liposomal agents were removed from the peritoneal cavity via the sub-diaphragmatic lymphatics, while others have shown the predisposition of liposomal

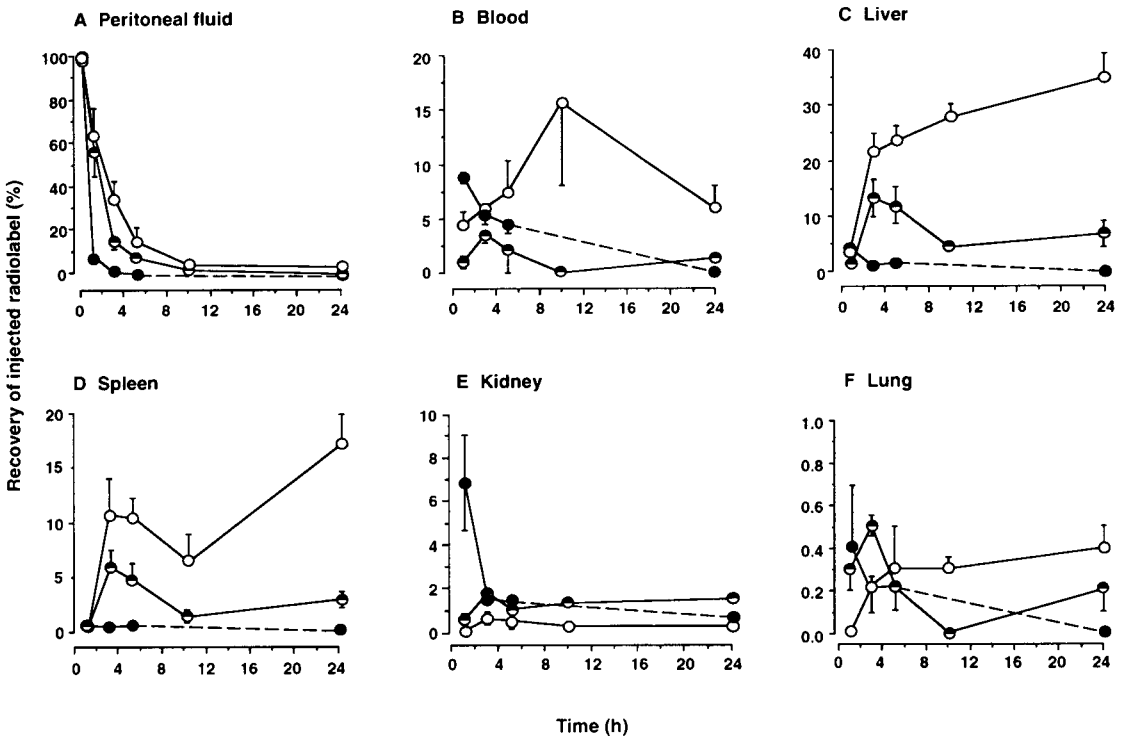

FIG. 1. Recovery of radiolabels from body fluids and various organs. ● Cefoxitin, ◓ liposomal cefoxitin, ○ liposomal lipid. Each point represents the mean percentage of recovered dose ± s.e.m. of 3–6 animals.

Table 2. Comparison of drug concentrations present in the peritoneal fluid, blood and liver of septic rats treated with free cefoxitin or liposomal cefoxitin.

		Cefoxitin concn		
Treatment	Time (h)	Peritoneal fluid ($\mu g\ mL^{-1}$)	Blood ($\mu g\ mL^{-1}$)	Liver ($\mu g\ g^{-1}$)
Free	1	36.0 ± 5.4	19.3 ± 1.1	135.0 ± 33.0
	3	4.0 ± 1.2	11.3 ± 1.9	39.0 ± 3.0
	5	3.0 ± 0.6	9.7 ± 1.8	13.0 ± 8.0
Liposomal	1	329.2 ± 67.0*	2.0 ± 1.2*	42.0 ± 12.0*
	3	84.6 ± 21.0*	7.8 ± 1.6	408.0 ± 99.0*
	5	40.2 ± 13.8*	4.6 ± 2.6	348.0 ± 108.0*

* $P < 0.05$ compared with corresponding values for free cefoxitin. n = 3–6.

agents for uptake by phagocytic cells (Kao & Juliano 1981; Dijkstra et al 1984, 1985; Hojo et al 1985; Alving 1986; Bakker-Woudenberg et al 1988a; Kume et al 1991). The translymphatic route is the same as that followed by bacteria in their initial (first 2–3 h) dissemination or clearance from the peritoneal cavity during intra-abdominal sepsis (Dunn et al 1985a, b, 1987; Dumont et al 1986; Skau et al 1986; Levine & Saltzman 1988). Ultimate clearance of intraphagocytic bacteria and liposomes has been shown to take place within organs functioning as reticuloendothelial system cell reservoirs, including the liver and spleen (Scherphof et al 1983; Cheslyn-Curtis et al 1988; Albright et al 1990; Katz et al 1991). This phenomenon has been well documented and has resulted in the implementation of liposomal drug formulations in the treatment of intraphagocytic infections including candidiasis (Gondal et al 1989), listeriosis (Bakker-Woudenberg et al 1988a,b) and brucellosis (Dees et al 1985). In our model of intra-abdominal sepsis, liposome-entrapped cefoxitin may have acted as a drug reservoir in the liver and spleen. However, whether liposomal cefoxitin was delivered into phagocytic cells, e.g. Kupffer cells of the liver, remains to be established.

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