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Administration of liposomal agents and the phagocytic function of the mononuclear phagocyte system

Irma A.J.M. Bakker-Woudenberg [a,*], M.T. ten Kate [a], G. Storm [b], E.W.M. van Etten [a]

[a] *Institute of Clinical Microbiology and Antimicrobial Therapy, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands*

[b] *Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Groningen Utrecht Institute for Drug Exploration (GUIDE), Utrecht, The Netherlands*

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Abstract

Liposomal drugs used in clinical practice are often administered to patients that are immunocompromised and hence, highly susceptible to develop systemic infections. The resident phagocytic cells of the MPS will clear the microorganisms from the blood and thus prevent generalization of the infection as well as mortality. As substantial MPS uptake of liposomes occurs, the question arises whether administration of liposomes, particularly those containing potentially toxic agents such as amphotericin B and doxorubicin, affect the phagocytic capacity of the MPS. In the present study, at first the effect of administration of three types of 'empty' liposomes (i.e. devoid of drug), differing in blood residence time, on carbon clearance and bacterial clearance from blood was studied in mice: (1) Classical EPC:PS:Ch (4:1:5) liposomes, 300 nm, (2) placebo liposomes with lipid composition as in AmBisome®, 100 nm, and (3) placebo liposomes with lipid composition as in Doxil®, 100 nm. Liposomes were administered i.v. as a single dose. Secondly, the effect of multiple dose administration of AmBisome® or Doxil® on bacterial clearance from blood was studied in rats. AmBisome® or Doxil® were administered at various dosage schedules. Blood clearance capacity of the MPS was monitored at different time points after the last liposome dose. The data obtained show that carbon blood clearance capacity of the MPS was impaired only at a high lipid dose of empty classical liposomes. Bacterial blood clearance capacity was not impaired, not even after multiple dose treatment with AmBisome® or Doxil® when administered in a clinically relevant regimen.

Keywords: Liposomes; AmBisome; Doxil; Phagocytosis; MPS

* Corresponding author. Tel.: +31 10 4087666; fax: +31 10 4364730.

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1. Introduction

Possible implications of intravenous administration of liposomes regarding the bacterial blood clearance capacity of the mononuclear phagocyte system (MPS) needs to be investigated thoroughly. Liposomal agents used in clinical practice are often administered to patients that are immunocompromised and as a result, highly susceptible to (opportunistic) infections. Systemic infections (septicemia) may develop easily due to spread of microorganisms from a locally infected site. Good functioning resident phagocytic cells of the MPS play a major role in the clearance of microorganisms from the blood. Malfunction of the MPS may result in generalization of the infection and hence, increased mortality. As liposomes are also cleared from the circulation by MPS cells, it is important to know whether intravenous administration of liposomes interferes with the clearance of infectious organisms from the blood. Binding of blood proteins to liposomes during circulation (Oja et al., 1996) might lead to reduced opsonization and clearance of microorganisms. Furthermore, saturation of phagocytic uptake capacity of the MPS after administration of relatively high doses of 'classical' liposomes that rapidly accumulate in the MPS has been demonstrated. Particularly when potentially toxic agents are encapsulated in these liposomes, serious damage of the MPS should be considered. Experimental evidence for this was recently published (Daemen et al., 1995). It was demonstrated that the administration of doxorubicin-containing classical liposomes to rats can result in toxicity towards liver macrophages in terms of impaired phagocytic functions, or even depletion of liver macrophages.

Liposomes exhibiting prolonged blood residence time are able to avoid uptake by the MPS substantially. The industrially-prepared formulations such as AmBisome® and Doxil® indeed show relatively prolonged blood circulation time, which is for AmBisome® dependent on the lipid dose. However, MPS uptake to a certain extent is still observed, and may have implications as these liposomes carry the potentially toxic agents amphotericin B and doxorubicin, respectively. Toxicity of drug released from the liposomes intracellularly may result in impaired phagocytic capacity of the MPS.

In the present study, the single dose effects of three types of empty liposomes, differing in blood residence time, on carbon clearance and bacterial clearance from blood were investigated in mice. In a second series of experiments, the effects of AmBisome® or Doxil® given at multiple dose schedules on bacterial clearance from blood were studied in rats.

2. Materials and methods

2.1. Animals

Female Balb/c mice (10–13 weeks old, specified pathogen free) were obtained from Iffa Credo (L'Arbresle, France). Female R-strain albino rats (20–25 weeks old, specified pathogen free) were obtained from Harlan CPB, Austerlitz, The Netherlands.

2.2. Liposomes

Classical liposomes composed of egg phosphatidylcholine, phosphatidylserine, cholesterol (molar ratio, 4:1:5) 300 nm. Placebo liposomes with lipid composition as in AmBisome®, hydrogenated soybean phosphatidylcholine, distearoylphosphatidylglycerol, cholesterol (molar ratio, 10:4:5) 100 nm. Placebo liposomes with lipid composition as in Doxil®, hydrogenated soybean phosphatidylcholine, polyethyleneglycol (PEG) 1900 derivative of distearoylphosphatidylet hanolamine, cholesterol (molar ratio, 57:5:38) 100 nm. Classical liposomes and placebo liposomes were prepared in our laboratory. AmBisome® was kindly provided by NeXstar Pharmaceuticals (San Dimas, CA). Doxil® was kindly provided by SEQUUS Pharmaceuticals (Menlo Park, CA). All placebo liposome formulations were prepared in our laboratory.

2.3. Monitoring of carbon clearance or bacterial clearance from blood in mice treated with empty liposomes

Liposomes were injected i.v. into mice at single doses of 400 μmol total lipid/kg (all liposome formulations) or 80 μmol total lipid/kg (classical liposomes only). At different time points after administration of liposomes or buffer (controls), blood clearance capacity of the MPS was determined, i.e. at 1 min after liposome administration ($>90\%$ of liposomes present in blood), and at the times that 50 or 10% of liposomes were present in blood. Carbon clearance was monitored by i.v. injection of carbon (Drawing ink FT, Pelikan AG, Hannover, Germany, diluted in phosphate-buffered saline) at a dose of 1 mg/mouse. At 1 and 10 min after administration, blood was collected from which carbon concentration was determined, after lysis of blood cells with lysis buffer (Isolator 10 blood culture system, Wampol laboratories, USA). The percentage of clearance within 9 min was calculated. Bacterial clearance was monitored by i.v. injection of *Klebsiella pneumoniae* at an inoculum of 1.4×10^5 bacteria/kg or *Staphylococcus aureus* at an inoculum of 1.4×10^9 bacteria/kg. At 1 and 10 min after administration, blood was collected, from which the number of viable bacteria was determined, after lysis of blood cells.

2.4. Monitoring of bacterial clearance from blood in rats treated with AmBisome® or Doxil®

AmBisome® was injected i.v. into rats at five or ten doses of 5 mg/kg, each at a 24 h interval. Doxil® was injected i.v. into rats at five doses of 1.5 mg/kg each at a 9 or a 4 day interval. At different time points after administration, i.e. 24 and 72 h (for Doxil® also 48 and 144 h) after the last liposome dose, blood clearance capacity of the MPS was determined. Bacterial clearance was monitored by i.v. injection of *Klebsiella pneumoniae* at an inoculum of 3.2×10^7 bacteria/kg. At 60 and 120 min after inoculation, blood was collected from which the number of viable bacteria was determined.

2.5. Statistical analysis

In the study with empty liposomes, results were expressed as the percentage of clearance within 9 min. In the study with AmBisome® and Doxil®, results were expressed as the geometric mean of numbers of viable bacteria at various time points. In both studies, differences in blood clearance between the various treatment groups were analyzed by the Mann–Whitney test.

3. Results

3.1. Effect of i.v. administration of empty liposomes on carbon clearance from blood in mice

The three types of liposomes showed different blood circulation times in mice. At 1 min after liposome administration, $>90\%$ of the liposome dose was present in blood. At the high dose of 400 μmol lipid/kg, 50% of the liposome dose was present in blood at 50 min for classical liposomes, at 4 h for placebo-AmBisome, and at 16 h for placebo-Doxil; 10% of the liposome dose in blood at 110 min for classical liposomes, at 40 h for placebo-AmBisome and at 48 h for placebo-Doxil. At the dose of 80 μmol lipid/kg for classical liposomes, 50% of the dose was present in blood at 10 min and 10% of the dose at 30 min.

Due to administration of classical liposomes at the high dose of 400 μmol lipid/kg the carbon clearance within 9 min was significantly reduced at all time points measured: being 46, 58 and 47% at 1 min, 50 and 110 min after liposome administration, respectively, whereas in control animals, carbon clearance was 72, 88 and 65% at the same time intervals after buffer administration. After administration of classical liposomes at the low dose of 80 μmol lipid/kg, the carbon clearance efficiency was unaffected. Administration of placebo-AmBisome or placebo-Doxil at the high dose of 400 μmol lipid/kg did not result in impaired carbon clearance capacity.

3.2. Effect of i.v. administration of empty liposomes on bacterial clearance from blood in mice

In buffer-treated control mice, an average of 56% of *Klebsiella pneumoniae* was cleared within 9 min. Administration of all three types of liposomes at the high dose of 400 μmol lipid/kg did not affect the bacterial clearance efficiency at all time points.

The clearance of *Staphylococcus aureus* within 9 min amounted to an average of 95% in buffer-treated controls. Again, the bacterial clearance efficiency was not different in animals treated with all three types of empty liposomes, with one exception: at 1 min after administration of placebo-AmBisome, the clearance capacity was slightly but significantly reduced to 80%.

3.3. Effect of i.v. administration of AmBisome® on bacterial clearance from blood in rats

In buffer-treated control rats, $>99\%$ of *Klebsiella pneumoniae* was cleared within 60 min. The elimination half-life of AmBisome® in rats is about 8 h (Proffitt et al., 1991). Administration of AmBisome® daily at five or ten doses of 5 mg/kg each did not affect clearance of *Klebsiella pneumoniae* within 60 or 120 min after inoculation, when determined at 24 or 72 h after the last dosage.

3.4. Effect of i.v. administration of Doxil® on bacterial clearance from blood in rats

The elimination half-life of Doxil® in rats is ~ 27 h. Administration of Doxil® at five doses of 1.5 mg/kg each every 9 days did not affect clearance of *Klebsiella pneumoniae* within 60 or 120 min after inoculation when determined at the four time intervals after the last dosage. Administration of the same dosage (and number of doses) in a higher frequency at 4 day intervals resulted in a significantly impaired clearance of bacteria within 60 and 120 min after inoculation.

4. Discussion

In the present study it was investigated for different types of liposomes whether i.v. administration of liposomes interferes with the blood clearance capacity of the MPS. Blood clearance capacity was assessed at various time points after liposome administration. As parameters for blood clearance capacity of the MPS, the clearance of carbon particles was determined as this is the classical parameter mostly used by other investigators (Ellens et al., 1982; Allen et al., 1984; Fichtner et al., 1992). We also monitored clearance of bacteria from blood, as this is the most relevant parameter from the clinical point of view. Two clinically relevant bacterial pathogens were chosen: *Klebsiella pneumoniae* (gram-negative bacteria) and *Staphylococcus aureus* (gram-positive bacteria).

Regarding the three types of empty liposomes that were used, their blood circulation half-lives were substantially different: In mice, 50% of the injected dose of liposomes were present in the blood for classical liposomes at 50 min, for placebo-AmBisome liposomes at 4 h, and for placebo-Doxil liposomes at 16 h after i.v. administration. For that reason we decided to measure blood clearance capacity of the MPS not at fixed time points after liposome administration, but at the time points that comparable amounts of liposomes were still circulating in the blood: for $>$ 90%, for 50%, and for 10% of the injected dose. In the studies dealing with the effects of AmBisome® or Doxil®, prolonged treatment schedules were applied using regimens resembling the clinical setting closely. In the latter experiments, bacterial blood clearance capacity was determined at various time intervals over a substantial period after the last liposome administration.

The experimental data obtained for empty liposomes show that after administration of 300 nm, classical liposomes at the high dose of 400 μmol total lipid/kg carbon clearance was significantly reduced at each of the three different time points. However, bacterial clearance was not reduced. At the low dose of 80 μmol total lipid/kg, which is near to clinically relevant lipid dosage, carbon and bacterial clearance were not affected. Al-

though the biodistribution of the classical liposomes was not investigated, substantial hepatosplenic uptake of classical liposomes, when administered in a high dose, can be expected and may explain the reduced hepatosplenic uptake of carbon particles from blood. At the time that most of the liposomes are still in the blood compartment, binding of blood proteins, in particular opsonins, to liposomes (Oja et al., 1996) might lead to reduced opsonization and thereby reduced clearance of carbon particles. Bacterial clearance from blood was not impaired in animals treated with the high dose of classical liposomes, demonstrating that the measurement of blood clearance capacity of the MPS is dependent on the type of particle that is used.

Administration of empty liposomes representing the size and lipid composition of AmBisome® and Doxil®, respectively, did not influence blood clearance capacity of the MPS. In view of the MPS-avoiding behaviour of these liposome formulations, particularly in the case of the sterically stabilized placebo-Doxil liposomes, these findings are not surprising.

An important question is whether long-circulating liposome formulations containing the potentially toxic agents amphotericin B or doxorubicin, show similar results. For that purpose, the industrially prepared formulations of AmBisome® and Doxil® were evaluated at a clinically relevant dose regimen. In man, AmBisome® is generally administered at 5 mg/kg daily, and has an elimination half-life of $\sim$32 h (NeXstar Pharmaceuticals, 1994). In rats, AmBisome® half-life is $\sim$8 h (Proffitt et al., 1991). In the present study, AmBisome® at 5 mg/kg was administered daily during a period of 5 or 10 days, as 10 i.v. injections were maximally tolerated by the rats. In man, Doxil® is generally administered at 60 mg/m^2 (which corresponds to $\sim$1.5 mg/kg) every 4 weeks, and elimination half-life is $\sim$48 h (Gabizon et al., 1994). In rats, half-life of Doxil® is $\sim$27 h (Working et al., 1994). Calculated from these data, a dosage of 1.5 mg/kg every 9 days in rats would be roughly equivalent to the human dose regimen. To measure bacterial clearance capacity, *Klebsiella pneumoniae* was inoculated as the blood clearance of this highly encapsulated bacterial strain is rather slow compared to other bacterial pathogens. In infections caused by highly encapsulated bacteria, maximal MPS function is very important. Bacterial clearance from blood was measured over a 120 min period. Although prolonged treatment with daily AmBisome® in rats is expected to result in high amounts of AmBisome®, particularly in the liver, impairment of bacterial blood clearance capacity was not observed at any of the observation time points. Also prolonged treatment, Doxil® appeared to be safe in this respect, provided the frequency of administration is not too high. Considering the observations obtained for Doxil® in the present study and the data published by Daemen et al. with respect to the toxicity (including impaired MPS function) of various liposome formulations containing doxorubicin, we can conclude the following. Impressive effects in terms of decreased bacterial clearance from blood were seen with classical liposomes containing doxorubicin. The toxic effects were dependent on the dose schedule, and most pronounced at the relatively high dose of 5 mg/kg (Daemen et al., 1995). The effects were related to a major depletion of the liver macrophage population, as revealed by both macrophage isolation and histology. Doxorubicin encapsulated in long-circulating liposomes was less toxic for the liver macrophage population, and had less impressive effects on the bacterial blood clearance capacity; in addition a faster recovery from liposomal-doxorubicin treatment was observed (Daemen et al., 1997). The present study shows that with the sterically-stabilized liposomes containing doxorubicin (Doxil®), which show even more prolonged circulation in blood toxicity in terms of decreased bacterial blood clearance was not observed, provided Doxil® was administered at relatively long intervals.

Summarizing, reduction of the phagocytic capacity of the MPS is a major concern, particularly in immunocompromised patients. In these patients potentially toxic agents in the liposome-encapsulated form are administered to achieve activity with reduced toxicity. However, the accumulation in MPS tissues, particularly the liver and spleen, is still a concern. In this regard, the dose

and dose regimen need to be carefully chosen. This is however, not well recognized. Potential toxic effects of the liposomal agents with respect to MPS function in terms of reduced bacterial blood clearance capacity, should be taken into consideration. The dosage schedule with respect to dose and dose frequency has to be chosen carefully. In addition, in the development of new liposomal agents, from a clinical point of view it is important to focus on MPS-avoiding liposomes, such as sterically-stabilized liposomes. With these liposome types, high lipid doses are not needed to obtain prolonged circulation in blood.

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