LIPOSOMAL TARGETING OF ANTIMICROBIAL AGENTS TO BACTERIAL INFECTIONS

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Liposomal targeting of antimicrobial agents to bacterial infections
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Ter herinnering aan mijn moeder

CONTENTS

1. General Introduction

Summary

Failure of antimicrobial treatment is observed frequently in hospitalized patients resulting in morbidity and mortality. A possible way to improve antimicrobial treatment is the targeted delivery of antimicrobial agents. This thesis describes a study on the use of long-circulating liposomes for the targeted delivery of antimicrobial agents to sites of bacterial infections. In this chapter an introduction is given on the use of targeted drug delivery in infectious diseases, which is followed by the aims and outline of this thesis.

1. Infectious diseases

Infectious diseases remain an important cause of morbidity and mortality in the Western world, especially in hospitalized patients. The long, invasive, and intensive hospital treatments lower the barriers to microbial invasion of host tissues and compromise the development of an adequate inflammatory/immune response. In addition, the host response may be weakened by administration of immunosuppressants or cytotoxic drugs or as a result of a pathology that affects the host defense. Under these circumstances antimicrobial treatment frequently fails. This thesis focuses on ways to increase the efficacy of antimicrobial treatment against bacterial infections.

Bacteria are unicellular prokaryotic organisms with a circular double-stranded deoxyribonucleic acid (DNA) molecule that bears the genetic information (Salyers, 1994). Additional genetic information may be present in the form of plasmids, small extrachromosomal, self-replicating, and transferable DNA molecules (Actis, 1999; Davison, 1999). Some components of the bacterial cell wall, like peptidoglycan, (lipo)teichoic acid and lipopolysaccharide (LPS), are toxic molecules known as endotoxins (Seydel, 2000; Wiese, 1999; Sriskandan, 1999). In addition, bacteria may also secrete soluble toxic proteins, known as exotoxins (Guerrant, 1999; Middlebrook, 1984, Lesieurs, 1997). These toxins have detrimental effects on the infected host, as they may destruct host cells or structures and may affect host homeostasis. The effects of toxins on host cytokine profiles may lead to disseminated intravascular coagulation, severe inflammatory response syndrome and ultimately septic shock and multiple organ failure by distorting the balance between pro-inflammatory and anti-inflammatory stimuli (Bone, 1997; Deitch, 1999; van der Meer, 1999).

2. Antimicrobial treatment

Impediment of bacterial contamination and colonization, for example by sterilization, disinfection, or isolation is a way to prevent the development of infectious diseases. Once the bacteria have invaded the host, the aspecific and specific host defense become activated to control the infection. For prevention or treatment of bacterial infections antimicrobial

agents may be used. Antimicrobial agents are molecules that are (relatively) selectively toxic to bacteria, by interfering with essential bacterial metabolic processes. Different classes of antibiotics show different modes of action. For example, the ß-lactam class of antibiotics (cephalosporins and penicillins) interfere with bacterial cell wall synthesis (Kidwai, 1999), whereas the aminoglycosides inhibit bacterial protein synthesis as a result of ribosome binding (Forge, 2000). The discovery of antibiotics, early in the 20th century, has had a tremendous impact on morbidity and mortality and even, according to some authors, the outcome of the Second World War (Bol, 1999; Demain, 1999). Less than a century later, the golden age of antibiotics seems to have passed (Trnobranski, 1998; Young, 1985). Clinical practice shows that failure of antimicrobial treatment is frequently encountered in hospitalized patients (Shlaes, 1993; Cunha, 1995; Sugarman, 1980; Butts, 1994; Barry, 1999; Sanchez-Navarro, 1999; Liu, 1999; O'Brien, 1997). Three prominent causes of failure of antimicrobial therapy can be distinguished:

- sub-effective drug concentrations at the infected site (as a result of limited drug access to the infected site by unfavorable pharmacokinetics of the drug, limited blood flow (as in osteomyelitis) or the presence of barriers (as in intracellular *Mycobacterium*-infections)
- low susceptibility of the microorganism towards the applied antimicrobial agent
- growing number of immunocompromised patients (as a result of long-term, invasive, and intensive hospital treatments, administration of drugs that affect the host defense, or as a result of a condition that compromises the host defense (e.g. human immunodeficiency virus infection).

These causes underlying treatment failure create a need for more efficacious treatment. Site-specific drug delivery could improve the therapeutic index by increasing drug

concentrations at the target site and by reducing side-effects in case of potentially toxic antibiotics.

3. Drug targeting

Site-specific drug delivery, or drug targeting, in its broadest sense, is practiced daily by millions of people. The application of iodine after a cut (Burks, 1998; Cho, 1998), eye, ear, and nose drops for local infection or hay fever (Vajpayee, 2000; Shikiar, 1999; Curran, 1998; Davies, 1996), or inhalation of drugs for asthma (Dhand, 2000; Volsko 2000) are just a few examples. The rationale for this site-specific delivery of drugs may be summarized as follows (Tomlinson, 1986):

- to administer the drug (almost exclusively) to a specific body compartment (to increase therapeutic efficacy)
- to protect body and drug from deposition at unwanted -non-target- sites (to reduce side effects or drug inactivation)
- to control rate and modality of drug delivery to the pathological site
- to reduce the amount of drug used

Whatever the rationale, the examples mentioned on application of iodine, eye, ear, and nose drops and inhalation of drugs, share one resemblance: they affect epithelial surfaces. In this case, the target site is readily accessible. If it is less accessible, drug targeting may serve an additional purpose:

- to obtain drug access to the pathological site

To reach the latter goal a drug delivery vehicle, or drug carrier, is generally needed for a drug to arrive at the target site. The term drug targeting in scientific literature is generally confined to this carrier-mediated drug delivery. The first study employing a drug carrier for

targeted drug delivery was published approximately 30 years ago, using antibodies as carriers of radioactivity for the specific recognition of tumor cells (Ghose, 1969).

3.1 Drug carriers

The ideal drug carrier ensures the timely release of the drug within the therapeutic window at the appropriate site, is neither toxic nor immunogenic, is biodegradable or easily excreted after action, and is preferably cheap and stable upon storage (Sinkula, 1988; Tomlinson, 1986). Many different kinds of drug carriers have been investigated (as reviewed by Pouton, 1985; Langer, 1998; Mrsny, 1997; Duncan, 1997):

either soluble macromolecular carriers (as reviewed by Hawiger, 1999; Takakura, 1996; Wadhwa, 1995; Rihova, 1997; Felt, 1998; Dubowchick, 1999; Trail, 1999), like proteins, lectins, hormones, polysaccharides, or DNA

or microparticulate carriers (as reviewed by Tomlinson, 1987; Kwon, 1998; Davis, 1997; Tenjarla, 1999; Rensen, 1996; Templeton, 1999; Storm, 1997; Yamaguchi, 1994; Allen, 1998; Tonetti, 1993; Alemany, 1999; Jones, 1999), like liposomes, lipoproteins, emulsions, nanoparticles, cells, or viruses.

The wide variety in investigated drug carriers indicates that "the ideal drug carrier" does not exist. The suitability of a drug carrier is determined by the disease that will be targeted, its access to the pathological site, and the carriers ability to achieve appropriate drug retention and timely drug release (Tomlinson, 1987). These points will be discussed in relation to the research presented in this thesis.

3.2 DART: Disease, Access, Retention, Timing (Tomlinson, 1987)

Reports on drug carriers with access to or localization at sites of bacterial infection, may be categorized according to the approach taken to improve the antibacterial efficacy. Four approaches may be distinguished:

- Local administration of carriers
- Passive targeting after i.v. administration of carriers which tend to be taken up by phagocytic cells
- Passive targeting after i.v. administration of carriers with a long-circulation time
- Active targeting after i.v. administration of carriers

The first approach is the use of drug carriers to increase the residence time of antibiotics after local administration of the drug-carrier complex at the site of infection. Examples are polymethylmethacrylate beads (Wahlig, 1978) or tubes (Bircher, 1985), glyceryl monostearate implants (Allababidi, 1998) poly-L-lactic acid conjugates (Teupe, 1992), bioerodible polyanhydrides (Laurencin, 1993), fibrin glue/Dacron grafts (Fujimoto, 1997), liposomes (Reimer, 1997; Lichtenstein, 1995), and nanoemulsions (Hamouda, 1999). These carriers are generally investigated with the intention to treat local infections in body parts with limited blood flow as in bone, joint, skin, and cornea. Their use is limited to accessible sites of infections with a clear infectious focus.

The second approach is the use of drug carriers to target intracellular infections. These infections are often difficult to treat as a result of limited ability of the antimicrobial agent to penetrate into cells. This approach makes use of the recognition of drug carriers as foreign material in the bloodstream by the phagocytic cells of the mononuclear phagocyte system (MPS), the cell type often infected with microorganisms. Examples of carriers that have been shown to be taken up by phagocytic cells are nanoparticles (Youssef, 1988, Fattal 1989; Pinto-Alphandary, 1994; Peters, 2000; Couvreur, 1991), lactide-glycolide microspheres (Barrow, 1998), acetylated low-density lipoproteins (Nicolas, 1990), and liposomes (Melissen, 1993; Bakker-Woudenberg, 1989; Majumdar, 1992; Couvreur, 1991).

Regarding the third and fourth approach, the targeting of infectious foci not restricted to MPS-tissues, reports on access of drug carriers to the infected site are mainly derived from

imaging studies and are either based on passive or active drug targeting. Passive drug targeting makes use of long-circulating drug carriers, whereas active targeting makes use of carriers that specifically bind to the infectious organism or host cells involved in the inflammatory response. Carriers that have been reviewed are: peptides and proteins (Weiner, 1999; Okarvi, 1999; van der Laken, 1998; Nibbering, 1998; Chan, 1999; Giacomuzzi, 1999), leukocytes (Chik, 1996), nanoparticles (Becker, 1995), and liposomes (Morgan, 1985; Boerman, 1998; Oyen, 1996; Bergers, 1995; Bakker-Woudenberg, 1994).
In particular long-circulating liposome systems have been intensively investigated as potential drug carriers in infectious or inflammatory pathologies, as substantial liposome localization at the target site has been demonstrated in a variety of models of infection and inflammation (Morgan, 1981; Dams, 1999; Crommelin, 1999; Rousseau, 1999; Dams, 1999(II); Klimuk, 1999; Awasthi, 1998; Awasthi, 1998(II); Corvo, 1999; Bakker-Woudenberg, 1995; Bakker-Woudenberg, 1993; Bakker-Woudenberg, 1992). The target localization has been shown to be dependent on the inflammatory response as the localization in uninfected/uninflamed controls is insignificant.
In addition, the sufficiency of drug retention and timing of the drug release determine the suitability of a drug carrier for targeted drug delivery. The optimal characteristics are to a high degree dependent on the class of antibiotics that is to be targeted. A number of papers on drug retention and release provided by liposomes show that the retention and release profiles can be modulated by variation of liposome characteristics (Kulkarni, 1995; Gregoriadis, 1993; Allen, 1998; Gregoriadis, 1988). These results provide further support for the choice of long-circulating liposomes as carriers for the targeted delivery of antibacterial agents to sites of bacterial infection.

3.3 Structure of liposomes

Liposomes are spherical vesicles, with particle sizes ranging between 30 nm up to several microns, consisting of one (unilamellar vesicle) or more (multi-lamellar vesicle) lipid bilayers surrounding an equal number of aqueous spaces (Figure 1). Liposomes can either incorporate hydrophilic drugs in the aqueous compartment(s), hydrophobic drugs in the lipid

bilayer(s), or amphiphilic compounds that partition between both phases. The bilayers usually consist of (natural or synthetic) phospholipids and cholesterol but incorporation of other sterols, fatty acids, glycolipids, and proteins is also possible. The composition of the lipid bilayer can be manipulated to influence the physicochemical characteristics of the liposome (e.g. surface charge, sensitivity to pH changes, and bilayer rigidity) (Vemuri, 1995; Jones, 1995; Gregoriadis, 1993). Liposomes were originally investigated as model membranes during the 1960's, until, in the early 1970's, the possibility to incorporate drugs was appreciated (Gregoriadis, 1971; Gregoriadis, 1973). The result has been an 'avalanche' of liposome-focused research.

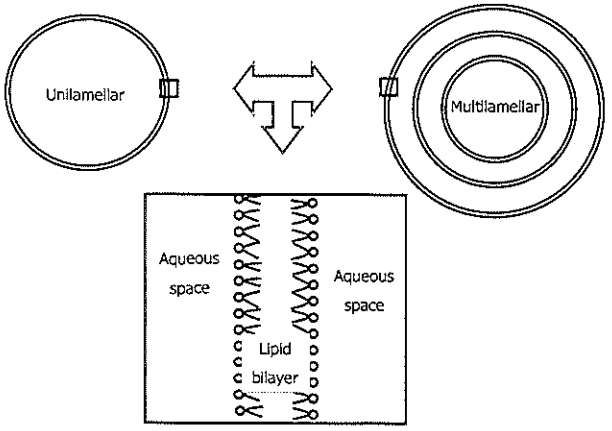

Figure 1. Schematic representation of the structure of liposomes. The lipid bilayer is composed of phospholipids, with their hydrophilic head group facing the aqueous spaces.

3.4 The search for a prolonged circulation time of liposomes

Information on liposome behavior in vivo is abundant. Soon after liposomes were first administered to experimental animals, it appeared that intravenously injected liposomes tend to rapidly accumulate in the MPS-cells, most prominently in those in the liver and

spleen (Alving, 1983; Kirsh, 1986; Senior, 1987; Gregoriadis, 1989; Kreuter, 1991). The efficient uptake of liposomes by this cell type is thought to occur as a result of liposome opsonization in the circulation (Patel, 1992; Devine, 1997). Opsonization may be viewed as a protective process by which foreign material is coated with proteins facilitating recognition and removal of this foreign material from the blood stream by the MPS. As a result, these liposomes have a short circulation time and they have only access to the cells of the MPS.

This conclusion stimulated research on the design of liposomes possessing a prolonged circulation time (Senior, 1987; Paphadjopoulos, 1990; Woodle, 1992; Woodle, 1993; Oku, 1994; Torchillin, 1996). Initially, it appeared that small (approximately 100 nm), neutral liposomes composed of rigid lipid bilayers display a prolonged circulation time compared to larger, charged liposomes with fluid lipid bilayers. One of the drawbacks of this approach to obtain long-circulating liposomes is that the ability to tailor liposome characteristics for optimal drug retention and release profiles is partly lost.

Subsequent research explored the possibility to increase circulatory half-life by inclusion of glycolipids. This method was supposed to mimic the red blood cell's constituents to evade uptake by the MPS (Gabizon, 1988; Allen, 1989). However, the apparent species dependency of the prolongation of circulation time (Liu, 1995), confinement to a rigid lipid bilayer structure, clinical acceptability and costs are important drawbacks in this approach.

A 'breakthrough' was achieved by coating of the liposome surface with soluble and flexible polymers such as poly(ethylene glycol) (PEG) (Klibanov, 1990; Senior, 1991; Allen, 1991; Papahadjopoulos, 1991) (Figure 2). The method is based on experience with protein:polymer adducts that display reduced plasma clearance compared to the native protein (Wieder, 1979; Pyatak, 1980). It is believed that the presence of a dense conformational polymer cloud grafted onto the liposome surface sterically hinders the adherence of opsonins to the liposome surface (Torchillin, 1996). Such long-circulating, PEG-coated, MPS-avoiding, Stealth® or sterically stabilized liposomes (SSL) offer a convenient way to obtain liposomes for which long circulation time is to a high degree independent of liposome characteristics as size, charge, and bilayer rigidity (Woodle, 1992). The value of PEG-coating was first illustrated in tumor models (Gabizon, 1994; Huang,

1992; Allen, 1992; Papahadjopoulos, 1991). In these experiments prolonged liposomal circulation time, increased tumor targeting, and increased therapeutic efficacy were demonstrated.

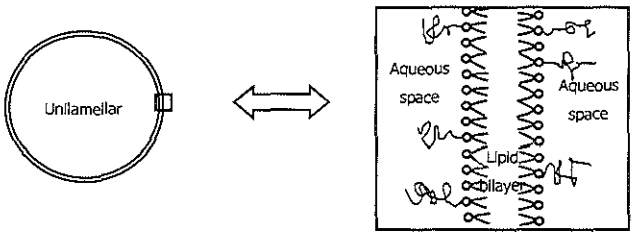

Figure 2. Structure of sterically stabilized liposomes. The lipid bilayer is composed of phospholipids, of which some (usually about 5 mol%) have been grafted with poly(ethylene) glycol.

4. Aims and outline of this thesis

The basis for the present study is the previous work of Bakker-Woudenberg et al. showing enhanced target localization of SSL in a rat model of a unilateral pneumonia caused by *Klebsiella pneumoniae* (Bakker-Woudenberg, 1993). The attractiveness of the unilateral *K. pneumoniae* pneumonia rat model for use in drug targeting studies is not only its resemblance of the pathology associated with human lobar pneumonia. The ability to selectively inoculate bacteria into the left lung, developing into a left-sided pneumonia, allows the right lung to serve as an intra-individual control organ. The previously mentioned study showed an increase in liposome localization in the infected left lung compared to the contralateral non-infected right lung. Moreover, a linear relationship was demonstrated between liposome left lung localization and infection intensity. Administration of a single dose of SSL-encapsulated gentamicin or SSL-encapsulated ceftazidime was shown to have an increased therapeutic efficacy compared to an equivalent dose of the free drug (Bakker-Woudenberg, 1995).

In conclusion, these two studies demonstrated that SSL localize preferentially at the site of bacterial infection and that the increased therapeutic efficacy of antibiotics was the result of encapsulation in SSL.

These studies have been continued. For optimization of liposome targeting to the site of infection it is essential to identify the critical factors that contribute to liposome target localization. In addition, in clinically relevant infection models in which antibiotic treatment fails, the value of SSL-encapsulated antibiotics should be evaluated.
Therefore, the present investigations aimed at:

- identifying the factors (on the side of the liposomes as well as on the side of the host) that influence the selective target localization of SSL in *K. pneumoniae* infected rat lung tissue
- evaluating the therapeutic efficacy of these liposomes, when loaded with antibiotics, in clinically relevant rat models, in particular addressing the issues of impaired host defense and reduced bacterial susceptibility.

Chapter 2 and 3 describe experiments in which liposome-related factors (Chapter 2) and host-related factors (Chapter 3) were studied. Liposome-related factors that were investigated were PEG-density, particle size, bilayer fluidity and negative surface charge. The investigation of host-related factors focussed on the components of the inflammatory response. The conclusions were used to suggest a general model for liposome localization at sites of infection based on the equation of Kedem and Katchalsky that describes protein influx into inflammatory tissue (Chapter 4).

Chapter 5 is a review of preclinical and clinical data on liposome-encapsulated aminoglycosides, and provides an introduction to our experiments with gentamicin encapsulated in SSL in the next chapters.

In Chapter 6 the therapeutic efficacy of two different liposomal gentamicin formulations was tested in clinically relevant rat models of serious *K. pneumoniae* infections. Both the clinically encountered problems of a low susceptibility of the bacteria as well as lack of adequate host defense were addressed.

Chapter 7 describes a new concept in the field of antimicrobial drug targeting: the co-encapsulation of antibiotics, which show synergistic interactions in vitro, in SSL. Therapeutic efficacy of site-specific co-delivered gentamicin and ceftazidime was evaluated in rats infected with an antibiotic-susceptible or an antibiotic-resistant strain of *K. pneumoniae*.

In Chapter 8 the results, presented in this thesis are summarized, discussed and suggestions are made for future research.

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2. Localization of sterically stabilized liposomes in *Klebsiella pneumoniae*-infected rat lung tissue: influence of liposome characteristics

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Summary

Sterically stabilized liposomes are able to localize at sites of infection and could serve as carriers of antimicrobial agents. For a rational optimization of liposome localization, the blood clearance kinetics and biodistribution of liposomes differing in poly(ethylene) glycol-density, particle size, bilayer fluidity or surface charge were studied in a rat model of a unilateral pneumonia caused by *Klebsiella pneumoniae.*

It is shown that all liposome preparations studied localize preferentially in the infected lung compared to the contralateral non-infected lung. A reduction of the poly(ethylene) glycol-density or rise in particle size resulted in a higher uptake by the mononuclear phagocyte system, lower blood circulation time and lower infected lung localization. Differences in bilayer fluidity did not affect blood clearance kinetics or localization in the infected lung. Increasing the amount of negatively charged phospholipids in the liposome bilayer did not affect blood clearance kinetics, but did reduce localization of this liposome preparation at the site of lung infection.

In conclusion, the degree of localization at the infected site is remarkably independent of the physicochemical characteristics of the PEG-liposomes. Substantial selective liposome localization can be achieved provided that certain criteria regarding PEG-density, size, and inclusion of charged phospholipids are met. These properties seem to be a direct consequence of the presence of the polymer coating operating as a repulsive steric barrier opposing interactions with biological components.

1. Introduction

Unfavorable location of an infectious organism, limited susceptibility to the applied antimicrobial agent, and/or decreased immune status of the infected host are all major factors complicating the efficacy of antimicrobial therapy [1, 2, 3]. The use of a drug carrier, like liposomes, could help to improve the efficacy of antibiotic treatment by modifying the pharmacokinetics and tissue distribution of the antibiotic. This strategy may increase drug concentrations at the target site and/or reduce toxicity to non-target tissues enabling higher dosing [4, 5].

The location of the infection is an important consideration for the choice of the liposome type to be used as targeted antibiotic carrier. As intravenously administered conventional liposomes display a high affinity for the mononuclear phagocyte system (MPS), a rapid localization of these liposomes in MPS-rich organs, most notably in the liver and the spleen, is observed. This property makes them appropriate for the specific targeting of antimicrobials to infectious organisms residing within MPS cells in these organs. In addition, since the MPS plays an important role in the non-specific defense against infections, MPS-directed targeting of liposomal immunomodulators may enhance the non-specific host resistance. Both strategies have been reviewed [6-7].

The limitations of these conventional liposomes as antibiotic carriers are clear, when targeting to infections outside the MPS is the main goal. Various strategies have been followed to reduce uptake of liposomes by the MPS and consequently to increase their blood residence time, allowing interaction with other tissues. At present, coating of the surface of liposomes with poly(ethylene glycol), is a popular strategy to increase the circulation time of liposomes. It is thought that the poly(ethylene) glycol coating provides a steric barrier against opsonization, thereby reducing the interaction with the MPS. Consequently, these liposomes, referred to as sterically stabilized liposomes (SSL) exhibit a prolonged circulation time (for reviews see [8, 9, 10]).

Previous studies of our group in a model of acute unilateral pneumonia caused by *Klebsiella pneumoniae* in rats demonstrated that SSL are capable to substantially localize in the infected left lung. The presence of a local active infectious process appeared to be required

as minimal liposome localization was observed in the contralateral non-infected right lung [11,12]. Furthermore, it was shown that encapsulated gentamicin was strongly effective in reducing bacterial numbers in the infected left lung as well as preventing mortality, likely due to local release of the drug from the SSL at the site of infection [13].

The phenomenon of preferential localization of SSL at pathological sites has also been described for other inflammatory conditions like adjuvant arthritis in rats, experimental colitis in rabbits, focal bacterial infection in the calf muscle of rats, and osteomyelitis in rabbits [14, 15, 16, 17]. However, only little information is available on the influence of liposome characteristics on the degree of localization of SSL at inflammatory foci. Insight into the liposomal properties favoring the localization in inflammatory tissue may help to optimize liposomal formulations for antimicrobial therapy. The aim of the present study is to evaluate the effects of poly(ethylene glycol) surface density, particle size, bilayer fluidity, and surface charge of SSL on circulation kinetics and localization in infected lung tissue.

2. Materials and Methods

2.1 Introduction of a unilateral pneumonia

Specified pathogen-free female RP/AEur/RijHsd strain albino rats (18-25 weeks of age, weighing 185-225 g) (Harlan, Horst, The Netherlands) were used in all experiments. Experiments were approved by the animal experiments ethical committee of the Erasmus *university* Medical Center Rotterdam. A unilateral pneumonia was induced in the left lung as described previously [18]. In brief, rats were anesthetized by an intramuscular injection of 0.1 ml fluanisone and fentanyl citrate (Hypnorm®) (Janssen Animal Health, Saunderton, UK) followed by an intraperitoneal injection of 0.3 ml 1:4 v/v diluted pentobarbital (Nembutal®) (Sanofi Santé b.v., Maassluis, The Netherlands). After intubation followed by cannulation of the left primary bronchus, 0.02 ml of a saline suspension containing 10^6 *K. pneumoniae* (ATCC 43816, capsular serotype 2) was inoculated in the lower left lung lobe. Bacterial inoculum was checked by culturing appropriate dilutions of the bacterial suspension on tryptone soy agar plates (Unipath Ltd., Basingstoke, UK). Following bacterial inoculation, rats received an intramuscular injection of nalorphine bromide to recover from anesthesia (Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands) and were housed individually with free access to water and SRMA chow (Hope Farms b.v., Woerden, The Netherlands).

2.2 Liposome preparation and characterization

Liposomes were prepared as described previously [12]. In brief, appropriate amounts of partially hydrogenated egg phosphatidylcholine (PHEPC, iodination value 40) (Asahi Chemical Industry Co. Ltd., Ibarakiken, Japan), cholesterol (Chol) (Sigma Chemical Co., St. Louis, Missouri), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethylene glycol-2000] (PEG-DSPE), egg L-α-phosphatidylcholine (EPC), egg L-α-phosphatidylglycerol (EPG) or distearoyl phosphatidylcholine (DSPC) (Avanti Polar Lipids, Alabaster, Alabama) were dissolved in chloroform:methanol in a round bottom flask. The solvent was

evaporated under reduced pressure in a rotary evaporator and the lipid mixture was dried under nitrogen for 15 min, redissolved in 2-methyl-2-propanol (Sigma Chemical Co., St. Louis, Missouri) and freeze-dried overnight. The dried lipids were hydrated for 2h in Hepes/NaCl buffer, pH 7.4 (10 mM N-[2-hydroxy ethyl] piperazine-N'-ethane sulfonic acid (Hepes) (Sigma Chemical Co., St. Louis, Missouri) and 135 mM NaCl (Merck, Darmstadt, Germany) containing 5 mM of the chelator deferoxamine mesylate (Desferal®) (Novartis, Basel, Switzerland). The chelator was added to enable labeling with ^{67}Ga (see section 2.3).
Liposomes of approximately 100 nm (range 80-120 nm) were obtained by sonication of the hydrated lipid dispersion for 8 min with an amplitude of 8 μ using a 9.5 mm probe in an MSE Soniprep 150 (Sanyo Gallenkamp PLC, Leicester, UK). To study the effect of particle size, liposome populations of three different mean sizes (i.e. 100, 280, 360 nm) were obtained by multiple extrusion of the hydrated lipid dispersion through two stacked polycarbonate membranes (Nuclepore, Pleasanton, California) with pore sizes of 100 and 100 nm for the 100 nm liposomes, 400 and 200 nm for the 280 nm liposomes, and 600 and 400 nm for the 360 nm liposomes.
Particle size distribution was measured using dynamic light scattering, detected at an angle of 90° to the laser beam on a Malvern 4700 System (Malvern Instruments Ltd., Malvern, UK). The polydispersity of the liposome population is reported by the system as a value between 0 and 1. A reported value of 1 indicates large variations in particle size, whereas a value of 0 indicates a complete monodisperse system. For all liposome preparations used in the experiments, the polydispersity index value was below 0.3.
The ζ-potential of 0, 5, or 30 mol% EPG containing liposomes was measured using a zetasizer equipped with PCS v1.35 software (Malvern Instruments Ltd., Malvern, UK). Liposomes were prepared in 5% aqueous Hepes/NaCl buffer and the instrument was calibrated with electrophoresis standard latex particles.

2.3 Liposome labeling
To remove non-encapsulated deferoxamine, liposomes were applied on a Sephadex G-50 column (Pharmacia, Uppsalla, Sweden) and eluted with Hepes/NaCl buffer followed by concentration via ultracentrifugation at 60,000 rpm for 2h at 4 °C in a Beckman ultracentrifuge L-70 (Beckman, Palo Alto, California). Liposomes were labeled with ^{67}Ga according to Gabizon et al. [19]. ^{67}Ga-citrate (1 mCi/ml) (Mallinckrodt Medical b.v., Petten, The Netherlands) was diluted 1:10 in aqueous 5 mg/ml 8-hydroxyquinone (Sigma Chemical Co., St. Louis, Missouri) and incubated for 1 h at 52 °C to yield ^{67}Ga-oxine. 1 ml of this mixture was added per 1000 μmol total lipid (TL) of liposomes. Since ^{67}Ga-oxine can pass the liposomal membrane and has a high affinity for the encapsulated chelator deferoxamine, the radioactive label becomes entrapped. ^{67}Ga-deferoxamine is an appropriate label for monitoring intact liposomes in the circulation as it is encapsulated in the liposome interior and it is excreted rapidly via the kidneys in case it leaks from circulating liposomes [19]. Free label was removed by gel filtration. Radiolabeled liposomes were concentrated by ultracentrifugation at 60,000 rpm for 2 h at 4 °C in a Beckman ultracentrifuge L-70 (Beckman, Palo Alto, California). Resulting specific activities were between $4{\cdot}10^4$ and $2{\cdot}10^5$ cpm/μmol TL. Phosphate concentration was determined colorimetrically according to Bartlett [20].

2.4 Blood volume and organ blood content
Total blood volume of the infected rats was 5.3% of the body weight. This percentage was determined by labeling syngeneic erythrocytes with ^{111}In-oxine according to Kurantsin-Mills [21]. Briefly, in an independent experiment, blood samples were taken at 10 min after injection of the labeled erythrocytes assuming that all erythrocytes were still present in the circulation. The dilution factor of the radioactive

^{111}In-label allowed calculation of the total blood volume as well as the blood content of the various organs.

2.5 Blood circulation kinetics and biodistribution
Experimental groups consisted of 6 rats. Liposomes were administered i.v. at the selected dose (indicated in the text) via the tail vein at 40 h after bacterial inoculation of the left lung. Blood samples of approximately 0.3 ml were taken from alternating groups of three rats by retro orbital bleeding using heparinized capillaries at 1, 4, 8, 12, and 16 h after liposome injection. All rats were bled at 24 h after injection. Blood sample volume was measured and radioactivity was counted in a Minaxi autogamma 5000 gamma counter (Packard Instrument Company, Meriden, Connecticut) allowing calculation of the radioactivity present in the blood (see section 2.5.).
At 24 h after injection, rats were sacrificed by CO_2 inhalation and infected left lung, right lung, spleen, kidneys, liver and heart were dissected. The organs were weighed and radioactivity was counted to assess the biodistribution of the liposomes. The contribution of radioactivity in the blood to the radioactivity measured in the organs was subtracted (see section 2.5.).

2.6 Statistical analysis
Pharmacokinetic studies have shown that SSL exhibit single first-order clearance rates independent of dose [9, 22]. Similarly, in our experiments the circulation kinetics of individual animals between 1 and 24 h after injection could be well described by a linear relationship on a semi-logarithmic plot ($0.88 < r < 1.00$).
Data were tested for homogeneity of variance using Levene's test. In case of significant differences in variance between groups, data were log transformed. Paired t-test was used to compare infected left lung and right lung localization. Comparisons between groups were made by one-way analysis of variance (ANOVA) corrected for multiple comparisons by the Bonferroni method using SPSS for Windows software release 7.5.2 (Statistical Product & Service Solutions Inc., Chicago, USA).

3. Results

At 40 h after bacterial inoculation of the left lung, at the time of liposome injection, 3 zones could be clearly distinguished in the lobar pneumonia area in the left lung: the early infected, hemorrhagic and consolidated zone. Furthermore, limited variation was noted in severity of infection between animals. At 40 h after bacterial inoculation, the inoculum of log 6 bacteria had multiplied to log 9.77 ± 0.24, whereas the original lung weight of 0.36 ±0.07 g had increased to 1.07 ±0.19 g (mean ± standard deviation, n=9).

3.1 Effect of PEG-density

The circulation kinetics and the biodistribution pattern of liposomes containing three different levels of PEG-DSPE with an approximate molecular weight of PEG of 2,000 (i.e. 1, 5, or 10 mol%) are shown in Figures 1A and 1B, respectively.

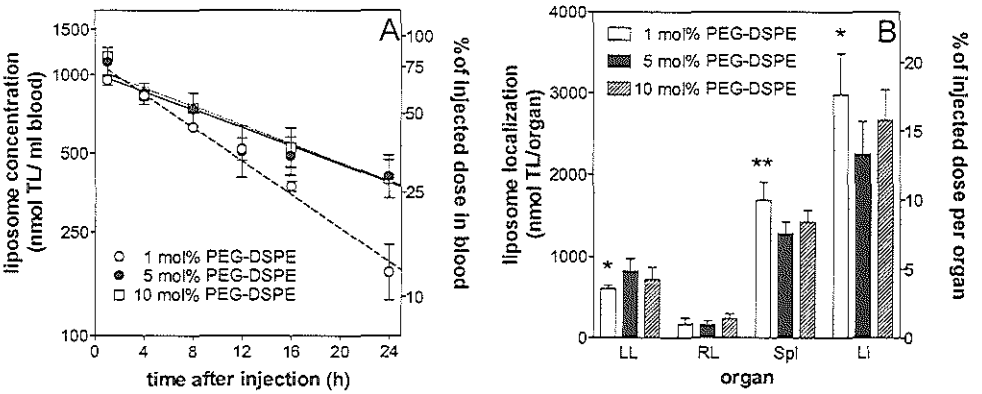

Figure 1. Effect of PEG-density on blood clearance and biodistribution of liposomes. ^{67}Ga-deferoxamine labeled liposomes were injected i.v. at 40 h after bacterial inoculation of the left lung with 10^6 *K. pneumoniae* at a dose of 75 µmol TL/kg body weight. The lipid composition was PHEPC:Chol:PEG-DSPE 1.85:1:0.15 mol:mol for the 5 mol% PEG-DSPE containing liposome preparation (particle size 104 nm, range 91-115 nm, n=8), 1.97:1:0.03 mol:mol for the 1 mol% PEG-DSPE preparation (particle size 107 nm, range 98-116 nm, n=2), and 1.7:1:0.3 mol:mol for the 10 mol% PEG-DSPE preparation (particle size 98 nm, range 96-100 nm, n=2).

A. Liposome concentration in nmol TL/ ml blood (left Y-axis) and % of injected dose remaining in the circulation (right Y-axis) at indicated time points after injection. Data were calculated from radioactivity recovered (n=3, mean±standard deviation).

B. Biodistribution of liposomes expressed as nmol TL/organ (left Y-axis) and % of injected dose/organ (right Y-axis) at 24 h after injection. Data were calculated from radioactivity recovered (n=6, mean±standard deviation). LL=infected left lung, RL=right lung, Spl=spleen, Li=liver. *=p<0.05, **=p<0.01, ***=p<0.001. Significant differences are noted against the 5 mol% PEG-DSPE containing preparation.

The 1 mol% PEG-liposomes disappeared from the bloodstream faster as compared to the 5 and 10 mol% PEG-liposomes ($p<0.01$) (Fig. 1A). An increase of the PEG content from 5 up to 10 mol% did not affect the circulation kinetics. The biodistribution data show that, for all three PEG-liposome preparations, the localization in the infected left lung (LL) at 24 h after injection was approximately 3 to 4-fold higher than the corresponding localization in the uninfected right lung (RL) ($p<0.001$) (Fig. 1B). Yet, the degree of localization in the infected left lung of the 1 mol% PEG-liposomes was somewhat lower (± 20%) compared to the 5 mol% PEG-liposomes ($p<0.05$), while the extent of localization of the 5 mol% and 10 mol%

PEG-liposomes in the infected lung was similar. The hepatosplenic uptake of the 1 mol% PEG-liposomes was significantly higher than the 5 mol% and 10 mol% PEG-liposomes ($p<0.05$ and $p<0.005$, respectively). The latter two liposome types displayed a similar degree of localization in the liver and spleen. Localization in the uninfected right lung was not significantly different for the three different liposome preparations. Very low levels of liposome localization were seen in heart (< 40 nmol TL) and kidneys (<200 nmol TL) (data not shown).

3.2 Effect of particle size

The circulation kinetics and the biodistribution pattern of liposomes differing in mean size (i.e. 100, 280, and 360 nm) are shown in Figures 2A and 2B, respectively.

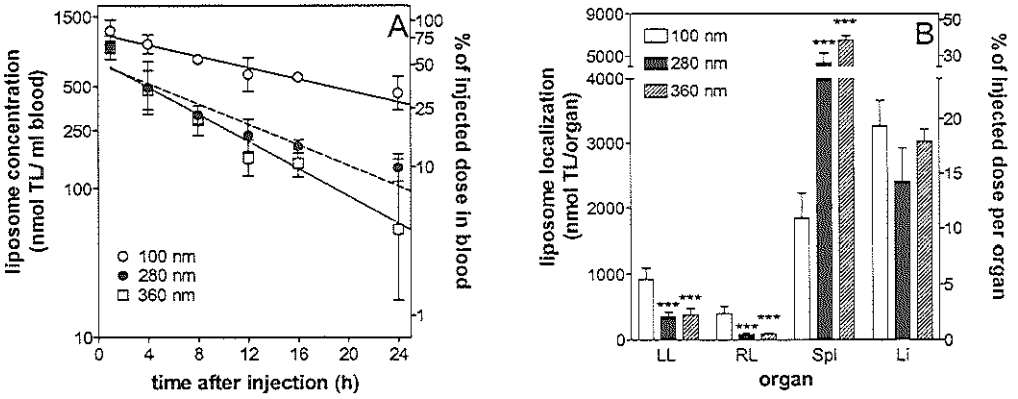

Figure 2. Effect of particle size on blood clearance and biodistribution of liposomes. See figure 1 for details. The lipid composition of the liposomes was PHEPC:Chol:PEG-DSPE 1.85:1:0.15 mol:mol. 100 nm formulation particle size 104 nm, range 91-115 nm, n=8; 280 nm formulation particle size: 277 nm, range 268-286 nm, n=2; 360 nm formulation particle size: 359, range 331-387 nm, n=2. Significant differences against the 100 nm sized liposome preparation are noted.

PEG-liposomes with a mean size of 100 nm were removed from the bloodstream at a much lower rate as compared to the 280 nm and 360 nm liposome formulations ($p<0.01$ and $p<0.05$, respectively) (Fig. 2A). Regarding the biodistribution pattern, it was observed that the localization in the uninfected right lung and the infected left lung was approximately 2-fold lower in case of the 280 nm and 360 nm liposome preparations compared to the 100 nm PEG-liposomes ($p<0.001$) (Fig. 2B). Importantly, for each liposome formulation the localization in the infected left lung was approximately 4-fold higher than in the uninfected right lung ($p<0.001$). Splenic uptake appeared to increase considerably with increasing size of the PEG-liposomes ($p<0.001$). The distribution to liver, heart (data not shown), and kidneys (data not shown) was not significantly different for any of the liposome preparations in this experiment.

3.3 Effect of bilayer fluidity

Bilayer fluidity is determined by the lipid composition of the liposome. Hydrogenated phospholipids yield rigid bilayers whereas more unsaturated phospholipids give bilayers a more fluid character [23]. Besides, cholesterol has also been shown to be an important modulator of bilayer fluidity by increasing bilayer rigidity and lateral packing [23]. The liposome preparations evaluated here vary, regarding bilayer fluidity, as follows (in order of increasing fluidity): DSPC:PEG-DSPE, PHEPC:PEG-DSPE, EPC:PEG-DSPE. One liposome preparation contains additionally cholesterol (i.e. PHEPC:Chol:PEG-DSPE) to examine the effect of inclusion of the steroid. The circulation kinetics and the biodistribution pattern of liposomes differing in bilayer fluidity are shown in Figures 3A and 3B, respectively.

The circulation times of all four liposome preparations evaluated were similar (Fig. 3A). Regarding the biodistribution profile, all four liposome formulations showed an approximately 4-fold higher localization in the infected left lung compared to the uninfected right lung ($p<0.001$) (Fig. 3B). Tissue distribution patterns of the various liposome formulations were not significantly different.

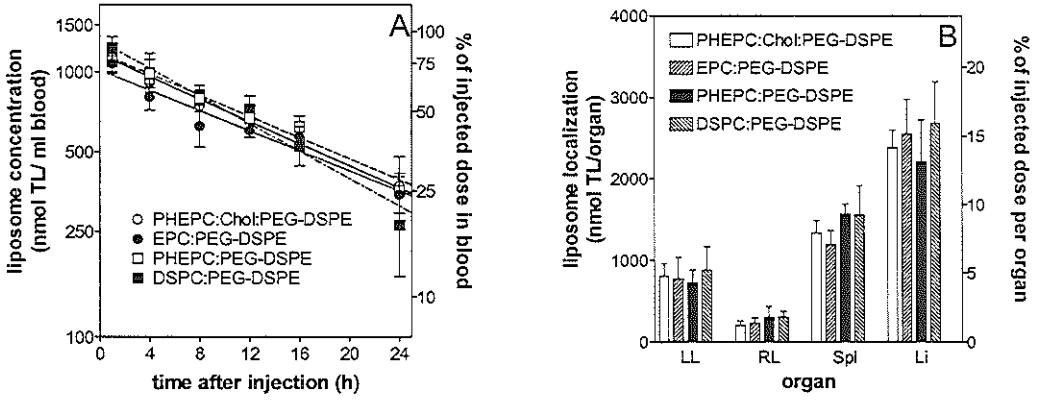

Figure 3. Effect of bilayer fluidity on blood clearance and biodistribution of liposomes. See figure 1 for details. The lipid composition of the PHEPC:Chol:PEG-DSPE liposome was 1.85:1:0.15 mol:mol (particle size 104 nm, range 91-115 nm, n=8), of the DSPC:PEG-DSPE 1.9:0.1 mol:mol (particle size 114 nm, range 95-133 nm, n=2), of the PHEPC:PEG-DSPE 1.9:0.1 mol:mol (particle size 121 nm, range 109-133 nm, n=2), and of the EPC:PEG-DSPE preparation 1.9:0.1 mol:mol (particle size 84 nm, range 76-92 nm, n=2).

3.4 Effect of charged phospholipids

The circulation kinetics and the biodistribution pattern of PEG-liposomes differing in EPG content (i.e. 0, 5, and 30 mol%) were determined to study the effect of charged phospholipids (Figures 4A and 4B).

The ζ-potentials of the 0, 5, and 30 mol% EPG containing liposomes were measured. ζ-potential decreased slightly with increasing EPG content, under the experimental conditions used, from -10.7 ± 1.1 mV for the 0 mol% EPG containing liposomes via −13.4 ±1.4 mV for the 5 mol% EPG preparation to −19.7 ±1.1 mV for the 30 mol% EPG containing liposomes (mean ± standard deviation, n=3 preparations).

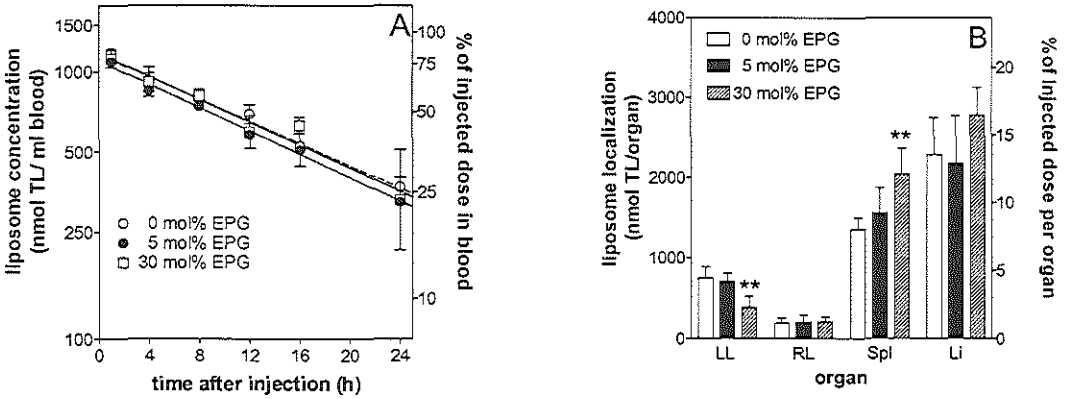

Figure 4. Effect of EPG-level on blood clearance and biodistribution of liposomes. See figure 1 for details. The lipid composition was PHEPC:Chol:PEG-DSPE:EPG 1.85:1:0.15:0 mol:mol for the 0 mol% EPG containing liposome preparation (particle size 104 nm, range 91-115 nm, n=8), 1.7:1:0.15:0.15 mol:mol for the 5 mol% EPG preparation (particle size 93 nm, range 87-99 nm, n=2), and 0.95:1:0.15:0.9 mol:mol for the 30 mol% EPG preparation (particle size 82 nm, range 78-86 nm, n=2). Significant differences against the 0 mol% EPG containing liposome preparation are noted.

Inclusion of the negatively charged phospholipid did not appear to influence the circulation characteristics of the PEG-liposomes (Fig. 4A). The degree of localization in the infected lung was not affected by the inclusion of 5 mol% EPG. Remarkably, inclusion of 30 mol% EPG resulted in an approximately 2-fold reduced target localization compared to the PEG-liposomes containing 0 mol% and 5 mol% EPG ($p<0.01$) (Fig. 4B). But, for all three types of PEG-liposomes differing in EPG-content the degree of localization in the infected left lung was higher than in the uninfected right lung ($p<0.01$). The 30 mol% EPG containing preparation showed a significantly increased splenic localization ($p<0.01$) compared to the 0 mol% and 5 mol% EPG containing PEG-liposomes. Distribution of the three PEG-liposome types to liver, kidneys and heart was not significantly different (heart and kidney data not shown). Total accumulation within the infected left lung, right lung, liver and spleen was not

significantly different between the liposome formulations differing in EPG-content. (4572 ± 623, 4655 ± 855, 5406 ± 618 nmol TL/tissues for the 0 mol%, 5 mol%, and 30 mol% EPG containing liposomes, respectively. Mean ± standard deviation, n=6).

4. Discussion

SSL, obtained by coating the liposome surface with PEG via inclusion of PEG-DSPE into the liposomes, display a considerably increased circulation time compared to conventional liposomes lacking the PEG coating [9, 10, 22, 24]. This long-circulating property has been exploited to reach infectious sites localized outside the major MPS organs, the liver and spleen [14, 15, 16, 17]. SSL-encapsulated gentamicin showed a superior therapeutic effect compared to the free drug in our rat model of acute unilateral pneumonia in the left lung caused by *Klebsiella pneumoniae* [13]. SSL are, therefore, attractive candidates for site-selective drug delivery of antimicrobial agents. In order to be able to rationally optimize liposomal preparations for targeted drug delivery to non-MPS infectious foci, it is important to gain insight into the liposome characteristics potentially affecting the process of liposome localization at the infected site. In this study the effects of PEG density, liposomal size, bilayer fluidity and charge on circulation kinetics and biodistribution of liposomes were evaluated in our rat pneumonia model.

Liposomes containing 1 mol% PEG displayed inferior localization at the infected site and a shorter circulation time compared to liposomes containing 5 or 10 mol% PEG. It has been shown previously in healthy animals that inclusion of 5-7.5 mol% PEG-DSPE is optimal for achieving maximal prolongation of the circulation time, in case of PEG with an approximate molecular weight of 2,000 [24]. This finding is consistent with the present observations in a rat model of bacterial infection, showing lack of effect of increasing PEG density from 5 to 10 mol% on blood circulation time and tissue disposition (Fig. 1A and 1B). Inclusion of 1 mol% PEG-DSPE is, apparently, less efficient in prolonging the circulation time as compared to inclusion of 5 or 10 mol% PEG-DSPE. The lower localization in the infected left lung of the 1 mol% PEG-DSPE SSL is most likely related to the faster removal from the bloodstream resulting in a reduced possibility to interact with the target site. However, it can not be

totally excluded that PEG has a direct effect as well. It has been shown in tumor bearing mice that PEG-coated liposomes have an intrinsically higher tumor vascular permeability coefficient than conventional liposomes [25]. In this view, the presence of 1 mol% PEG-DSPE may have resulted in a suboptimal PEG-density to facilitate extravasation. Future experiments will focus on the relative importance of circulation time versus the presence of a PEG-coating.

A positive correlation between circulation times and infected lung localization is also apparent from the experiments on the effect of particle size on circulation kinetics and biodistribution of SSL. SSL with mean particle sizes of 280 nm or 360 nm show an approximately two-fold lower target localization compared to the 100 nm PEG-liposomes. Again, this may be related to differences in circulation times as 100 nm SSL are cleared more slowly from the bloodstream compared to the SSL with larger mean sizes. On the other hand, the reduced localization at the site of infection of the two liposome types with the larger mean size may also be a direct consequence of the particle size of the liposomes, which may have been too large to achieve equally efficient extravasation compared to the 100 nm PEG-liposome preparation. Boerman and co-workers studied the effect of particle size of SSL on liposome localization in a focal infection in the calf muscle of rats. The studied particle sizes were between 90 and 220 nm. Their study showed that particle sizes, within this range, do not affect the degree of target localization [26]. On the basis of the present results, we speculate that a further increase of the particle size to sizes well above 220 nm is required to observe a significant reduction in localization at the site of infection. The higher splenic uptake of the larger PEG-coated liposomes, noticed in this study, has also been observed by Litzinger and colleagues for SSL of approximately 300 nm, and was suggested to be due to a filtration effect within the splenic collagen fiber meshwork [27]. Although the infected lung localization of the two larger SSL is relatively low, it can nonetheless be argued that these liposomes are still attractive for the delivery of antimicrobials. The present results demonstrate that SSL with a mean particle size up to 360 nm are still able to localize preferentially in infected tissue, though less efficient in this regard than the 100 nm SSL. If the liposome-associated drug load is increased sufficiently

for the larger liposomes, it can be envisaged that the net drug concentration at the target site may even be increased, when larger SSL with suboptimal localization characteristics are used.

Since bilayer fluidity can be an important determinant of the release of liposome-encapsulated compounds, this liposome characteristic should also be considered regarding its effect on SSL localization at the site of infection. Generally, it is reported that release of encapsulated compounds increases with increasing fluidity of the liposome bilayer, which has also been shown to affect therapeutic efficacy [28-32]. Minimal variations were noticed in circulation kinetics and tissue distribution of the liposome formulations differing in bilayer fluidity. This finding is in agreement with previous results reported by Woodle et al. [10, 33]. Apparently, bilayer fluidity of SSL may be chosen to optimize drug retention in the blood stream and release profile at the site of infection without compromising target localization.

The only factor that appeared to adversely affect target site localization without compromising circulation kinetics was incorporation of 30 mol% EPG into the SSL formulation. Charged phospholipids are often added to a liposomal preparation to improve drug loading and/or stability of the formulation against aggregation during storage. The PEG-coating has a distinct effect on liposomal surface charge. The PEG-coating moves the hydrodynamic plane of shear from the charged surface of the liposome to the edge of the PEG coating. The ζ-potential measurements as well as Gouy-Chapman calculations on the 0 mol%, 5 mol%, and 30 mol% EPG containing PEG-liposomes suggested that in physiological ionic strength the ζ-potential of the 30 mol% EPG SSL would be negligible at 2-3 nm from the surface, as the Debye length in this milieu is only 0.8 nm. Estimates for the thickness of the PEG-coating, for PEG with a molecular weight of 2,000, range from 3 to 5 nm [34, 35]. Therefore inclusion of EPG was not expected to have an effect on the circulation kinetics or biodistribution of SSL. However, the biodistribution profile of the 30 mol% EPG-containing preparation shows an approximately 2-fold lower target localization compared to the 0 mol% and 5 mol% EPG-containing liposomes, but similar circulation kinetics as the 0 mol% and 5 mol% EPG-containing liposomes. It is speculated that the inclusion of 30 mol% EPG

may have conferred a small but detectable negative ζ-potential at the edge of the PEG-coating, allowing interaction with biological components.

The results of the present study are to a certain degree in accordance with studies on pharmacokinetics and target localization of SSL in experimental tumor models. With respect to tumor localization of SSL, it is generally accepted that prolongation of liposomal circulation time is beneficial for target localization [5, 8, 9, 10, 25, 27, 36]. The results regarding the effect of PEG-density, particle size and bilayer fluidity in the present study as well as previous results point in the same direction [12]. It has been demonstrated that tumor localization of SSL is a result of the increased microvascular permeability in malignant tissue [9, 25]. As increased capillary permeability is also one of the hall marks of inflamed tissue, we suggest that selective SSL localization in infected tissue is a result of a similar effect of microvascular permeability changes.

In conclusion, SSL offer interesting possibilities for delivery of antibiotics to sites of bacterial infection localized outside the major MPS-organs. It is shown here that the degree of localization at the infected site is remarkably independent of the physicochemical characteristics of the PEG-liposomes. Substantial selective liposome localization can be achieved provided that certain criteria regarding PEG-density, size, and inclusion of charged phospholipids are met. In view of the differences in pharmacodynamics of the different classes of antibiotics, the rate and extent of release of encapsulated antibiotic from the PEG-liposome extravasated into the infected site is an important issue. The present findings indicate that manipulation of the release profile by variation of the lipid composition may be possible without compromising the prolonged circulation and target localization properties. These properties seem to be a direct consequence of the presence of the polymer coating operating as a repulsive steric barrier opposing interactions with biological components.

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3. Host factors influencing the preferential localization of sterically stabilized liposomes in *Klebsiella pneumoniae*-infected rat lung tissue

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ABSTRACT

Purpose. To gain insight into the host factors influencing liposome localization at sites of bacterial infection. **Methods.** In a unilateral *Klebsiella pneumoniae* pneumonia rat model, capillary permeability and number of circulating leukocytes was quantified and related to the degree of liposome target localization. **Results.** Liposome localization was highest in the hemorrhagic zone of infection, a zone characterized by markedly increased capillary permeability and high bacterial numbers. Both liposome localization and capillary permeability correlated positively with severity of infection. Lung instillation of other inflammatory stimuli, such as lipopolysaccharide or 0.1 M HCl inducing increased capillary permeability, also promoted liposome localization. As liposomal target localization in leukopenic rats was similar to that in immunocompetent rats, contribution of circulating leukocytes seems limited. Intrapulmonary distribution of liposomes shows that leukocytes at the target site are involved in liposome uptake after extravasation. **Conclusions.** Increased capillary permeability plays a crucial role in liposome localization at the infected site, whereas contribution of leukocytes is limited. These results suggest the inflammatory conditions that could benefit from liposomal drug delivery. The involvement of leukocytes in liposome uptake at the target site could be important information in the selection of appropriate drugs.

1. Introduction

Targeted delivery of antibiotics may improve antibacterial therapy by increasing the concentration of the drug at the site of infection. Liposomes have been investigated as targeted drug carriers for this purpose. The avid uptake of conventional liposomes after intravenous administration, by the cells of the mononuclear phagocyte system (MPS), has been employed to deliver substantial amounts of antibiotics selectively to the cells of this system (1-2). However, the prompt uptake by MPS-cells strongly limits the chance of these conventional liposomes to interact with sites of infections located outside the major MPS-organs.

The development of liposomes coated with poly(ethylene) glycol (PEG), also known as sterically stabilized liposomes (SSL), potentially permits liposomal drug delivery to tissues beyond the MPS-cells (3-6). SSL display a reduced affinity for the MPS, compared to liposomes lacking the PEG-coating. It is believed that the PEG-coating reduces the interaction of opsonins with the SSL surface, thereby reducing MPS recognition and uptake. As a result SSL display a prolonged circulation time. In experimental models representing a variety of inflammatory and infectious foci outside the MPS, substantial SSL localization at the site of infection has been demonstrated. The presence of an infectious process is essential as SSL localization in uninfected controls is limited (7-13). Previous reports have suggested that presence of the long circulation property is also essential to achieve significant localization at the target site (5-6, 14). In addition, SSL-encapsulated gentamicin showed improved efficacy compared to the free drug in a rat model of *Klebsiella pneumoniae* pneumonia (15). These data warrant further investigations on SSL as carriers of antibiotics to sites of infection beyond the cells of the MPS.

At present, limited information is available on the host factors that are responsible for the selective SSL localization at inflammatory sites, compared to non-infected control organs. It has been suggested that the increased capillary permeability at inflammatory foci allows SSL to extravasate (14). Besides, the infiltration of inflammatory cells may actively or passively promote SSL localization, either by taking up liposomes in the circulation and subsequent

infiltration into the inflamed tissue, or by facilitating liposome extravasation as a result of leukocyte-mediated tissue injury (16).

The present study was designed to gain more insight into the mechanism of localization of SSL at sites of infection. In a rat model of a unilateral pneumonia caused by *Klebsiella pneumoniae* the requirement of increased capillary permeability for achieving SSL localization was investigated by correlating the extent of capillary permeability to the degree of SSL localization. The correlation was also studied after lung-instillation of other inflammatory stimuli (lipopolysaccharide (LPS) and 0.1 M HCl). The involvement of leukocytes in SSL extravasation was investigated by leukocyte depletion. Microscopic visualization of colloidal-gold labeled SSL was performed to obtain information on the intrapulmonary localization of SSL after extravasation in the infected tissue.

2. Materials and Methods

2.1 Animals

RP/AEur/RijHsd strain albino rats, with a specified pathogen-free status (18-25 weeks of age, weighing 185-225 g) (Harlan, Horst, The Netherlands), were used in the experiments. Rats were housed individually with free access to sterilized water and SRMA chow (Hope Farms, Woerden, The Netherlands)

2.2 Bacterial inoculation of the left lung

Bacteria were inoculated in the left lung as described previously (17). Briefly, rats were anaesthetized by an intramuscular injection of fluanisone and fentanyl citrate (Janssen Animal Health, Saunderton, UK) followed by an intraperitoneal injection of pentobarbital (Sanofi Santé b.v., Maassluis, The Netherlands). The left primary bronchus was subsequently intubated and 0.02 ml of a saline suspension containing the indicated number of *K. pneumoniae* (ATCC 43816, capsular serotype 2) was inoculated in the lower left lung lobe. Following the inoculation, rats received an intramuscular injection of nalorphine bromide as an anesthetic antagonist (Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands)

When indicated, leukopenia was effectuated by intraperitoneal injection of 60 mg/kg cyclophosphamide (Sigma, St. Louis, MO) every 4 days starting at 5 days before bacterial inoculation, according to Leenders et al. (18). Leukopenia was ascertained by measuring leukocyte counts in fresh blood samples, obtained by retro-orbital bleeding just before SSL injection. Leukocytes were counted on a Cobas Minos Stex (Roche Haematology, Montpellier, France) using Minotrol™ 16 standards (Roche Haematology, Montpellier, France) to verify proper functioning of the instrument.

2.3 Instillation of other inflammatory stimuli in the left lung

Rats were anesthetized and intubated as described above. 0.02 ml of a saline solution containing 2 mg LPS (from *Escherichia coli*, serotype 0111:B4 (Sigma, St. Louis, MO) or 0.1 ml aqueous 0.1 M HCl (Fluka, Buchs, Switzerland) were instilled in the lower left lung lobe. Nalorphine bromide was injected as an anesthetic antagonist.

2.4 SSL preparation

SSL were prepared as described previously (13, 15). In brief, appropriate amounts of partially hydrogenated egg phosphatidylcholine (PHEPC) (Asahi Chemical Industry Co. Ltd., Ibarakiken, Japan), cholesterol (Chol) (Sigma Chemical Co., St. Louis, MO), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethylene glycol-2000] (PEG-DSPE) (Avanti polar lipids, Alabaster, Alabama) in a molar ratio of 1.85: 1.00:0.15, respectively, were dissolved in chloroform:methanol in a round bottom flask, followed by evaporation of the solvent in a rotary evaporator. The lipid mixture was dried under nitrogen for 15 min, dissolved in 2-methyl-2-propanol (Sigma Chemical Co., St. Louis, MO) and freeze-dried overnight. The resulting lipid film was hydrated for 2 h in Hepes/NaCl buffer, pH 7.4 (10 mM N-[2-hydroxy ethyl] piperazine-N'-ethane sulfonic acid (Hepes) (Sigma Chemical Co., St. Louis, MO) and 135 mM NaCl (Merck, Darmstadt, Germany) containing 5 mM of the chelator deferoxamine mesylate (Desferal®) (Novartis, Basel, Switzerland). SSL were sized by subjecting the hydrated lipid dispersion to a sonication procedure for 8 min with an amplitude of 8 μ using a 9.5 mm probe in an MSE Soniprep 150 (Sanyo Gallenkamp PLC, Leicester, UK).

2.5 Radiolabeling of liposomes

Non-encapsulated deferoxamine was removed by gel filtration of the SSL over a Sephadex G-50 column (Pharmacia, Uppsalla, Sweden) using Hepes/NaCl buffer as an eluens. The SSL were subsequently concentrated via ultracentrifugation at 365,000 x g for 2 h at 4 °C in a Beckman ultracentrifuge L-70 (Beckman, Palo Alto, CA). SSL were labeled with ^{67}Ga according to Gabizon et al. (19). ^{67}Ga-citrate (1 mCi/ml) (Mallinckrodt Medical, Petten, The Netherlands), diluted 1:10 in aqueous 5 mg/ml 8-hydroxyquinone (Sigma Chemical Co., St. Louis, MO), was incubated for 1 h at 52 °C to obtain ^{67}Ga-oxine. 1 ml of this mixture was added per 1000 μmol total lipid (TL) of SSL. ^{67}Ga-oxine can pass the liposomal membrane and has a high affinity for the encapsulated chelator deferoxamine, with the result that the radioactive label becomes entrapped. ^{67}Ga-deferoxamine is a suitable label for studying intact liposomes in the circulation as it is excreted rapidly via the kidneys in case it leaks from the liposomes (19). Unencapsulated ^{67}Ga was removed by gel filtration and radiolabeled SSL were concentrated by ultracentrifugation. Resulting specific activity was approximately 10^5 cpm/μmol TL.

2.6 SSL characterization

Particle size distribution of the SSL was measured using dynamic light scattering, detected at an angle of 90° to the laser beam on a Malvern700 System (Malvern Instruments Ltd., Malvern, UK). The polydispersity of the liposome population is reported by the system as a value between 0 and 1. A reported value of 1 indicates large variations in particle sizes, whereas a value of 0 indicates a complete monodisperse system. For all SSL preparations used in the experiments, the mean liposomal size was approximately 100 nm and the polydispersity was below 0.3. Phosphate concentration was determined colorimetrically according to Bartlett (20).

2.7 Bacterial counts

At indicated time points after bacterial inoculation, rats were sacrificed by CO_2 inhalation, since this form of euthanasia does not affect bacterial counts (unpublished observations). The right and left lung were excised and kept on ice and the left lung was divided into the three distinct zones of the lobar pneumonia: the early infected, hemorrhagic, and consolidated zone. Lung (zones) were homogenized in 20 ml of phosphate buffered saline (4°C), appropriate dilutions were cultured (overnight, 37 °C) on tryptone soy agar plates (Oxoid, Basingstoke, UK) and bacterial colonies were counted.

2.8 Evan's blue dye extravasation
Evan's blue dye extravasation is a measure for the capillary permeability (21-22) and was determined as described previously by Zhang et al. (21). A saline suspension of Evan's blue dye (40 mg/kg) (Merck, Darmstadt, Germany) was injected in the tail vein at indicated time points after inoculation of the left lung. Rats were sacrificed 24 h later. A blood sample was taken via retro-orbital puncture, the lungs were excised and, when indicated, the left lung was divided into the three distinct zones of the lobar pneumonia. Blood sample volume was measured and the right lung and the three zones of the left lung were weighed and put in 7 ml of formamide (Sigma, St. Louis, MO) to extract the dye. Absorbance was measured on an LKB Ultrospec Plus spectrometer (Pharmacia, Uppsalla, Sweden) at a wavelength of 623 nm. Standards (0-30 µg Evan's blue/ml) were also dissolved in formamide. Corrections were made for the amount of Evan's blue present in the blood (see SSL localization).

2.9 Lung wet-to-dry weight ratio
Another measure of capillary permeability is the wet-to-dry weight ratio (23). Animals were sacrificed at 64 h after bacterial inoculation, the lungs were excised and left and right lung weight was determined. Lungs were subsequently dried in a stove for 3 days at 70 °C, the dry weight of the lungs was determined and the wet-to-dry weight ratio was calculated.

2.10 SSL localization
SSL (75 µmol TL/kg) were administered via the tail vein 24 h before the rats were sacrificed by an i.v. overdose of pentobarbital, at indicated time points after inoculation of the left lung. A blood sample was taken via retro-orbital puncture, lungs were excised and, when indicated, the left lung was divided into the three distinct zones of a lobar pneumonia. The right lung and the three zones of the left lung were weighed and radioactivity was counted in a Minaxi autogamma 5000 gamma counter (Packard Instrument Company, Meriden, CT) to assess the degree of localization of the SSL. Blood content of the tissues was determined in independent experiments with ^{111}In-oxine labeled syngeneic erythrocytes, as described by Kurantsin-Mills et al. (24). Labeled erythrocytes were injected 10 min before the animals were sacrificed. Assuming that all erythrocytes are still present in the circulation, the dilution factor of the label allows determination of total blood volume and the blood content of the tissues of interest. Blood content values were used to correct for the contribution of labeled SSL in the circulation to total tissue radioactivity.

2.11 Blood cell-associated SSL
To determine the amount of cell-associated SSL in the circulation, blood samples were obtained from infected animals via retro-orbital bleeding in heparanized tubes at indicated time points after injection of radioactively labeled SSL. Blood cells (0.5 ml sample) were washed three times in 50 ml PBS (4 °C) by centrifugation at 1500 x g for 10 min (Hettich Rotanta, Germany). Radioactivity of pellet and supernatants was counted as described above.

2.12 Colloidal gold-labeling of SSL
Colloidal gold-labeled SSL were prepared as described by Daemen et al. (25). Briefly, the lipid film was prepared as described above. A 1.1% (w/v) aqueous solution of $AuCl_2$ (Sigma, St. Louis, MO) was four-fold diluted with sodium citrate (28 mM)/ potassium carbonate (7 mM) buffer, filtered (0.2 µm), and used to hydrate the lipid film at 4 °C. SSL were prepared by multiple extrusion of the hydrated lipids through two stacked 100 nm membranes (Nuclepore, Pleasanton, CA). The resulting yellow SSL suspension was placed at 37 °C, after which the color of the suspension turned purple. Unencapsulated colloidal gold was removed by gel filtration of the SSL suspension over a Sephacryl SF S1000 column (Pharmacia, Uppsalla, Sweden) using Hepes/NaCl buffer as the eluens.

2.13 Localization of colloidal-gold labeled SSL
Colloidal-gold labeled SSL (approx. 5 µmol TL/kg) were injected at 40 h after bacterial inoculation of the left lung. 24h after injection both right and left lung were excised, washed in three changes of 2.5 % buffered formaldehyde (Merck, Darmstadt, Germany), fixed in 10% buffered formaldehyde and embedded in paraffin. 5 µm sections were cut on a microtome and mounted on slides. Sections were deparaffinized in two changes of xylene and hydrated. Next, colloidal gold was silver-enhanced using a silver-enhancement kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Eosin/haematoxylin was applied as a counterstain, when indicated.
For the immunohistochemical staining procedure, slides were deparaffinized and hydrated as described above. Endogenous peroxidase activity was blocked by incubation of slides for 10 min in 3% H_2O_2 (Merck, Darmstadt, Germany) in methanol (Fluka, Buchs, Switzerland) followed by rinsing with PBS. Slides were incubated with 5% goat serum for 30 min. Then 0.4 µg/ml of mouse anti-rat CD43 (Pharmingen, San Diego, CA) was applied and slides were incubated overnight at 4 °C in a humid container. Slides were rinsed in 3 changes of PBS and the secondary polyclonal horseradish peroxidase conjugated goat anti-mouse IgG (10 µg/ml) (Pharmingen, San Diego, CA) was added, incubated for 30 min at room temperature and rinsed in 3 changes of PBS. Peroxidase activity was detected using the 3-amino-9-ethylcarbazole kit (Sigma, St. Louis, MO) according to manufacturers instructions.

2.14 Statistical analysis
Indicated statistical analyses were performed using SPSS for Windows software release 7.5.2 (Statistical Product & Service Solutions Inc., Chicago, IL).

3. Results

3.1 Characteristics of the lobar K. pneumoniae pneumonia

At 64 h after inoculation of the left lung of the rats with 10^5 *K. pneumoniae* a lobar pneumonia has developed (Figure 1). Three zones characteristic for this type of pneumonia can be clearly distinguished. The consolidated zone (C) is the lower part of the lung where the bacterial inoculum was deposited and the infectious process started. As the active infection gradually moves upward, the consolidated area is characterized by gray hepatization. Microscopic evaluation (data not shown) indicated disintegration of alveolar walls, cellular debris, limited blood flow and the deposition of connective tissue. The hemorrhagic zone (H) is the active area of the infection featured by a dark red appearance. Microscopic observations (data not shown) indicate the presence of edema fluid, a large number of bacteria, infiltrating leukocytes, and hemorrhagic areas. The early-infected zone (EI) appears macroscopically as normal lung tissue, however, microscopic evaluation (data not shown) reveals the presence of bacteria and leukocytes indicates the early involvement in the infectious process. As the three zones of the lobar pneumonia can be clearly distinguished at 64 h after inoculation, and these zones showed limited variation in size and

macroscopic appearance between animals, this time-point was chosen to examine bacterial counts, capillary permeability and SSL localization.

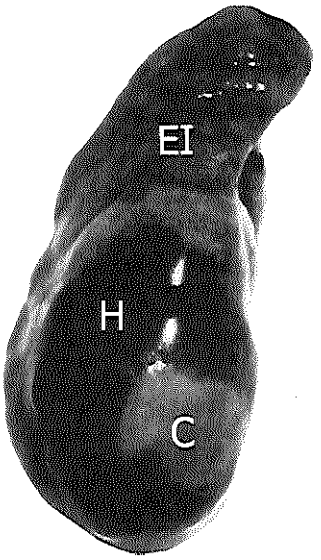


Figure 1. Acute lobar pneumonia in a rat. The consolidated (C), hemorrhagic (H), and early infected (EI) zone of an acute lobar pneumonia in the left lung of a rat at 64 h after inoculation of the left lung with 10^6 *K. pneumoniae*.

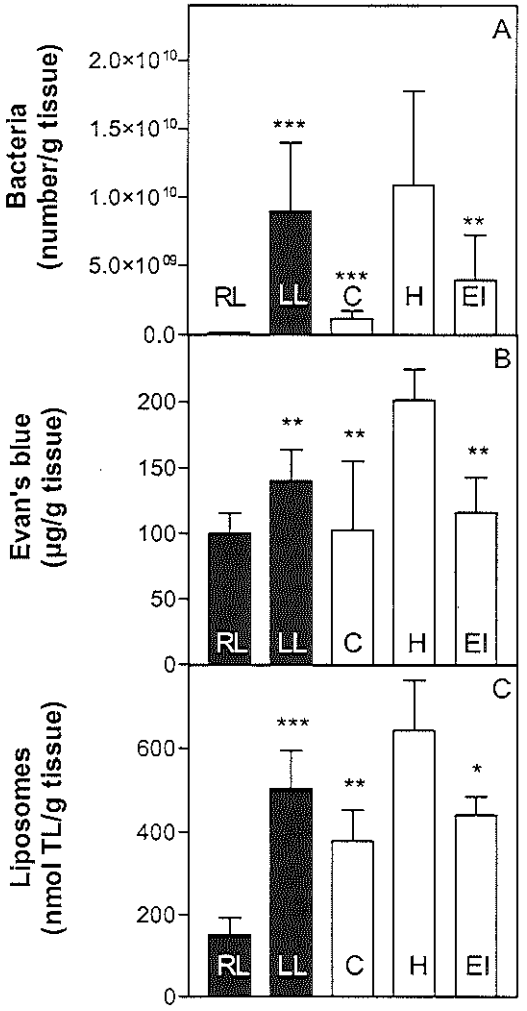
As expected, significantly higher bacterial numbers were observed in the infected left lung (LL) per gram organ weight compared to the uninfected right lung (RL) at 64 h after inoculation. The distribution of the bacteria over the three different zones of the lobar pneumonia shows that the number of bacteria was significantly higher in zone H of the pneumonia compared to C and EI (Figure 2A). The localization of a marker for capillary permeability -Evan's blue- shows a similar pattern at 24 h after injection (Figure 2B). A

significantly higher level of Evan's blue was seen in the infected left lung compared to the contralateral non-infected right lung. The level of Evan's blue in the right lung was comparable to the levels measured in lungs of uninfected rats (data not shown). Within the infected lung a significantly higher level of Evan's blue was present in zone H compared to C and EI. Another measure for capillary permeability is the tissue wet-to-dry weight ratio. The wet-to-dry weight ratio was lower for the uninfected right lung (4.81 ± 0.05) compared to the infected left lung (5.84 ± 0.06) (mean ± standard deviation, n=10) ($p<0.0001$, paired t-test). Localization of ^{67}Ga-labeled SSL shows the same profile at 24 h after injection (Figure 2C). Significantly higher levels were seen in the infected left lung compared to the right lung. Within the infected lung the highest level of SSL localization was seen in zone H compared to C and EI.

3.2 Effect of increase in inoculum size on localization of SSL and Evan's blue

To test whether the severity of infection has an effect on capillary permeability and SSL localization, three different inocula of *K. pneumoniae* were applied and compared to sterile buffer-inoculated animals (Figure 3). Tissues were dissected at 48 h after inoculation as the highest inoculum already induced mortality at 64 h after inoculation. Evan's blue dye localization and ^{67}Ga-labeled SSL localization measured at 24 h after injection, increased with an increasing inoculum ($r=0.91$, $p<0.0001$, $r=0.91$, $p<0.0001$, both Spearman's correlation test).

Figure 2. Localization of bacteria (A), Evan's blue (B), and ^{67}Ga-labeled SSL (C) in right lung (RL) and infected left lung (LL) (closed bars) (n=6, mean ± standard deviation). Localization within the LL in the consolidated (C), hemorrhagic (H) and early infected (EI) zone of the lobar pneumonia (open bars) (n=6, mean ± standard deviation). * $p<0.05$, ** $p<0.01$, ***$p<0.001$, left lung compared to right lung (paired t-test), consolidated zone and early infected zone each compared to hemorrhagic zone (repeated measures ANOVA corrected for multiple comparisons by the Bonferroni method). Tissues were dissected at 64 h after inoculation of the left lung with 10^5 *K. pneumoniae*, at 24 h after injection of liposomes and Evan's blue. ⇨


A
Bacteria
(number/g tissue)
2.0×10¹⁰
1.5×10¹⁰
1.0×10¹⁰
5.0×10⁰⁹
0.0
***
***
**
RL
LL
C
H
EI
B
Evan's blue
(µg/g tissue)
200
150
100
50
0
**
**
**
RL
LL
C
H
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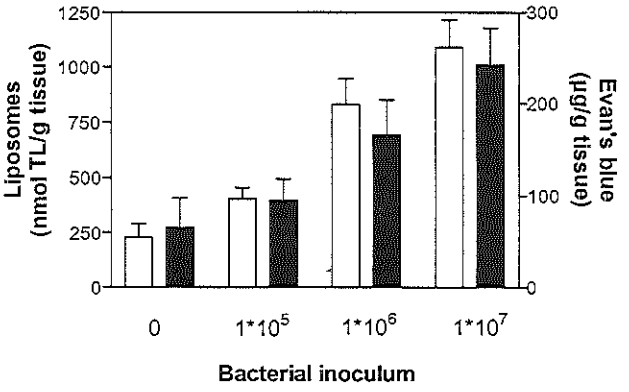


Figure 3. Effect of increasing *K. pneumoniae* inoculum on degree of localization of SSL (open bars) and Evan's blue (closed bars) in the inoculated left lung (LL) (n=6, mean ± standard deviation). Tissues were dissected at 48 h after bacterial inoculation of the left lung, at 24 h after injection of liposomes and Evan's blue.

3.3 Effect of local administration of LPS and HCl on left lung localization of SSL and Evan's blue

LPS, a component of the Gram-negative bacterial cell wall, has been shown to increase capillary permeability in lung tissue after local administration (26). To test whether an increase in capillary permeability induced by LPS in the absence of living bacteria also produces increased ^{67}Ga-labeled SSL localization, 2 mg LPS was inoculated in the left lung. After instillation of the LPS a significantly increased localization of ^{67}Ga-labeled SSL and Evan's blue in the lung was noted at 24 h after injection (Figure 4). Similarly, inoculation of the left lung with 0.1 ml 0.1 M HCl, a model for adult respiratory distress syndrome featured by an increased capillary permeability (23), resulted in an increased localization of ^{67}Ga-labeled SSL and Evan's blue at 24 h after injection (Figure 4).

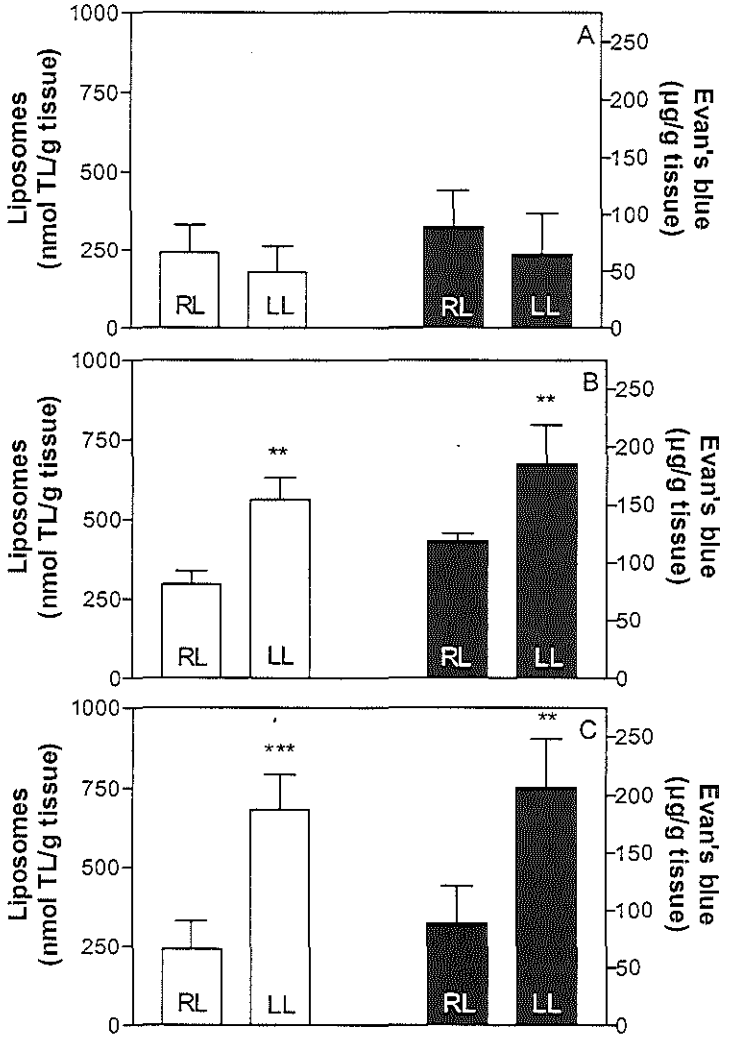


Figure 4. Localization of liposomes (open bars) and Evan's blue (closed bars) in right lung (RL) and left lung (LL) of rats inoculated with buffer (A), 2 mg LPS per left lung (B), and 0.1 ml 0.1 M HCl per left lung (C). Liposomes and Evan's blue were injected 15 min after inoculation. Tissues were dissected at 24 h after injection of liposomes and Evan's blue. (n=6, mean ± standard deviation). * $p<0.05$, ** $p<0.01$, ***$p<0.001$ compared to right lung (paired t-test).

3.4 Effect of leukocytes on localization of SSL

To investigate whether SSL are taken up by circulating leukocytes, ^{67}Ga-labeled SSL were injected i.v. Blood samples were washed three times in PBS by centrifugation. The amount of cell-associated radioactivity was determined. In blood samples obtained at 1 h, 4 h and 24 h after SSL injection 0.28 ± 0.12 %, 0.35 ± 0.16% and 0.17 ± 0.08% of the recovered radioactivity was pelletable with the blood cell fraction, respectively (mean ± standard deviation, n=3).

To establish whether extravasation of inflammatory cells is a key factor contributing to SSL localization in the left lung, the number of leukocytes in the circulation was reduced by i.p. injections of cyclophosphamide. As a result of the cyclophosphamide treatment, the number of leukocytes in the blood was reduced 6-fold from $5.8 \times 10^9 \pm 1 \times 10^9$ for the buffer treated controls to $1 \times 10^9 \pm 8 \times 10^8$ for the cyclophosphamide treated rats (mean ± standard deviation, n=3) ($p<0.01$, unpaired t-test). Tissues were dissected at 48 h after inoculation as the infection produced mortality in the leukopenic animals already at 64 h after inoculation. At 24 h after injection of ^{67}Ga-labeled SSL, SSL localization in the infected left lung of either leukopenic animals or animals with an intact host defense was significantly higher compared to ^{67}Ga-labeled SSL localization in the lungs of uninfected animals (Figure 5). Cyclophosphamide treatment did not result in a significant difference in the degree of ^{67}Ga-labeled SSL localization in infected animals.

3.5 Microscopic evaluation of colloidal gold-labeled SSL within the infected site

Localization of SSL in infected left lung and uninfected right lung tissue was visualized using silver-enhancement of colloidal gold-labeled SSL. Figure 6 A and B show dense clusters of silver-enhanced colloidal gold in the connective tissue surrounding the primary bronchus and major bronchioles of the infected left lung. These clusters were not observed in the uninfected right lung. Magnification of these clusters show that the silver-enhanced colloidal-gold is present in mononuclear cells (Figure 6 C). These cells stained positive immunohistochemically for CD43 (Figure 6 D). One aspect of SSL localization that could only be clearly visualized in slides unstained or with just a very light counterstain is the presence

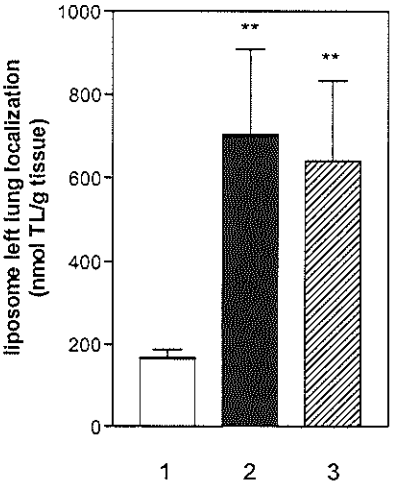
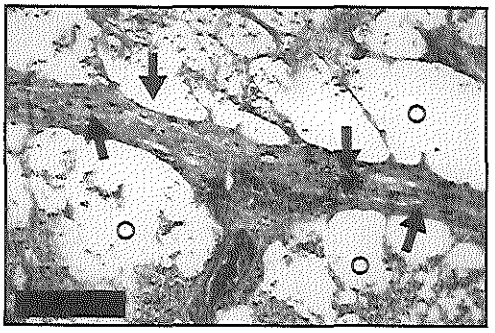


Figure 5. Localization of liposomes in the left lung of uninfected rats with intact host defense (1), infected rats with intact host defense (2), and infected leukopenic rats (3) (n=6, mean ± standard deviation). Tissues were dissected at 48 h after inoculation of the left lung inoculated with 10^6 *K. pneumoniae*, at 24 h after injection of liposomes and Evan's blue. ** $p<0.01$ infected rats compared to uninfected rats. No significant differences were noted between leukopenic infected rats and infected rats with an intact host defense (ANOVA corrected for multiple comparisons by the Bonferroni method).

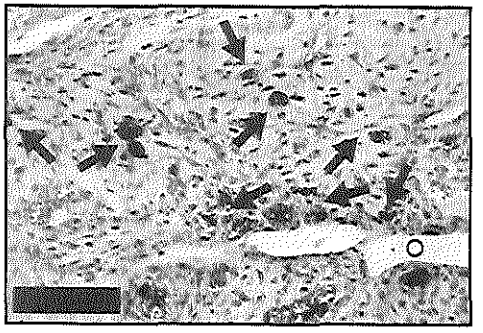
of SSL in and around alveolar capillaries. Micrographs show silver granula in and around the capillaries in the alveolar walls, most prominent in the hemorrhagic zone of infected left lung tissue (Figure 6 E and F). Silver granula were not seen surrounding capillaries or alveolar walls in the uninfected right lung tissue.

Figure 6. Localization of colloidal gold-labeled liposomes in infected left lung tissue.



⇦
A. Presence of silver-enhanced colloidal gold-labeled SSL in large clusters (arrows) in the connective tissue surrounding the large bronchioles and blood vessels (asterisk). Inflamed alveolar spaces with high numbers of leukocytes surround the connective tissue (circles). Counterstain haematoxylin/eosin. Bar = 1125 µm.

⇨
B. Magnification of clusters, as shown in A. Separate silver-enhanced colloidal gold-labeled clusters (arrows) can be clearly distinguished from surrounding tissue, in which a bronchiolar lumen (circle) and blood vessels (asterisks) are identified. Counterstain haematoxylin/eosin. Bar = 450 µm.



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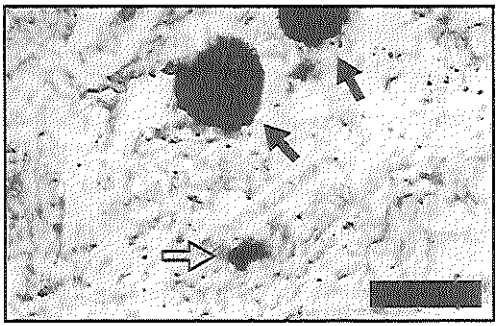
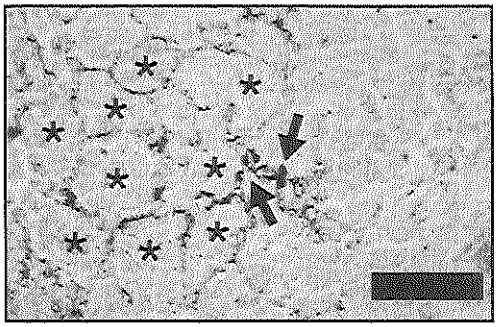
C. Magnification of clusters, as shown in B. Clusters appear intracellular silver-enhanced colloidal-gold in a mononuclear cell. Bar = 45 µm.

⇨

D. Positive immunohistochemical staining for CD43 of cells containing silver-enhanced colloidal gold-labeled SSL (closed arrows). Not all positively stained leukocytes show colloidal-gold accumulation (open arrow). Peroxidase activity was detected by 3 amino-9-ethylcarbazole . Bar = 45 µm.





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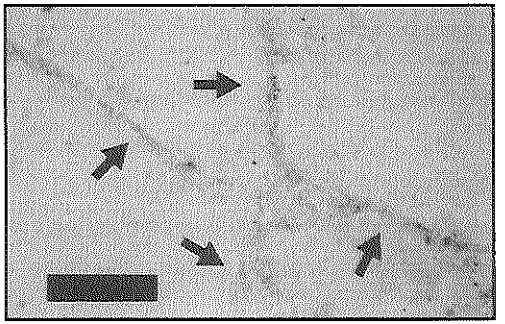
E. Localization of silver-enhanced colloidal gold-labeled SSL in and around alveolar capillaries in the alveolar walls, surrounding the alveolar spaces (asterisks) in a hemorrhagic area of the infected lung. Clustered silver-enhanced colloidal-gold may indicate cellular uptake (arrows). Lightly counterstained with haematoxylin/eosin. Bar = 450 µm.

⇨

F. Localization of silver-enhanced colloidal gold labeled SSL (arrows) in and around alveolar capillaries in the alveolar walls. No counterstain. Bar = 45 µm



4. Discussion

Preferential localization of SSL at sites of infection or inflammation that have developed outside the MPS has been reported by a number of investigators (7-13). Which host factors contribute to selective extravasation of SSL at infectious or inflammatory foci is not clear. In the present study, the involvement of increased capillary permeability and the role of inflammatory cells in the selective localization of SSL in bacterially infected lungs was studied in a rat model of unilateral pneumonia caused by *K. pneumoniae*.

Three distinct zones of infection characterize the lobar unilateral *K. pneumoniae* pneumonia: the early infected, hemorrhagic, and consolidated zone. When the distribution of bacteria was studied at 64 h after inoculation, a higher number of bacteria was present in the inoculated left lung compared to the right lung. Within the infected left lung, the highest number of bacteria is present in the active hemorrhagic zone of the infection compared to the consolidated zone and early-infected zone. It is shown that the high number of bacteria in the hemorrhagic zone of the infected lung correlates with a pronounced inflammatory response, featured by a significantly increased capillary permeability as reflected by Evan's blue dye levels and lung wet-to-dry weight ratio. Furthermore, by raising the bacterial inoculum and thereby aggravating the severity of the infection, the increase in capillary permeability is enlarged. The increase in capillary permeability was invariably seen along with a proportional increase in SSL localization illustrating that capillary permeability is an important determinant for SSL extravasation. The observed correlation between capillary permeability and SSL extravasation is in line with studies showing preferential accumulation of SSL in tumor tissue, possessing angiogenic blood vessels exhibiting increased permeability, as has been reviewed (4-6). It also supports the observation of Klimuk et al. that increase in vascular permeability and liposome localization occur simultaneously in a model of delayed type hypersensitivity (8).

Increased capillary permeability has been described after intratracheal administration of LPS and HCl in rats (23, 26). Also in our experiments, both inflammatory stimuli promoted an increase in capillary permeability as well as SSL localization. Apparently, the nature of the inflammatory stimulus is not important for SSL localization as long as a sufficient increase in

capillary permeability is effectuated. Preferential SSL localization at the target site has been demonstrated in a wide variety of models of experimental inflammation and infection, including pathology as a result of administration of guinea-pig spinal cord (allergic encephalomyelitis) (7), 2,4-dinitrofluorobenzene (delayed type hypersensitivity) (8), trinitrobenzene sulfonic acid (acute colitis) (9), sodium-morrhuate and *Staphylococcus aureus* (osteomyelitis) (10), *Mycobacterium butyricum* (adjuvant arthritis) (11), turpentine or *Staphylococcus aureus* (focal infection or inflammation) (12), and *K. pneumoniae* (unilateral pneumonia) (13). These findings strongly suggest that the etiology or location of the pathological process is not decisive for achieving SSL localization. Instead, increased capillary permeability, as part of the inflammatory response is likely to be the decisive parameter.

Previous studies in models of hypersensitivity, colitis and encephalomyelitis have reported preferential SSL localization as well as leukocyte influx at the site of inflammation (7-9). Also in the present model, the inflammatory response involves leukocyte infiltration. It has been suggested that there is a correlation between SSL and leukocyte localization (14). In the present study, it is shown that the amount of cell-associated SSL-encapsulated radioactivity in the circulation is negligible. In addition, a 6-fold reduction of the number of leukocytes in the circulation by cyclophosphamide injections, did not result in a reduced degree of SSL localization in the infected lung compared to rats with normal levels of leukocytes. In addition, Dams et al. showed that SSL still localized preferentially at sites of infection in granulocytopenic rats (27). These results strongly suggest that the contribution of circulating leukocytes to SSL localization at the site of infection is limited. An important implication would be that liposomal drug delivery could also be beneficial to leukopenic patients. These patients are highly susceptible to infections that are difficult to treat as a result of a reduced support by the host defense (28).

Microscopic evaluation of silver-enhanced colloidal gold-labeled SSL in lung tissue showed that SSL could be visualized principally in the infected left lung. In the left lung, SSL were present in dense clusters in the connective tissue surrounding the primary bronchus and main bronchioles. These clusters appeared to be uptake by CD43-positive mononuclear

leukocytes. SSL were also seen surrounding capillaries in alveolar walls, especially in the hemorrhagic zone of infection, supporting the observation that increased capillary permeability allows liposome extravasation. To our knowledge, the localization of SSL around capillaries and liposome uptake at the site of bacterial infection by leukocytes has not been demonstrated before. Our results are in line with studies obtained in other models showing that SSL are taken up by inflammatory cells present at the inflamed tissue. In the FSN mouse (having a gene mutation producing inflammatory lesions resembling psoriasis) (29), in mice having been injected with substance P (30), and in mice overexpressing the HIV *tat* gene showing lesions resembling Kaposi's sarcoma (31), selective SSL localization at the target site was demonstrated together with uptake of colloidal gold labeled SSL by phagocytic cells. It is tempting to speculate that phagocytic cells at the pathological site are involved in the processing of SSL and release of encapsulated agents. Still, the quantitative contribution of SSL uptake by local phagocytes to the fate of SSL localizing within the infected area remains unclear.

In conclusion, our observations indicate that the selectivity of SSL localization is a result of the local inflammatory response, as the locally increased capillary permeability seems decisive for SSL localization. The localization process appears independent of the nature of the inflammatory stimulus and number of circulating leukocytes. Microscopic observations indicate that phagocytes are to a certain extent involved in SSL uptake at the site of infection. In addition, extravasation of liposomes is observed in the alveolar walls. Further elucidation of the processes that determine the fate of SSL after target site localization, may help to design SSL-antibiotic formulations with increased antibacterial activity.

Acknowledgements

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4. Localization of sterically stabilized liposomes in experimental rat *Klebsiella pneumoniae* pneumonia: dependence on circulation kinetics and presence of poly(ethylene) glycol-coating

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Summary

Preferential localization of liposomes at sites of infection or inflammation has been demonstrated in a variety of experimental models. Most studies report enhanced localization at the target site of poly(ethylene) glycol (PEG)-coated liposomes as compared to conventional non-coated liposomes. It is generally accepted that the prolonged circulation time of PEG-coated liposomes increases target site exposure, which results in increased target localization. A quantitative relationship between circulation kinetics and localization at the pathological site has not been defined as yet. Besides, an effect of the PEG-coating itself has been suggested, as theoretically the PEG-coating may facilitate liposome extravasation. In the present study, in a rat model of an acute unilateral *Klebsiella pneumoniae* pneumonia, circulation kinetics of PEG-coated liposomes were manipulated by incorporation of different amounts of phosphatidylserine (PS) and variation of lipid dose, additionally allowing evaluation of the saturability of the localization process. In addition, this paper addresses the effect of the PEG-coating, by comparing the circulation kinetics and target localization of long-circulating 'PEG-free' and PEG-coated liposomes. It is shown that the degree of liposome localization at the target site is positively linearly related to the area under the blood concentration time curve (AUC) of the liposome formulations, irrespective of PEG-coating. This finding is discussed in relation to the equation of Kedem and Katchalsky, which describes protein influx into sites of infection or inflammation.

1. Introduction

In a variety of experimental models of infection or inflammation, liposomes have been demonstrated to localize at the pathological site [1-7]. The localization is dependent on the inflammatory response as the localization at comparable anatomical sites in uninfected control animals is generally insignificant. As a result, liposomes have attracted considerable interest as targeted drug carriers for application in infection and inflammation. In order to maximize the degree of liposome localization at the target site, it is essential to identify which factors contribute to the passive targeting of liposomes to inflamed sites.

With respect to the inflamed target site, this area is characterized by an increased blood flow, and an increased capillary permeability causing edema. The fluid flux into the target site facilitates movement of the phagocytes and promotes plasma protein localization supporting the host defense. The influx of plasma proteins into the inflamed site has been mathematically described by Kedem and Katchalsky [8] as:

$$J_s = J_v (1-\sigma) C_s + P_c S_c \Delta C \qquad (1)$$

where J_s and J_v are the fluxes of the studied protein and of fluid, respectively, σ is the reflection coefficient (fraction of the protein that is stopped by the microvascular barrier), C_s is the average protein concentration in the capillary, $P_c S_c$ is the capillary permeability-capillary surface area product, and ΔC is the protein concentration difference across the endothelium. The first term of the equation describes the convective transport component ($J_v (1-\sigma) C_s$), whereas the second term describes the diffusion component ($P_c S_c \Delta C$). Assuming that liposome flux into the target site can also be described by this equation, two liposome related factors would then determine the degree of liposome localization: the ability of the liposomes to cross the vascular endothelium (determined by σ) and the liposome concentration in the blood (C_s). The $P_c S_c \Delta C$ component of the equation is negligible due to the low diffusion coefficient of particulate systems. The fluid flux (J_v) is not related to the particulate system but to the inflammatory response. Therefore, only C_s and σ seem to be the main determining parameters in the liposome localization process.

Several studies have shown an improved target site localization of liposomes coated with poly(ethylene) glycol (PEG), also known as sterically stabilized liposomes (SSL), compared to

conventional liposomes lacking the PEG-coating [1-6]. It is generally accepted that the higher degree of localization is enabled by the prolonged circulation time of SSL. The increased average liposome concentration in the capillaries at the inflamed area, yields increased exposure of the target site to the liposomes, suggesting that the liposome concentration (or C_s) in time is important for target localization. Besides, permeability studies in tumor tissue suggest that the PEG-coating itself can promote target localization [9]. A less interactive liposome surface, conferred by the PEG-coating, which could also be interpreted as a reduced reflection coefficient (or σ), may facilitate their extravasation at sites of increased capillary permeability.

Up to now, a limited number of qualitative studies has appeared on the relationship between circulation kinetics (i.e. involvement of the C_s parameter) and degree of localization of SSL in the pathological target tissue, as has been discussed by Storm and Woodle [10]. In the present study, this relationship is explored in more detail. In a rat model of an acute unilateral *Klebsiella pneumoniae* pneumonia in rats, SSL circulation kinetics were manipulated by incorporation of different amounts of phosphatidylserine (PS) [11, 12]. Circulation kinetics were also manipulated by variation of lipid dose, additionally allowing evaluation of the saturability of the localization process. A relationship between liposomal circulation time and target site localization was established on the basis of the collected data. In addition, this paper addresses the effect of the PEG-coating itself (i.e. involvement of the σ parameter) by comparing the circulation kinetics and target localization of long-circulating 'PEG-free' liposomes and SSL.

2. Materials and Methods

2.1 Liposome preparation and characterization

Liposomes were prepared as described previously [2] using appropriate amounts of the following lipids: partially hydrogenated egg phosphatidylcholine (PHEPC) (Asahi Chemical Industry Co. Ltd., Ibarakiken, Japan), cholesterol (Chol), L-(-phosphatidyl-L- serine (PS) (Sigma Chemical Co., St. Louis, Missouri), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethylene glycol-2000] (PEG-DSPE), or distearoyl phosphatidylcholine (DSPC) (Avanti Polar Lipids, Alabaster, Alabama). In brief, lipids were dissolved in a mixture of chloroform and methanol. The solvent was evaporated and the lipids were dried, redissolved in 2-methyl-2-propanol (Sigma Chemical Co., St. Louis, Missouri), frozen, and freeze-dried overnight. The resulting lipid film was hydrated in Hepes/NaCl buffer, pH 7.4 (10 mM N-[2-

hydroxy ethyl] piperazine-N'-ethane sulfonic acid (Hepes) (Sigma Chemical Co., St. Louis, Missouri), 135 mM NaCl (Merck, Darmstadt, Germany), and 5 mM of the chelator deferoxamine mesylate (Desferal ®) (Novartis, Basel, Switzerland).
The liposome dispersion was sonicated for 8 min with an amplitude of 8 µm using a 9.5 mm probe in an MSE Soniprep 150 (Sanyo Gallenkamp PLC, Leicester, UK) to obtain liposomes with a mean particle size of 100 nm. Dynamic light scattering, detected at an angle of 90° to the laser beam on a Malvern 4700 System (Malvern Instruments Ltd., Malvern, UK), was measured to evaluate the particle size distribution. In addition to the mean particle size, the system reports a polydispersity index (a value between 0 and 1). A polydispersity index of 1 indicates large variations in particle size, a reported value of 0 means that size variation is absent. All liposome preparations used had a polydispersity index below 0.3. The DSPC:Chol preparation with a mean size of 80 nm was obtained by multiple extrusion at 60 °C of the liposome dispersion through two stacked polycarbonate membranes (Nuclepore, Pleasanton, California) with a final pore size of 50 nm. Mean particle size was 80 nm.

2.2 Radiolabeling of liposomes
Liposomes were radiolabeled with ^{67}Ga, as described previously [2]. In brief, non-encapsulated deferoxamine was removed by gel filtration. Liposomes were concentrated via ultracentrifugation. ^{67}Ga-citrate (1 mCi/ml) (Mallinckrodt Medical b.v., Petten, The Netherlands) was diluted 1:10 in aqueous 5 mg/ml 8-hydroxyquinone (Sigma Chemical Co., St. Louis, Missouri) and incubated for 1 h at 52 °C to yield ^{67}Ga-oxine. 1 ml of this solution was added per 1000 µmol total lipid (TL). 67Ga-oxine can pass the liposomal membrane and form a complex with the entrapped chelator deferoxamine. The complex is an appropriate label for monitoring intact liposomes in the circulation as it is rapidly renally excreted once it is released from circulating liposomes [13]. Free label was removed by gel filtration and labeled liposomes were concentrated by ultracentrifugation. Resulting specific activities were between $1*10^4$ and $2*10^5$ cpm/µmol TL. Phosphate concentration was determined spectrophotometrically according to Bartlett [14].

2.3 Unilateral pneumonia
The animal experiments ethical committee of the Erasmus university Medical Center Rotterdam approved the experiments described in this study. Female albino RP/AEur/RijHsd strain albino rats, 18-25 weeks of age, body weight 185-225 g (Harlan, Horst, The Netherlands) with a specified pathogen free status were used. A left-sided unilateral pneumonia was induced as described previously [15]. In brief, rats were anesthetized and the left primary bronchus was intubated. Through the tube, 0.02 ml of a saline suspension containing 106 *K. pneumoniae* (ATCC 43816, capsular serotype 2) was inoculated in the left lung lobe. Rats were housed individually with free access to water and SRMA chow (Hope Farms b.v., Woerden, The Netherlands).

2.4 Blood clearance and biodistribution
Experimental groups consisted of 6 rats. At indicated time points after bacterial inoculation of the left lung, liposomes were injected at the indicated dose in the tail vein. At indicated time-points after injection blood samples of approximately 0.3 ml were taken, by retro-orbital puncture, from alternate groups of 3 rats. After measuring sample volume, radioactivity was counted in a Minaxi autogamma 5000 gamma counter (Packard Instrument Company, Meriden, Connecticut). To determine the tissue distribution of the liposomes, rats were sacrificed by CO_2 inhalation. Subsequently, organs were dissected, weighed and radioactivity was counted. Organ radioactivity was corrected for radioactivity present in the blood (see below).

2.5 Total blood volume and blood content of tissues

Total blood volume of infected rats was determined, in an independent experiment, as being 5.3% of the total body weight. Syngeneic erythrocytes labeled with ^{111}In-oxine according to Kurantsin-Mills were used [16]. Blood samples were taken at 10 min after injection of the labeled erythrocytes assuming that all erythrocytes were still present in the circulation. The dilution factor of the radioactive label allowed calculation of the total blood volume. Sample volume and blood volume allowed calculation of total blood radioactivity content. This technique was also used to determine blood content of the tissues at appropriate time-points after inoculation.

2.6 Statistical analysis

SSL display single first-order clearance rates, independent of dose [17, 18]. Similarly, in our study blood clearance profiles of individual animals could be well described by a linear relationship on a semi-logarithmic plot ($0.88 < r < 1.00$), which allowed estimation of individual area under the blood concentration-time curve (AUC) values.

Liposome localization data were tested for homogeneity of variance using Levene's statistic. Data were log transformed in case of significant differences. Infected left lung and right lung localization was compared using the paired t-test. Comparisons between groups were made by one-way analysis of variance (ANOVA). The Bonferroni method was used to correct for multiple comparisons. All analyses were performed using SPSS for Windows software release 7.5.2 (Statistical Product & Service Solutions Inc., Chicago, USA).

3. Results

3.1 Manipulation of AUC by incorporation of PS and effect on degree of infected left lung localization of SSL

Incorporation of PS was used to manipulate the circulation time of SSL, as PS is a strong recognition signal for macrophage uptake, which cannot be prevented by the presence of PEG-DSPE at the usually applied amount of 5 mol% [11, 12]. The circulation kinetics and biodistribution profiles of SSL containing either 0, 1, or 10 mol% PS in the bilayer are shown in Figure 1 A and B, respectively. The $AUC_{0\text{-}24h}$ of the 1 and 10 mol% PS-SSL was 2.0-fold and 2.6-fold lower, respectively, compared to that of the 0 mol% PS-SSL. Regarding the biodistribution profile at 24 h after injection, the three liposome preparations displayed an approximately 4-fold higher localization in the infected left lung compared to the uninfected right lung ($p<0.001$, Fig. 1 B). The degree of localization in the infected lung was 2.5-fold higher for the 0 mol% PS containing SSL compared to the 1 and 10 mol% PS containing SSL ($p<0.001$). The differences in localization in the right lung between the SSL types showed a similar pattern ($p<0.05$; $p<0.001$, respectively).

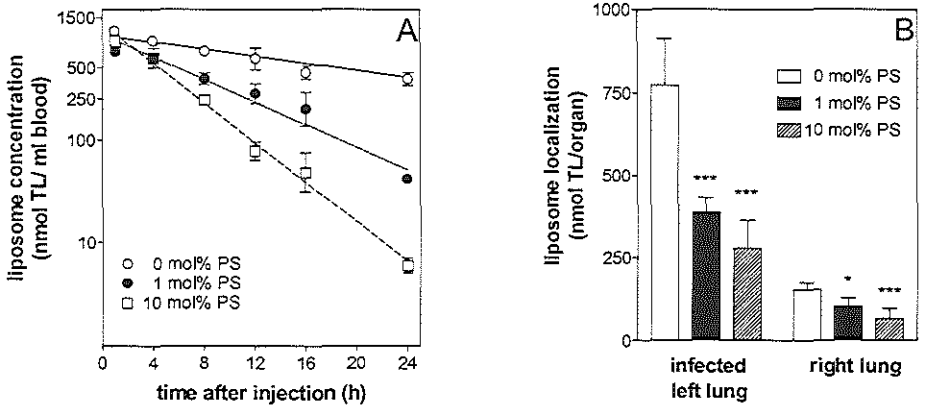

Figure 1. Effect of incorporation of 0, 1, or 10 mol% PS on blood clearance and degree of localization of SSL in infected left lung and right lung tissue. ^{67}Ga-labeled liposomes were injected i.v. at 40 h after bacterial inoculation of the left lung at a dose of 75 µmol TL/kg. Lipid composition was PHEPC:Chol:PEG-DSPE:PS 1.85:1.00:0.15:0 mol:mol for the 0 mol% PS containing liposomes, 1.82:1.00:0.15:0.03 for the 1 mol% PS preparation, and 1.55:1.00:0.15:0.30 for the 10 mol% PS preparation.

A. nmol TL/ml blood was calculated from radioactivity recovered (n=3, Mean ± S.D.)

B. nmol TL/lung at 24 h after injection was calculated from radioactivity recovered (n=6, mean ± standard deviation).

$p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Significant differences versus the 0 mol% PS preparation are indicated.

3.2 Manipulation of AUC by variation of lipid dose and effect on degree of infected left lung localization of SSL

SSL were injected i.v. at lipid doses from 25 up to 375 µmol TL/kg. The resulting values for $AUC_{0\text{-}24h}$ and corresponding infected left lung localization at 24 h after injection are presented in Figure 2 A and B, respectively. The $AUC_{0\text{-}24h}$ value as well as degree of left lung localization increased linearly with escalating dose. For all doses tested, the localization in the infected lung was approximately 3-fold higher than the localization in the right lung (data not shown, $p<0.001$).

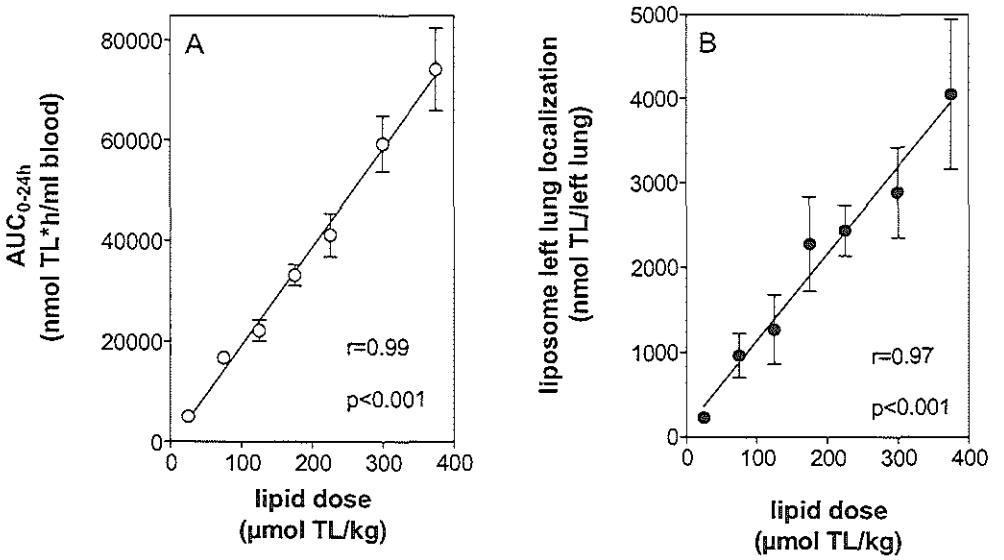

Figure 2. Effect of lipid dose on AUC_{0-24h} (A) and degree of left lung localization (B) of ^{67}Ga-labeled SSL. Lipid composition was PHEPC:Chol:PEG-DSPE 1.85:1.00:0.15 mol:mol. See the legend of Figure 1 for experimental conditions.

3.3 Effect of PEG-coating on degree of infected left lung localization of liposomes

To assess whether the presence of a PEG-coating has a facilitating effect on target site extravasation, two liposome types were studied: 1- long-circulating small rigid liposomes (LCL) without a PEG-coating (DSPC:Chol, 2:1, mol:mol) and 2- SSL (PEG-DSPE:PHEPC:Chol 0.15:1.85:1.0 mol:mol). The circulation kinetics and biodistribution profiles are shown in Figure 3 A and B, respectively. The slow clearance profiles of the long-circulating PEG-free liposomes and the SSL were similar, as is reflected by approximately equal AUC_{0-24h}-values. In line with these results, the degree of localization in the infected left lung was similar for both long-circulating liposome types. Both formulations demonstrated an approximately 3-fold higher degree of localization in the infected left lung compared to the degree of localization in the uninfected right lung ($p<0.001$, Fig. 3 B).

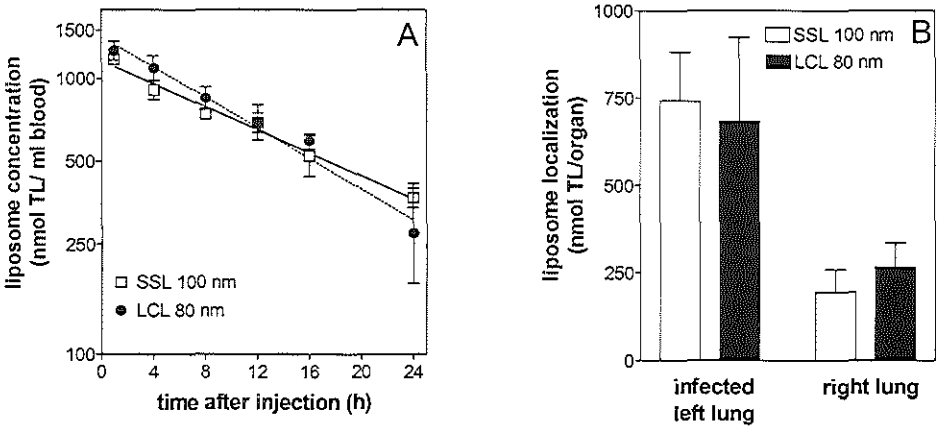

Figure 3. Blood clearance (A) and degree of lung localization (B) of SSL and LCL. Blood clearance and degree of localization in infected left lung and right lung tissue of ^{67}Ga-labeled SSL (lipid composition PHEPC:Chol:PEG-DSPE 1.85:1.00:0.15 mol:mol) and PEG-free small rigid long-circulating liposomes (LCL) (lipid composition DSPC:Chol 2:1 mol:mol). See the legend of Figure 1 for experimental conditions.

3.4 Correlation between $AUC_{0\text{-}24h}$ and degree of infected left lung localization of liposomes

Collected data regarding $AUC_{0\text{-}24h}$-values and regarding degree of infected lung localization in individual animals taken from Figures 1, 2 and 3, are presented in Figure 4. A positive linear correlation was obtained ($r=0.92$, $p<0.001$)

3.5 Relationship between infected left lung weight, AUC, and degree of infected left lung localization of SSL versus time after bacterial inoculation

An implication of liposome concentration as a driving force for target localization would be that the driving force gradually weakens in time as a result of reduced blood levels due to liposome clearance. Therefore, left lung weight (as a measure of fluid influx), liposomal. AUC, and degree of infected left lung localization of SSL was determined at different time-points after liposome injection (Figure 5 A-C). To obtain a clear effect of liposome clearance from the bloodstream, of these long-circulating SSL, data had to be collected over a 48 h period. To be able to collect data over this time period, liposomes were injected at 24 h

after bacterial inoculation as some animals died shortly after 72 h after inoculation. Figure 5 A shows that the increase in left lung weight is linear in time ($r=0.92$, $p<0.001$). Both liposomal AUC and degree of liposome left lung localization show a hyperbolic curve (Fig 5 B and C, respectively). Over 70% of the liposome AUC and degree of left lung localization is achieved in the first 24 h.

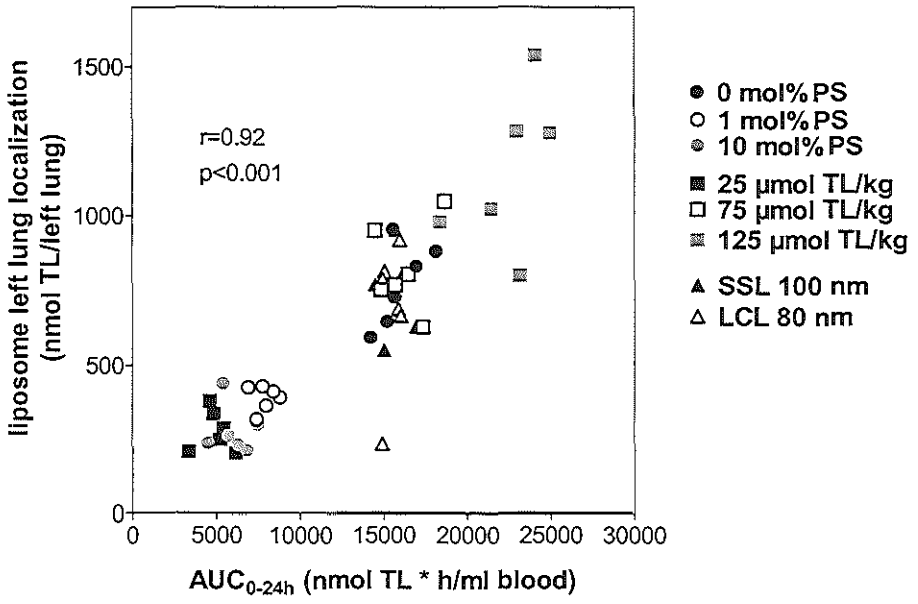

Figure 4. Relationship between $AUC_{0\text{-}24h}$-values and corresponding degree of left lung localization of individual animals for indicated liposome formulations.

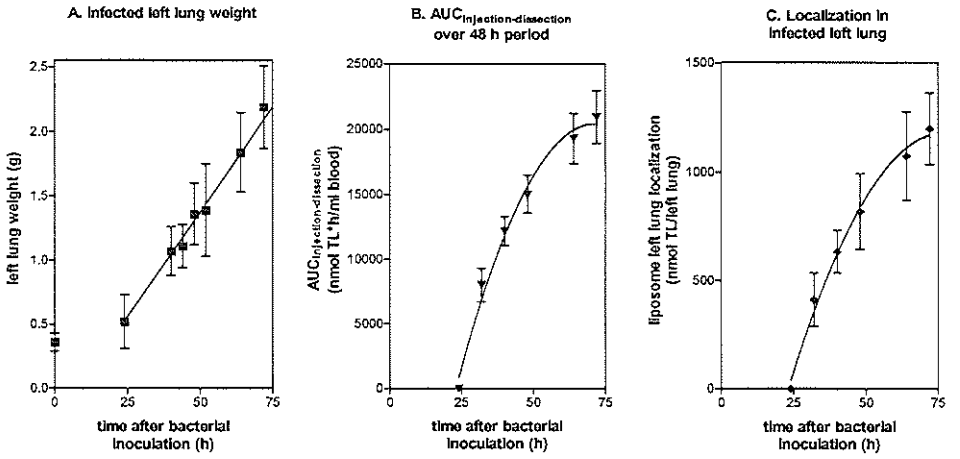

Figure 5. Infected left lung weight (A), degree of infected left lung localization of liposomes (B), and AUC-value (C) of SSL at different time points after liposome injection. ^{67}Ga-labeled liposomes were injected i.v. at 24 h after bacterial inoculation of the left lung at a dose of 75 µmol TL/kg. Rats were dissected at indicated time-points and left lung weight, degree of SSL left lung localization, and SSL blood concentration was determined. The AUC-values were calculated from individual blood clearance kinetics. n = 3-6 animals per time-point. Mean ± S.D.

4. Discussion

Similarly as plasma proteins, long-circulating SSL have been shown to localize preferentially at sites of infection or inflammation [1-7]. The equation of Kedem and Katchalsky (see Introduction) has been used to describe the influx of proteins into inflamed areas [8]. If the same equation also applies to liposome influx into inflammatory foci, then target localization would be dependent on the ability of liposomes to cross the vascular endothelium, determined by the reflection coefficient (σ), and liposome concentration in the bloodstream (C_s). SSL with prolonged circulation times generally show a higher degree of target site localization compared to non-coated liposomes with shorter circulation times, which points at the involvement of C_s [1-6]. In addition, the PEG-coating of SSL may have a promoting effect on target site localization itself by conferring a less interactive surface to the liposome, which implies a reduction of σ [9]. At present, a systematic investigation of the

effect of circulation time and PEG-coating on the degree of liposome localization at inflamed areas is lacking. The approach taken in the present study to investigate the effect of circulation kinetics of SSL on target localization was to incorporate PS in SSL and to vary SSL dose. The effect of PEG-coating was studied by comparing circulation kinetics and degree of target site localization values of long-circulating liposomes without PEG-coating to those of SSL.

The rate of SSL removal from the blood increased with an increasing amount of PS in the liposomal bilayers. A similar PS-effect has been observed by Boerman et al. [11]. Increased clearance from the circulation was paralleled by a diminished target site accumulation. The incorporation of PS could have an intrinsic effect on SSL biodistribution as a result of increased negative surface charge or induction of defects in the PEG-coating. More likely the reduced circulation time of PS-containing SSL reduces the number of liposomes able to interact with the target site over time (reduction in C_s), leading to a diminished target site localization. The results reveal that the reduction in AUC is directly proportional to the reduction in degree of target localization.

Liposomal AUC was also manipulated by variation of lipid dose. The rise in AUC-values was proportional to the increase in administered lipid dose, showing that the pharmacokinetics of SSL are independent of dose between 25 and 375 μmol TL/kg. Dose-independent pharmacokinetics have also been observed earlier by Allen et al. [18]. Here we show that the degree of target site localization increases proportionally to the administered dose, which points out that saturation of target localization does not occur within the dose range of 25 to 375 μmol TL/kg and further supports the notion that the liposome concentration (or C_s) 'drives' the localization in the infected left lung.

It has been argued that the presence of a PEG-coating facilitates liposome extravasation at sites of increased capillary permeability, which could be interpreted as a reduction of the reflection coefficient (σ). This issue was addressed by comparing the circulation kinetics and target site localization values of long-circulating liposomes without a PEG-coating to SSL. Since the long-circulating liposome formulation lacking the PEG-coating shows similar AUC and target localization values as the SSL formulation, it appears that the presence of a PEG-

coating does not contribute significantly to the degree of liposome localization at the site of infection and that the value for σ is equal for both liposome types. Experiments, similar to those described by Waypa et al. [19] to measure the actual value of σ for liposomes in inflammatory conditions could yield important information to maximize the degree of liposome target localization.

The linear relationship obtained by plotting the AUC-values of the liposome formulations tested and corresponding target localization values in individual animals emphasizes that liposome concentration is the prime determinant of liposome extravasation at the target site and supports the usefulness of the Kedem and Katchalsky equation for describing liposome influx at the site of infection.

Liposome concentration as a driving force for target localization implies that the liposome influx at the target site gradually slows down as a result of liposome clearance. Experimental support for this notion was obtained by determining lung weight, and degree of left lung localization of SSL at different time-points after injection and calculation of the AUC-values between liposome injection and tissue dissection. It is shown that the lung weight increase is linear in time, which indicates that fluid influx into the site of infection (J_v) occurs at an approximately constant rate ($r=0.92$, $p<0.001$). Both cumulative AUC and liposome localization show a hyperbolic curve, indicating that a gradual decrease in liposomal blood concentration results in a proportional decrease in target localization, which again confirms that AUC is the main factor driving the passive targeting effect.

A theoretical implication of the Kedem and Katchalsky equation is that, during fluid flux into the site of infection ($J_v>0$) and a liposomal reflection coefficient that allows passage through the vascular endothelium ($\sigma<1$), target localization of long-circulating liposomes will occur and will not be saturable. Therefore, the design of liposomes with a longer circulation time compared to that of the liposome formulations used in the present study can be expected to provide for a higher degree of target localization, indicating that there is still room for improvement. Our results deviate from those of Longman and co-workers who investigated the localization of i.v. administered liposomes with various circulation times in peritoneal fluid [20]. Localization of long-circulating liposomes in peritoneal fluid was not increased

compared to conventional liposomes, despite significant differences in circulation times. Limited fluid transport (J_v) due to an intact microvascular barrier in the abdomen (possessing a high reflection coefficient (σ)) likely explains these results.

In conclusion, the present study clearly shows for the first time that the blood concentration is the primary factor driving localization of liposomes into the *K. pneumoniae*-infected target site. In addition, the PEG-coating itself does not contribute significantly to the degree of liposome target localization. The design of liposomes with longer circulation times as compared to those of the SSL used in this study can be predicted to result in a higher degree of target localization of liposomes.

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5. Liposome-encapsulated aminoglycosides in preclinical and clinical studies

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Summary

Liposome-encapsulated amikacin has recently entered clinical trials. The rationale for liposome-encapsulation of aminoglycosides is the possibility to increase the therapeutic index of this class of antibiotics by increasing aminoglycoside concentrations at the site of infection and/or by reducing the toxicity of these drugs. Three approaches can be distinguished: the use of liposomes as a depot formulation for local drug administration, targeting of (relatively) short circulating conventional liposomes to the cells of the mononuclear phagocyte system (MPS) for treating intracellular bacterial infections, and targeting of long-circulating liposomes to infectious foci localized outside the MPS. This review discusses the preclinical and clinical data in connection with recent developments in liposome technology.

1. Aminoglycosides

After the introduction of streptomycin in 1944, aminoglycosides developed into an important class of antibiotics. Their wide antimicrobial-activity, post-antibiotic effect, synergism with ß-lactam antibiotics, rapid concentration-dependent bactericidal activity, low rate of resistance, and low cost contributed to their success [1-4]. However, the poor oral uptake of aminoglycosides requires parenteral administration. Moreover, the pharmacodynamics in relation to the dose-related adverse effects on kidneys and audio-vestibular apparatus demand that the plasma concentrations are maintained within a narrow therapeutic window [5-8]. Therefore, aminoglycosides are currently used for treating severe (nosocomial) Gram-negative and Gram-positive infections, especially in immunocompromised patients, and for treating mycobacterial infections [9-12].

A drug delivery system may help to increase the therapeutic index of the aminoglycosides by increasing the concentration of the drug at the site of infection and/or reducing the nephro- and ototoxicity. With regard to drug delivery, liposomal encapsulation of aminoglycosides has attracted considerable interest.

2. Liposomes

Liposomes are spherical vesicles, with particle sizes ranging between 30 nm up to several microns, consisting of one or more lipid bilayers surrounding aqueous spaces. Hydrophilic drugs, like aminoglycosides, can be encapsulated in the internal aqueous compartment, whereas hydrophobic drugs may bind to or are incorporated in the lipid bilayer. The bilayers are usually composed of (natural or synthetic) phospholipids and cholesterol but the incorporation of other lipids (and derivatives) as well as proteins is also possible [13-15]. The physicochemical characteristics of the liposome, like particle size, surface charge, sensitivity to pH changes, and bilayer rigidity, can be manipulated. Manipulation of these characteristics can have marked effects on the in vivo behavior of liposomes and therefore have a major impact on therapeutic success. Liposomes have also been studied as model

membranes regarding the interaction of aminoglycosides with phospholipids in relation to aminoglycoside toxicity [16-19]. The present review will exclusively focus on liposomes as a drug delivery system for aminoglycosides.

3. In vitro data

3.1 Extracellular bacteria

The first literature, reporting on liposome-encapsulated aminoglycosides, appeared approximately twenty years ago. Regarding the antibacterial activity of liposomal antibiotics against extracellular bacteria, variable results have been reported. It is generally shown that the concentration of the liposome-encapsulated aminoglycoside to obtain growth inhibition and killing needs to be substantially higher compared to the free drug [20-22]. The encapsulation of the antibiotic reduces the antibacterial activity, because the bacteria are separated from the antibiotic by the liposomal bilayer. The variability in the in vitro data is probably the consequence of the variations in liposome lipid compositions used, leading to various release profiles for the encapsulated agents.

In contrast to this generally observed reduction of antibacterial activity, Beaulac et al. reported that a liposome formulation composed of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylglycerol encapsulating tobramycin showed a considerable antibacterial effect against a range of Gram-positive and Gram-negative bacteria at concentrations below the minimum inhibitory concentration of the free antibiotic in vitro [23]. They reported that the enhanced antibacterial effect may be due to a fusion mechanism of this liposome formulation with bacteria [24].

3.2 Intracellular bacteria

In vitro studies using intracellularly infected phagocytic cells demonstrated that the phagocytosis of aminoglycoside-loaded liposomes yielded therapeutic intracellular drug concentrations [25], and consequently enhanced killing of intracellular micro-organisms such as *Staphylococcus aureus* [26, 27], *Escherichia coli* [28], *Brucella abortus* [29-31], *Brucella canis* [30], and *Mycobacterium avium* complex (MAC) [32-35]. A recent report addressed

the possibility to further improve liposomal drug efficacy towards infected cells. Liposomes encapsulating gentamicin composed of pH-sensitive dioleoylphosphatidylethanolamine-based bilayers, showed an improved antibacterial effect against intracellular *Salmonella typhimurium* and *Listeria monocytogenes* in murine macrophage-like J774A cells as compared to non pH-sensitive liposome formulations [36]. It is believed that the pH-sensitivity of the liposomes promotes drug release in the acidic environment of the lysosomes after phagocytosis by the infected cells.

4. Local application

Local application of large, multilamellar aminoglycoside-containing liposomes exploits the possibility to utilize liposomes as a reservoir from which the encapsulated drug can be slowly released, resulting in therapeutically active drug concentrations that are present at the site of infection for prolonged periods of time. Research in this area has focussed on intravitreal or subconjunctival injection or topical application of liposomes for treatment of bacterial endophtalmitis or keratitis [37-43]. All studies reported prolonged presence of therapeutic aminoglycoside concentrations compared to administration of the free drug, offering the opportunity to reduce the number of injections necessary for successful treatment. Besides, systemic drug levels remained low. Research has been done mainly in rabbits but a single study reported excellent therapeutic results regarding eye-infections in AIDS patients [44].
Similar results, as obtained in the ophthalmic studies, were reported after (prophylactic) application of aminoglycoside-loaded liposomes in models of soft tissue infection, burn wounds, prosthetic vascular grafts or surgical wound infections [45-50], and intrabronchial/intratracheal administration of liposomal aminoglycosides in rodents [51-54]. After intrabronchial administration, liposome-encapsulated tobramycin was shown to eradicate mucoid *Pseudomonas aeruginosa* in a model of chronic pulmonary infection [53]. Interestingly, treatment results were dependent on the lipid composition of the liposomal formulation. Free tobramycin as well as tobramycin encapsulated in liposomes with rigid lipid bilayers showed no bactericidal effect, whereas tobramycin in liposomes composed of fluid lipid bilayers was able to eliminate the bacteria. These data are in agreement with data

from in vitro experiments that have shown that fluid liposomes tend to release encapsulated aminoglycosides faster compared to their rigid counterparts [54].

5. Intravenous administration

5.1 Conventional liposomes

5.1.1 Circulation kinetics and Tissue distribution

Extensive research on liposome behavior after i.v. administration has shown that many liposome types rapidly accumulate in the cells of the mononuclear phagocyte system (MPS), particularly in the liver and spleen [55-57]. It is believed that the relatively rapid clearance of the liposomes is the result of opsonization in the bloodstream facilitating MPS-recognition and uptake [58, 59]. Such liposomes are generally termed 'conventional' liposomes. The rate by which conventional liposomes are taken up by the MPS can be manipulated by controlling the liposome dose, but also by variation of liposomal characteristics such as charge, size, and lipid composition. Generally, large, charged liposomes composed of fluid lipid bilayers tend to accumulate in the MPS more rapidly than small, neutral, rigid liposomes [60]. With the objective to reduce the MPS-uptake of conventional liposomes, it has been shown that by increasing the liposome dose, the amount of liposomes that remain in the circulation can be increased due to saturation of MPS-uptake [61]. However, saturation of the MPS needs to be avoided as it will impair the system's ability to clear infectious organisms from the circulation, which is exceedingly important in patients with severe infections [62, 63].

The pharmacokinetics of i.v. administered conventional liposome-encapsulated aminoglycosides generally show that plasma half-lifes are prolonged compared to the free drug [64-68]. The blood levels reported in some representative studies of (liposomal) aminoglycosides are shown in figure 1. Free and liposome-encapsulated drug were administered at equivalent doses. It is important to realize that when injected in the free form the aminoglycoside is completely therapeutically active, while after injection of the liposome-encapsulated form only the released part is expected to show antimicrobial activity. The tissue distribution of aminoglycosides is remarkably changed upon liposomal

encapsulation as is illustrated in figure 2, again free and liposome-encapsulated drug were administered at equivalent doses. Renal concentration of aminoglycosides is approximately similar after administration in either the free or liposome-encapsulated form, whereas much higher concentrations are observed in liver and spleen after injection of the liposome-encapsulated aminoglycosides. The absolute uptake of the liver exceeds that of the spleen considering its weight. Swenson et al. reported measurable gentamicin levels in liver and spleen up to 2 and 15 weeks after injection of a single dose of 20 mg/kg liposomal gentamicin, respectively [66]. Concentrations in other organs achieved with these conventional liposomes are generally insignificant. However, a few reports indicated increased concentrations in the lung [65, 68]. Interestingly, Ladigina and Vladimirski showed that in the lungs of mice infected with *Mycobacterium tuberculosis*, a six-fold increase in the amount of drug localizing in the infected lungs was seen for the liposome-encapsulated aminoglycoside [65]. However, absolute drug concentrations remained low.
It has been suggested that after liposome uptake and processing by the MPS-cells, the drug may be released in the blood, prolonging drug blood levels. Bermudez et al. showed that substantial urinary excretion of amikacin continued for up to 7 days after injection of 50 mg/kg liposomal amikacin, whereas mice that received an equivalent dose of the free drug excreted most of the administered dose within the first day and had an undetectable level in the urine by day 4 [69]. Similar results were obtained by Swenson et al., showing increasing cumulative gentamicin urinary excretion up to 10 days after injection of 20 mg/kg liposomal gentamicin. Even at that time point, only 80% of the injected dose was cumulatively excreted [66].

5.1.2 Safety

Considering the prolonged presence of aminoglycosides in the body, it is unfortunate that studies on nephro- or ototoxicity of 'conventional' liposomal formulations of aminoglycosides are lacking. There are, however, reports comparing the acute toxicity (characterized by convulsions or death as a result of neuromuscular blockade) of free versus liposome-

encapsulated aminoglycosides. Without exception all studies showed a substantial reduction in acute toxicity for the liposome-encapsulated drug [67, 69-71].

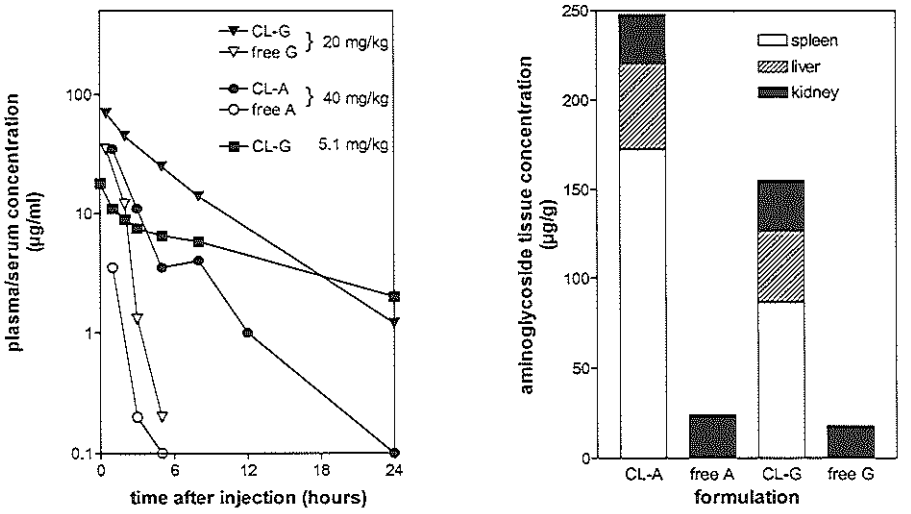

Figure 1. **A (left). Circulation kinetics of conventional liposome (CL)-encapsulated aminoglycosides (closed symbols) and free aminoglycosides (open symbols).** Mean aminoglycoside concentrations at indicated time-points after injection of a single dose of 20 mg/kg gentamicin in rats (triangles), 40 mg/kg amikacin in mice (circles), or 5.1 mg/kg gentamicin in AIDS-patients (squares). Adapted from references [66, 68, 80], respectively.
B (right). Tissue distribution of conventional liposome (CL)-encapsulated aminoglycosides and free aminoglycosides. Mean concentrations in tissues at 24 h after injection of a single dose of 20 mg/kg gentamicin (G) in rats and 40 mg/kg amikacin (A) in mice. Adapted from references [66, 68], respectively.

5.1.3 Therapeutic efficacy

Generally, aminoglycosides are not the drug of choice for treating intracellular infections inside phagocytic cells due to their polar character. However, conventional liposomes readily accumulate in the MPS [72-74]. Therefore, aminoglycoside-loaded conventional liposomes were initially studied in in vivo models of intracellular infections inside the MPS-cells. An

overview of treatment results achieved with conventional liposome formulations is presented in Table 1.

Promising results are reported regarding a bactericidal effect in the liver and spleen in intracellular infections caused by *Mycobacterium* spp., *Salmonella* spp., and *Brucella* spp. [26, 67-71, 75-87]. A pH-sensitive liposome formulation further increased therapeutic efficacy in liver and spleen in a murine intracellular *Salmonella typhimurium* infection [88]. In some studies a reduced bacterial load in lungs, blood, and/or kidneys was also reported, but the antibacterial effects in these organs were always less pronounced and were only achieved at higher dosages. These results illustrate the 'strength and weakness' of conventional liposomes as carrier system of antibiotics. On the one hand, liposome-encapsulated aminoglycosides are very efficiently transported into the MPS-cells in liver and spleen and consequently high intracellular concentrations can be achieved resulting in good therapeutic efficacy as shown by prolonged survival and the opportunity to increase the dosing interval. On the other hand, due to the relatively fast and efficient uptake of the liposomes by the MPS-cells relatively low therapeutically active drug levels are seen in organs outside the liver and spleen. As a result, only moderate therapeutic effects are observed in these organs.

A limited number of reports describe the therapeutic efficacy of conventional liposomes encapsulating aminoglycosides directed against infectious foci outside the cells of the MPS. The prolonged presence of drug in the body after administration of conventional liposome-encapsulated aminoglycosides has been the rationale to study the prophylactic activity against extracellular bacterial infections. Swenson et al. showed that the dose of liposome-encapsulated gentamicin needed for protection against a lethal i.p. infection caused by *Klebsiella pneumoniae* or *Escherichia coli* was substantially lower than for the free drug, when administered from 7 up to 2 days before bacterial inoculation [66]. This result is not surprising, as the free drug is almost completely excreted within 24 h after injection. In a single dose study in a murine model of a *Klebsiella pneumoniae* a single dose of 20 mg/kg liposome-encapsulated gentamicin was more effective than an 80 mg/kg dose of free drug

[89]. The prolonged residence time of gentamicin in the body by liposome-encapsulation is probably responsible for the enhanced efficacy.

5.2 Long-circulating liposomes

5.2.1 Circulation kinetics and Tissue distribution

To enable the liposomes to reach infectious sites outside the major MPS-organs, such as the liver and spleen, it is necessary to decrease the liposome uptake by the phagocytic cells. One way to reduce MPS uptake is to prepare small, neutral vesicles with a rigid bilayer. Using this approach, NeXstar Pharmaceuticals (currently Gilead Sciences Inc.) have developed MiKasome®, a small (approx. 50 nm) unilamellar liposome formulation containing amikacin. This formulation is currently in clinical trials. Another approach to prolong circulation time of liposomes is the incorporation of poly(ethylene glycol) (PEG) coupled to phosphatidylethanolamine in the liposome bilayers. It is believed that the hydrophilic PEG provides a layer of steric hindrance around the liposome reducing liposome opsonization and thereby rapid recognition and uptake by the MPS-cells. These liposomes are therefore termed 'sterically stabilized liposomes' (SSL). The low MPS-uptake of the SSL is to a high degree irrespective of liposome lipid composition, which is an important advantage when tuning the liposome lipid composition for optimal targeting, retention, and release [90-97]. Using this approach in our laboratory, we have developed a long-circulating sterically stabilized liposome formulation containing gentamicin [98]. In the case of MiKasome®, such flexibility in tailoring the liposome characteristics does not apply, as the lipid composition of MiKasome® is restricted to a rigid membrane structure to retain its long-circulation behavior.

Studies with aminoglycosides encapsulated in both types of LCL show that drug plasma half-lifes are markedly prolonged. Blood levels obtained for MiKasome® and SSL-gentamicin are shown in Figure 3. Studies in rats receiving 50 mg/kg MiKasome® demonstrated that the AUC in plasma is increased approx. 130-fold compared to the AUC of an equivalent dose of free amikacin [99]. These findings were generalized to rabbits, dogs, rhesus monkeys and humans [100, 101]. In man, the plasma half-life is 114 h. After 1 week of daily dosing with

2.5 mg/kg/d or 5 mg/kg/d plasma concentrations were 120 and 215 µg/ml, respectively. One week later, plasma concentrations still amounted to 10-20 µg/ml. Yet, the concentrations of free amikacin released from the liposome never exceeded 4 µg/ml. Our experimental studies with SSL-gentamicin showed a similar picture: a 70- to 130-fold increase in AUC compared to the free drug in rats [98].

The tissue distribution of aminoglycosides is remarkably changed after administration in the

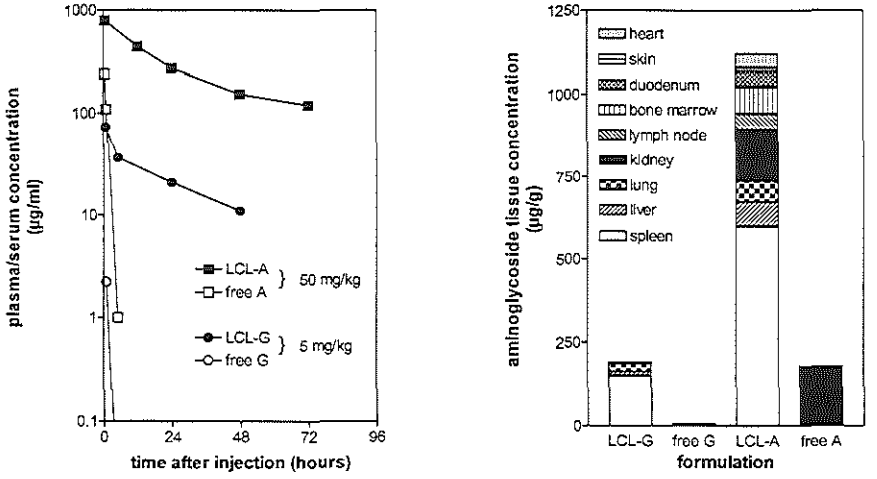

Figure 3. A (left). Circulation kinetics of long-circulating liposome (LCL)-encapsulated aminoglycosides (closed symbols) and free aminoglycosides (open symbols). Mean aminoglycoside concentrations at indicated time-points after injection of a single dose 5 mg/kg gentamicin in rats (circles) or 50 mg/kg amikacin in rats (squares). Adapted from ref [98, 99], respectively.

B (right). Tissue distribution of long-circulating liposome (LCL)-encapsulated aminoglycosides and free aminoglycosides. Mean concentrations in tissues at 24 h after injection of a single dose of 5 mg/kg gentamicin (G) in rats (data are based on liposome distribution, note the increased liposome levels in the infected lung tissue; organs shown are the only organs investigated) or 50 mg/kg amikacin (A) in rats. Adapted from ref [98, 99] respectively.

liposome-encapsulated form for both types of LCL, as is illustrated in Figure 4. Equivalent doses of free and liposome-encapsulated drug were administered. Relatively high tissue concentrations are seen in the liver and spleen compared to free drug. In addition, higher drug concentrations are observed in other organs as bone marrow, lungs, intestine, lymph nodes, skin, and heart. MiKasome® has been recovered from microvacuolated macrophages in most tissues after injection, which suggests that phagocytic cells could serve as a depot of amikacin. The urinary recovery of unchanged amikacin after injection in the MiKasome® formulation is dramatically reduced compared to that in case of the free drug. Whereas practically all amikacin is excreted within 24 h after injection of the free drug, MiKasome® showed less than 40% recovery in urine by day 10 [100].

In addition to the reduced affinity of LCL for the MPS and increased localization in other organs, it was demonstrated in our laboratory by Bakker-Woudenberg et al. that in a rat model of a unilateral pneumonia caused by *Klebsiella pneumoniae*, the localization of SSL in the infected left lung was approximately 4-fold higher than the localization in the contralateral non-infected right lung [102]. In the same model, 10-fold lower levels of infected lung localization were observed when liposomes with a relatively short circulation time were used [103]. Recent studies, indicate that a prolonged liposomal circulation time is essential for substantial target site localization. An increase in AUC of the liposome formulation, achieved by tuning the lipid composition, was reflected by a proportional increase in localization at the infectious focus [97]. Similar findings of selective liposome localization at the target site in models of inflammation such as adjuvant arthritis, osteomyelitis, intraabdominal abscesses, colitis, allergic encephalomyelitis, focal thigh infection, and contact hypersensitivity have been reported [104-113]. The selectivity of the localization of LCL at the site of infection or inflammation is mediated by the locally increased capillary permeability as a result of the inflammatory response [114-116]. The nature of the inflammatory stimulus seems not important as lung instillation of HCl and lipopolysaccharide also induced increased capillary permeability and effected liposome localization [116]. A contribution of infiltrating inflammatory cells to selective liposome target site localization has been suggested by some authors [105, 115]. Studies in the

unilateral *K. pneumoniae* model indicate that the contribution of infiltrating inflammatory cells is not required for substantial liposome target site localization, as the degree of localization was similar in leukopenic rats as well as in immunocompetent rats [116]. This is an important observation as these results would indicate that targeted liposomal drug delivery could also be beneficial to immunocompromised patients, who suffer from severe infections and show a high risk of antimicrobial treatment failure.

5.2.2 Safety

Regarding the safety of long-circulating liposomal aminoglycosides, much work has been done on MiKasome®. Parameters tested in a one month study with daily or every third day injection of MiKasome® in Beagle dogs were based on clinical chemistry, hematology, urine analysis, and coagulation together with body weights, clinical observations, and vital signs. Gross necropsy and histopathologic examination of tissues was performed at the end of the study period [100]. Daily doses of 20 mg/kg or every third day doses of 60 mg/kg were not associated with the occurrence of adverse effects despite steady state plasma concentrations above 750 µg/ml and trough levels above 600 µg/ml. Surprisingly, kidney concentrations above 1 mg/g did not lead to elevation of blood urea nitrogen or creatinine concentrations. The study shows that the ratio of cortical to medullary amikacin was substantially reduced by liposome encapsulation compared to the free drug. Therefore, it appears that liposome-encapsulation results in a different kidney localization, preventing aminoglycoside-induced nephrotoxicity [100].

A clinical safety-study in HIV-positive patients showed that after one week of daily dosing of 2.5 mg/kg and 5 mg/kg, plasma levels were approximately 120 µg/ml or 215 µg/ml, respectively. Plasma amikacin levels of 10-20 µg/ml persisted for two weeks after the last dose. However, no renal or audiovestibular toxicity was noted in any of the subjects participating in the study [100].

Administration of SSL-gentamicin in rats, showed acute toxicity after a single dose of 40 mg/kg, characterized by convulsions. A similar dose of SSL-gentamicin showed no acute toxicity [117].

5.2.3 Therapeutic efficacy

Results of the treatment studies with aminoglycosides encapsulated in LCL are shown in Table 2 [117-122]. The overall conclusion is that the efficacy of LCL-encapsulated aminoglycosides is superior over the free drug or conventional liposome-encapsulated aminoglycosides. Most studies relate to the use of MiKasome®. The long half-life of LCL in the circulation allows for prolonged dosing intervals or even single dose treatments. A clinical trial in urinary tract infection (UTI) patients shows that a single dose of 40 mg/kg MiKasome® produced a high cure rate and the efficacy was comparable to seven daily infusions of 10 mg/kg [118]. In two rabbit models of endocarditis, it was shown that single daily doses of MiKasome® improved survival and were as efficient in reducing bacterial numbers as twice daily doses of the free drug [119, 120]. In contrast, the rate of vegetation sterilization was higher in the animals treated with the free drug, probably as a result of the complete therapeutic availability of the free drug in the circulation. In immunocompromised mice, the relatively high tissue concentrations of MiKasome® are probably responsible for the enhanced prophylactic activity of the liposomal drug in prolongation of survival and reduction in bacterial numbers (also outside the liver and spleen) [121]. The studies related to SSL-gentamicin demonstrated in a rat *Klebsiella pneumoniae* pneumonia model that the therapeutic efficacy was clearly superior to the free drug in a single dose schedule [98]. Evaluation of its efficacy in a multidose schedule in leukopenic rats showed that addition of a single dose of SSL-gentamicin to free gentamicin treatment showed complete survival, using a 7-fold lower cumulative amount of gentamicin compared to treatment with free gentamicin alone. In leukopenic rats infected with low gentamicin-susceptible *K. pneumoniae*, free gentamicin at the maximum tolerated dose did not result in survival. Addition of SSL-gentamicin was needed for therapeutic success. Complete survival was obtained adding an SSL-gentamicin formulation with a fluid lipid bilayer, adding a rigid SSL gentamicin formulation showed only 50% survival. The increased gentamicin release from the fluid liposomes, improved rat survival, thus showing the importance of liposome lipid composition for therapeutic efficacy [117].

Table 1. Clinical and preclinical therapeutic efficacy of aminoglycosides in conventional liposomes.

INFECTION	STUDIED IN	DRUG USED	RESULT	COMMENTS
Intracellular *Brucella canis* [30], *B. abortus* [30, 86], and *B. melitensis* [87] (liver and spleen)	Swiss mice [30, 86, 87], Hartley strain guinea pigs [30]	Gentamicin [86, 87], Streptomycin [30]	Compared to free drug: reduction of number of bacteria in spleen [30, 87], liver [87] and other organs [30], prolonged survival [86], and high drug levels in liver and spleen [87]	Empty cationic liposomes did also prolong survival [86]
Intracellular *Salmonella typhimurium* [66], *S. dublin* [71], and *S. enteritidis* [67, 85] (liver and spleen)	BALB/c [66, 71], Swiss [85], C57BL/6 [67] mice	Gentamicin [66, 71], Streptomycin [67, 85]	Compared to free drug: prolonged survival [66, 67, 71, 85], reduced acute toxicity [67, 71], and high drug levels in liver and spleen [85]	No reduction of number of bacteria in lung compared to free drug [85]
Klebsiella pneumoniae sepsis, pneumonia, and thigh infection [66, 89] *Escherichia coli* sepsis [66]	(neutropenic) CD-1 mice [66, 89]	Gentamicin [66, 89]	Compared to free drug: enhanced therapeutic efficacy in *Klebsiella pneumoniae* pneumonia and thigh infection in neutropenic animals [89], prolonged survival when administered prophylactically [66], prolonged dosing interval allowed [89]	Similar efficacy of free and liposomal drug when administered immediately after inoculation [66]
Intracellular *Mycobacterium avium-intracellulare* complex [68, 69, 75-84], *M. tuberculosis* [70] (lung, liver, spleen)	AIDS patients [80, 81], Beige [68, 69, 75-79], SCID [82], C57BL/6 [82, 83], BALB/c [70, 82, 84] CBB6F1 [70] mice	Gentamicin [69, 76, 79-81], Amikacin [68, 69, 75, 79, 82, 83], Streptomycin [70, 77, 78], Kanamycin [84]	Compared to free drug: reduction of number of bacteria in liver, spleen [68-70, 75-79], blood [80], lung [84] and kidneys [75, 84], prolonged survival [70, 82, 83], reduced acute toxicity [69, 70], prolonged dosing interval allowed [82, 83] reduction in pulmonary lesions [84]	No reduction of number of bacteria in lung [68, 70, 75-79, 82, 83] or lymph nodes [75]. Transient renal insufficiency in 1 patient [80], no reduction of number of bacteria in any of the bone marrow core biopsy specimens [81]

Table 2. Clinical and preclinical therapeutic efficacy of aminoglycosides in long-circulating liposomes.

INFECTION	STUDIED IN	DRUG USED	RESULT	COMMENTS
(low-susceptible) *Klebsiella pneumoniae* pneumonia [98, 117]	(neutropenic) RP rats [98, 117]	Gentamicin [98, 117]	Compared to free drug: prolonged survival [98, 117], reduction of number of bacteria in lung [98, 117] and blood [117]	In this model selective liposome localization in the infected tissue was demonstrated [102]
Complicated UTI [118]	UTI patients [118]	Amikacin* [118]	Good bacterial and clinical cure rates. High dose single infusion as efficient as low dose daily infusions. No significant side-effects noted [118]	Trial is ongoing with two fixed doses of 2 g and 3 g amikacin in MiKasome® formulation [118]
Staphylococcus aureus endocarditis [119], *Pseudomonas aeruginosa* endocarditis [120]	New Zealand White rabbits [119, 120]	Amikacin* [119, 120]	Compared to free drug: prolonged survival [120], prolonged dosing interval allowed regarding vegetation density, relapse, reduction of renal and splenic abscesses [119, 120]	Treatments were combined with suboptimal doses of oxacillin. Both combinations preserved myocardial function [119]. Rate of vegetation sterilization was higher for free drug compared to liposome-encapsulated drug [120]
Klebsiella pneumoniae sepsis [121]	neutropenic mice [121]	Amikacin* [121]	Compared to free drug: prolonged survival, superior prophylactic activity, reduction of number of bacteria in liver and lung [121]	
Mycobacterium avium complex (lung, liver, and spleen) [122]	Beige mice [122]	Streptomycin [122]	Conventional and long-circulating liposomes were equipotent in reduction of number of bacteria in spleen, liver and lung [122]	Liposomal circulation times not investigated [122]

* the liposomal form of amikacin used in these studies was MiKasome ®.

Only one single study failed to show a superior effect of LCL-encapsulated aminoglycoside compared to conventional liposomal drug in the treatment of MAC infection [122]. Unfortunately, the preparations used in this study were not characterized with respect to their circulation time as well as their tissue distribution, so the underlying cause of the disappointing results cannot be traced.

6. Concluding remarks

Liposome-encapsulated aminoglycosides offer interesting prospects to increase the therapeutic index of this class of antibiotics. The local application of liposomes may provide a reservoir to prolong therapeutic drug concentrations at the site of infection. Accessible infected tissues such as eye, wound, and lung could benefit from this local administration. In order to optimize therapeutic efficacy it is of importance to balance drug release from and drug retention in the liposome. Specific liposome compositions may enhance bacterial killing by interacting with the infectious organism.

As the largest number of conventional liposomes is taken up by the MPS after intravenous administration, the targeted delivery of drugs to MPS-cells in the liver and spleen seems to be the most relevant application of this liposome type. Treatment of intracellular infections in the MPS-cells may benefit from the high amounts of aminoglycosides that can be delivered intracellularly. By making liposomes pH-sensitive, the therapeutic availability of the liposome-encapsulated phagocytized drug may even be increased. Research is needed on the nephro- and ototoxicity of conventional liposomal aminoglycosides, with respect to their prolonged presence in the body. This research should also include the potential danger of promoting microbial resistance as a result of the prolonged exposure of the resident microbial flora to the drug.

In case the infectious focus is located outside the MPS, conventional liposomes are of limited use. Therefore, research has been aimed at decreasing the MPS-uptake of liposomes and consequently increasing their circulation time. LCL were the result of these efforts. Intravenous administered LCL potentially offer drug targeting to sites of infection not

restricted to the MPS. A number of reports have demonstrated enhanced therapeutic efficacy of LCL-encapsulated aminoglycosides compared to free drugs or conventional liposomes. Unfortunately however, most studies with liposome-encapsulated aminoglycosides have, up to now, been performed in animal models with an intact host defense infected with high antibiotic-susceptible bacteria. Treatment failure in clinical practice, however, particularly occurs in patients having an impaired host defense or in patients infected with a low antibiotic-susceptible infectious organism. A single study addressed both issues in determining the efficacy of SSL-gentamicin [117]. These issues should be incorporated more in animal models to demonstrate the value of liposomes in clinically relevant settings. So far, MiKasome has shown an excellent safety profile. Yet, similarly as for the conventional liposome formulations, the effects that the prolonged tissue drug concentrations have on development of resistance need to be addressed. The results that have been reviewed sketch promising prospects for liposome-encapsulated aminoglycosides and warrant further clinical investigations into the use of these formulations for the treatment of severe infections.

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6. Therapeutic efficacy of liposome-encapsulated gentamicin in rat *Klebsiella pneumoniae* pneumonia in relation to impaired host defense and low bacterial gentamicin-susceptibility

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Summary

Long-circulating liposomes may be used as targeted antimicrobial drug carriers as they localize at sites of infection. As a result, liposome-encapsulated gentamicin (LE-GEN) has demonstrated superior antibacterial activity over the free drug (GEN) in a single dose study in immunocompetent rats with a *Klebsiella pneumoniae* pneumonia. In the present study, the therapeutic efficacy of LE-GEN was evaluated, by monitoring rat survival and bacterial counts in blood and lung tissue, in clinically relevant models, addressing the issue of impaired host defense and low bacterial antibiotic susceptibility. Results show that in immunocompetent rats infected with the high gentamicin-susceptible *K. pneumoniae*, a single dose of LE-GEN is clearly superior over an equivalent dose of free GEN. Yet, complete survival can also be obtained with multiple dosing of free GEN. In leukopenic rats infected with the high gentamicin-susceptible *K. pneumoniae*, free GEN at the maximum tolerated dose (MTD) was needed to obtain survival. However, with addition of a single dose of LE-GEN to free GEN-treatment complete survival can be obtained using a 7-fold lower cumulative amount of gentamicin compared to free GEN-treatment alone. In leukopenic rats infected with low gentamicin-susceptible *K. pneumoniae*, free GEN at the MTD did not result in survival. The use of LE-GEN is needed for therapeutic success. Increasing LE-GEN bilayer fluidity, resulted in an increased gentamicin release from the liposomes and hence improved rat survival, thus showing the importance of liposome lipid composition for therapeutic efficacy. These results warrant further clinical studies of liposomal formulations of aminoglycosides in immunocompromised patients with severe infections.

1. Introduction

Clinical practice shows that failure of antimicrobial treatment is not uncommon. Two major risk factors can be identified: an impaired host defense and a moderate to low antibiotic-susceptibility of the infectious organism(s) (4, 6, 8, 18). An impaired host defense increases the patients' susceptibility to infections. In addition, a limited ability of the host defense to support antimicrobial treatment increases the chance of treatment failure (15). A low antibiotic-susceptibility of bacteria can result in sub-effective drug concentrations at the site of infection despite high drug doses (8).

Targeted antibacterial drug delivery may increase drug concentrations at the infectious focus and therefore help to reduce the treatment failure risks imposed by the impaired host defense or low bacterial susceptibility. Besides, in case of potentially toxic antibiotics, toxicity to non-target tissues may be reduced which enables the use of higher doses. Liposomes have attracted considerable interest as targeted drug carriers in infectious diseases. A number of studies have convincingly demonstrated that so-called long-circulating liposomes tend to localize preferentially at foci of infection or inflammation after intravenous administration (1, 9, 11, 17, 19). The preferential localization appears to be the result of the inflammatory response provoking a locally increased capillary permeability allowing liposome extravasation. Generally, intravenously administered liposomes that are rapidly opsonized and taken up by the mononuclear phagocyte system (MPS), hardly localize at foci of infection outside the MPS. By coating the liposomal surface with poly(ethylene) glycol (PEG), opsonization and subsequent MPS-uptake is reduced thus prolonging circulation time and interaction with the infectious target site. As a result, these long-circulating PEG-coated liposomes (LCL) show superior target localization characteristics (19). Therefore, LCL have attractive prospects for the site-specific delivery of antimicrobial agents. Previous research has shown that LCL-encapsulated gentamicin (LE-GEN) demonstrates superior antibacterial efficacy compared to free gentamicin (free GEN) in a single dose study in rats with an intact host defense with a unilateral *Klebsiella pneumoniae* pneumonia (3).

In the present study, the antibacterial efficacy of LE-GEN was evaluated in clinically more relevant models addressing the issue of impaired host defense and low antibiotic susceptibility. Rat survival and bacterial counts in lung tissue and blood were monitored in immunocompetent as well as leukopenic rats with a pneumonia caused by high or low gentamicin-susceptible *Klebsiella pneumoniae*.

2. Material and Methods

2.1 Liposome preparation and characterization

Liposomes were prepared as described previously (3). In brief, appropriate amounts of partially hydrogenated egg phosphatidylcholine (PHEPC) (Asahi Chemical Industry Co. Ltd., Ibarakiken, Japan), cholesterol (Chol) (Sigma Chemical Co., St. Louis, Missouri), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethylene glycol-2000] (PEG-DSPE) (Avanti Polar Lipids, Alabaster, Alabama), in a molar ratio of 1.85:1:0.15 respectively, were dissolved in a mixture of chloroform and methanol to obtain 'rigid' liposomes. To obtain 'fluid' liposomes, egg phosphatidylcholine (EPC) (Asahi Chemical Industry Co. Ltd. Ibarakiken, Japan) and PEG-DSPE were dissolved in a molar ratio of 2.85:0.15, respectively. After evaporation of the solvent under constant rotation and reduced pressure, the lipid mixture was dried under nitrogen, dissolved in 2-methyl-2-propanol (Sigma Chemical Co., St. Louis, Missouri) frozen, and freeze-dried overnight. The resulting lipid film was hydrated for 2 h in 2.5 ml aqueous 200 mg/ml gentamicin (Duchefa Biochemie b.v., Haarlem, The Netherlands), subsequently 7.5 ml Hepes/NaCl buffer (10 mM Hepes) (Sigma Chemical Co., St. Louis, Missouri) and 135 mM NaCl (Merck, Darmstadt, Germany)) pH 7.4 was added. The hydrated lipids were sonicated for 8 minutes with amplitude of 8 µ using a 9.5 mm probe in an MSE Soniprep 150 (Sanyo Gallenkamp PLC, Leicester, UK). Dynamic light scattering, detected at an angle of 90° to the laser beam on a Malvern 4700 System (Malvern Instruments Ltd., Malvern, UK), was used to evaluate the particle size distribution. Liposomes were obtained with a mean particle size of 100 nm. In addition to the mean particle size, the system reports a polydispersity index (a value between 0 and 1). A polydispersity index of 1 indicates large variations in particle size, a reported value of 0 means that size variation is apparently absent. All liposome preparations used had a polydispersity index below 0.3. Non-encapsulated GEN was removed by ultracentrifugation in two changes of Hepes/NaCl buffer at 265,000 x g for 2 h at 4 °C in a Beckman ultracentrifuge L-70 (Beckman, Palo Alto, California). Phosphate concentration was determined spectrophotometrically according to Bartlett (5). Total (encapsulated and free) and unencapsulated (free) GEN was measured using a diagnostic sensitivity test agar (Oxoid, Basingstoke, UK) diffusion test with *Staphylococcus aureus* Oxford strain (ATCC 9144) as the indicator organism and standards ranging from 4 µg/ml to 0.25 µg/ml GEN, as described previously (3). For total (unencapsulated and encapsulated) GEN measurements, liposomes were destructed by 0.1% v/v (final concentration) Triton X-100 (Janssen Chimica, Geel, Belgium). Less than 10% of the GEN in the liposome dispersion was shown to be unencapsulated after ultracentrifugation. Specific activity was between 70-80 µg GEN/µmol total lipid.

2.2 Bacterial strains

The high gentamicin-susceptible *K. pneumoniae* (ATCC 43816, capsular serotype 2, MIC=0.5 µg/ml) was used. The MIC was determined by plating an inoculum of 10^4 cfu per spot on Mueller-Hinton (MH) agar (Difco laboratories, Detroit, MI, USA) plates containing two-fold dilutions of gentamicin, according to Woods et al. (20). The low gentamicin-susceptible *K. pneumoniae* (MIC=4 µg/ml) was obtained by

culturing the high-susceptible strain in Mueller-Hinton (MH) broth (Difco laboratories, Detroit, MI, USA) in the presence of increasing concentrations of gentamicin. The low gentamicin-susceptible strain appeared to be stable in vitro after repeated culture in antibiotic-free MH-broth.

2.3 Unilateral pneumonia

The animal experiments ethical committee of the Erasmus *university* Medical Center Rotterdam approved the experiments described in this study. Female RP/AEur/RijHsd strain albino rats, 18-25 weeks of age, body weight 185-225 g (Harlan, Horst, The Netherlands) with a specified pathogen free status were used. When indicated, rat host defense was impaired by i.p. injection of 60 mg/kg cyclophosphamide (Sigma, St. Louis, Missouri) every 4 days, starting at 5 days before bacterial inoculation. The leukopenic status of cyclophosphamide-treated animals was ascertained in separate experiments by measuring leukocyte counts in fresh blood samples, obtained by retro-orbital bleeding in EDTA-coated tubes. White blood cells were counted on a Cobas Minos Stex (Roche Haematology, Montpellier, France) using Minotrol™ 16 standards (Roche Haematology, Montpellier, France) to verify proper functioning of the instrument. As a result of the cyclophosphamide treatment, the number of leukocytes in the circulation at the day of bacterial inoculation was reduced 6-fold from approx. $6 \times 10^9 \pm 1 \times 10^9$ (buffer treated controls) to $1 \times 10^9 \pm 8 \times 10^8$ (mean ± standard deviation, n=3, $P<0.01$). Leukocyte counts remained reduced throughout the study period.

From 5 days to 1 day before bacterial inoculation, drinking water of the leukopenic rats was supplemented with 1 g/l cephalexin monohydrate (Dopharma, Raamsdonksveer, The Netherlands) to prevent superinfections. Drinking water in the remaining study period, and drinking water of immunocompetent rats was autoclaved water pH 3. At the time of bacterial inoculation, cephalexin concentrations were less than 1 µg/ml in the blood and lung tissue of rats that had received the drinking water supplemented with cephalexin (n=3 rats), as measured by agar diffusion test using an *Escherichia coli* as the indicator organism, as described previously (3).

A left-sided unilateral pneumonia was induced as has been described in detail elsewhere (2). In brief, rats were anesthetized and the left primary bronchus was intubated. Through the tube, 0.02 ml of a saline suspension of *K. pneumoniae* was inoculated in the left lung lobe. Inoculated bacteria were in the logarithmic phase of growth. Inoculum was adjusted such that the median survival of rats was comparable between the models. Rats with intact host defense were inoculated with 10^6 high gentamicin-susceptible *K. pneumoniae* (ATCC 43816, capsular serotype 2), leukopenic rats were inoculated with 10^5 high gentamicin-susceptible *K. pneumoniae*, or 10^7 low gentamicin-susceptible *K. pneumoniae*. The cephalexin-containing water did not have an effect on rat survival and did not have an effect on bacterial outgrowth. Rats were housed individually. In vivo stability of the phenotype of the low-susceptible *K. pneumoniae* was checked by culturing dilutions of homogenized left lung tissue obtained at 24 h after bacterial inoculation (the starting point of treatment) on MH-plates. Colonies were isolated and MIC was determined on MH-plates as described above (20). All out of 100 tested colonies had a stable low gentamicin susceptible phenotype after inoculation in vivo. The same procedure was applied to bacteria isolated at the end of the study period (after death of the rats or after 14 days). None of the treatments in this study resulted in a change of the MIC of the *K. pneumoniae* strains pre- and post-exposure.

2.4 Antimicrobial treatment

Indicated treatments were started at 24 h after bacterial inoculation. GEN was administered twice daily every 12 h (q12h) and LE-GEN was administered once daily every 24 h (q24h). The formulations were injected intravenously in the tail vein.

2.5 Survival
10 rats were used per experimental group. Survival of rats was examined every day until 14 days after bacterial inoculation. Blood of dead rats was cultured on Columbia III agar supplemented with 5 % sheep blood (Beckton-Dickinson) overnight at 37 °C. Substantial numbers of, exclusively, *K. pneumoniae* were recovered in the blood samples of dead rats.

2.6 Quantification of bacterial numbers in blood
At indicated time points after bacterial inoculation, blood samples were taken via retro-orbital bleeding in heparinized tubes on ice. Serial dilutions were prepared on ice and 0.2 ml of each dilution was applied on tryptone soy agar (TSA)-plates. Plates were incubated overnight at 37 °C and colonies were counted.

2.7 Quantification of bacterial numbers in left lung tissue
At indicated time points after bacterial inoculation, rats were sacrificed by CO_2-inhalation. The infected left lung was dissected and homogenized in 20 ml PBS (4 °C), supplemented with aminoglycoside acetylating enzyme, and 2 mM acetyl coenzyme A (sodium salt) (Sigma Chemical Co. St Louis, USA) to inactivate residual gentamicin present in the tissues, according to Den Hollander et al. (13). Serial dilutions were prepared and 0.2 ml of each dilution was applied on TSA-plates. Plates were incubated overnight at 37 °C and colonies were counted.

2.8 Pharmacokinetics and tissue concentrations of free GEN or LE-GEN
Free GEN or LE-GEN was injected i.v. in healthy rats at the indicated doses, used in the survival experiments, via the tail vein. Blood samples were taken at indicated time-points after injection from alternate groups of 3 rats via retro-orbital bleeding under CO_2-anaesthesia. Blood samples were collected in heparinized tubes and after centrifugation the plasma was collected. Drug concentrations were analyzed using the agar diffusion test as described above. The sample was divided into two portions, one was analyzed directly to determine the free (i.e. non-liposomal) drug concentration. The other was incubated with Triton X-100, as described above, to disrupt the liposomes in order to determine total (free + encapsulated) drug concentrations. Both liposome types used in the study did not show substantial drug leakage when mixed with plasma, and subsequently brought in agar wells and incubated overnight at 37 °C. As a result, free gentamicin concentrations could accurately be determined separately from the total gentamicin concentrations.
Total GEN-concentrations in lung tissue at different time-points after injection were analyzed by dissection of the infected left lung and uninfected right lung, homogenization of the tissue in PBS and subsequent incubation with 0.1 % Triton X-100 (final concentration) followed by agar diffusion test as described above.

2.9 Statistical analysis
Survival between experimental groups and controls was compared by the log-rank test (Graph Pad software Inc., San Diego, CA, USA).

3. Results

3.1 Rats with intact host defense infected with the high gentamicin-susceptible K. pneumoniae

In the first model, rats with an intact host defense were infected with the high gentamicin-susceptible *K. pneumoniae* (MIC=0.5 µg/ml). Treatment with either free GEN or LE-GEN was started at 24 h after bacterial inoculation. The survival rates are shown in Figure 1.

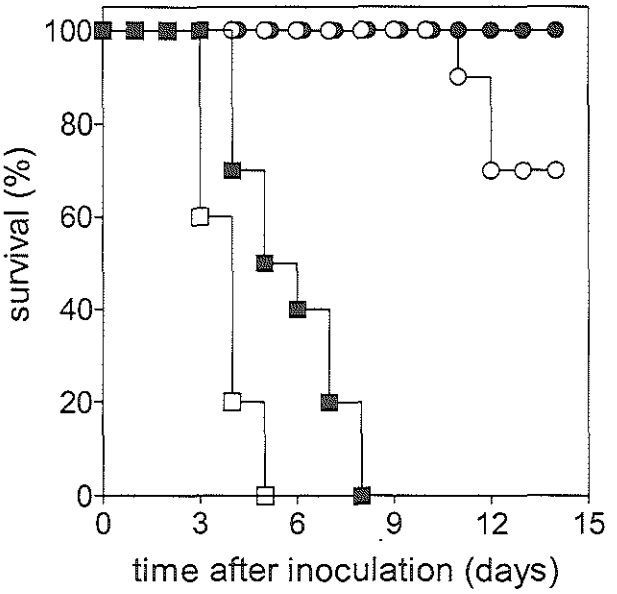

Figure 1. Effect of free GEN and LE-GEN on survival of rats with intact host defense infected with a high gentamicin-susceptible *K. pneumoniae*. Treatments: control (no drug treatment) (□), free GEN 5 mg/kg single dose (■), free GEN 5 mg/kg/d q12h for 3 days (●), or LE-GEN 5 mg/kg single dose (○).

Untreated control animals died between day 3 and day 5 after bacterial inoculation. A single dose of free GEN 5 mg/kg slightly prolonged survival ($P<0.01$ compared to controls) but all animals still rapidly died before day 8. An equivalent dose of LE-GEN yielded 70% survival after two weeks ($P<0.001$, compared to a single dose free GEN 5 mg/kg). The difference in efficacy between these two treatments is paralleled by the differences in gentamicin

concentrations in the lung tissue after injection. Already at 5 h after a single dose of GEN 5 mg/kg, gentamicin levels are below 1.5 µg/lung in either infected left lung or uninfected right lung. In contrast, an equivalent dose of LE-GEN results in total gentamicin concentrations of 7.9±0.8 µg/left lung and 4.0±2.1 µg/right lung at 5 h after injection. At 24 h, concentrations of 12.9±4.3 µg/left lung versus 5.5±1.2 µg/right lung were noted, whereas after 48 h concentrations were 16.2±3.9 µg/left lung versus 3.3±2.8 µg/right lung (mean ± S.D., n=5 to 9 rats per time point).

Treatment of rats with free GEN 5 mg/kg/d 2 daily doses every 12 h (q12h) for 3 days resulted in 100% survival.

3.2 Leukopenic rats infected with the high gentamicin-susceptible K. pneumoniae

Rat host defense was impaired, in the second model, by cyclophosphamide injections resulting in a 6-fold reduction in number of circulating white blood cells. These leukopenic rats were infected with the high gentamicin-susceptible *K. pneumoniae* (MIC=0.5 µg/ml). Treatment with either free GEN, LE-GEN, or a combination of both was started at 24 h after bacterial inoculation. The survival rates are shown in Figures 2 and 3.

Figure 2 shows that, similarly as in the previous model, untreated control animals died between day 2 and 4 after bacterial inoculation. The therapeutic regimen that resulted in complete survival in the previous model (free GEN 5 mg/kg/d q12h for 3 days) hardly prolonged survival in leukopenic rats. Free GEN 5 mg/kg/d q12h for 5 days prolonged survival during treatment, but after termination of treatment only 10% of the rats survived up to 14 days ($P<0.0001$ compared to control animals). Increasing the dose of free GEN to 20 mg/kg/d q12h for 5 days, showed 70% survival on day 14. A further increase to 40 mg/kg/d q12h for 5 days, which is the maximum tolerated dose (MTD), resulted in complete survival.

Figure 3 shows that addition of a single dose LE-GEN 5 mg/kg on day 1 to a 5-day treatment with free GEN 5 mg/kg/d q12h also resulted in 100% survival. The complete survival, obtained with that therapeutic regimen, is the result of the addition of liposomal gentamicin as the addition of free GEN on day 1 to the 5-day treatment with free GEN

showed only 30% survival on day 14 ($P<0.001$, addition of free GEN versus addition of LE-GEN). Treatment with LE-GEN only at 5 mg/kg/d once daily (q24h) for 5 days was not effective as the majority of rats already died during treatment.

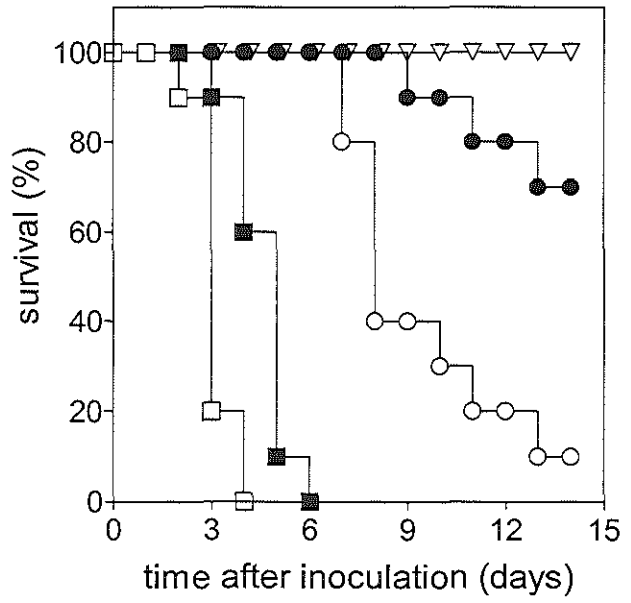

Figure 2. Effect of free GEN on survival of leukopenic rats infected with a high gentamicin-susceptible *K. pneumoniae*. Treatments: control (no drug treatment) (□), free GEN 5 mg/kg/d q12h for 3 days (■), free GEN 5 mg/kg/d q12h for 5 days (○), free GEN 20 mg/kg/d q12h for 5 days (●), or free GEN 40 mg/kg/d q12h for 5 days (▽).

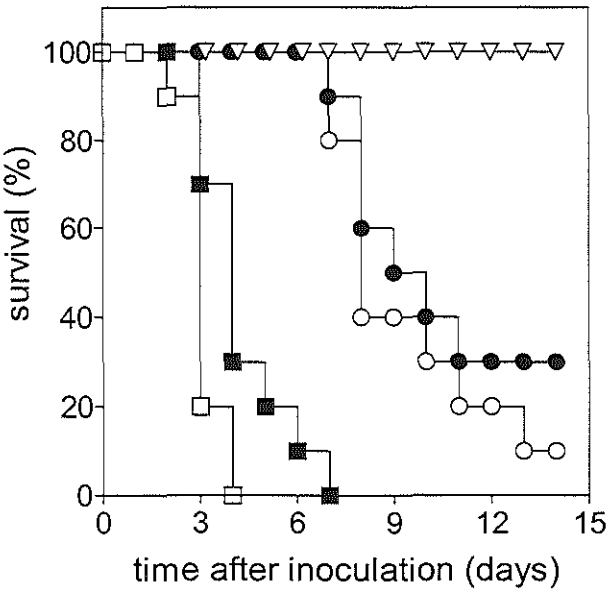

Figure 3. Effect of GEN and LE-GEN on survival of leukopenic rats infected with a high gentamicin-susceptible *K. pneumoniae*. Treatments: control (no drug treatment) (□), free GEN 5 mg/kg/d q12h for 5 days (○), free GEN 5 mg/kg/d q12h for 5 days plus a single dose of LE-GEN 5 mg/kg on day 1 (▽), free GEN 5 mg/kg/d q12h for 5 days plus a single dose of free GEN 5 mg/kg on day 1 (●), or LE-GEN 5 mg/kg/d q24h for 5 days (■).

The median number of bacteria (± range) recovered from the left lung and blood on day 0, 1, 6, 10, and 14 after bacterial inoculation of leukopenic rats infected with the high-gentamicin-susceptible *K. pneumoniae* are shown in Table 1. Rats received a 5-day treatment with free GEN 5 mg/kg/d q12h with addition of either a single dose of LE-GEN 5 mg/kg or free GEN 5 mg/kg on day 1. Median bacterial counts, 24 h after the last dose (day 6), in the left lung of rats treated with the combination free GEN + LE-GEN were 10-fold lower compared to rats treated with equivalent doses of only free GEN. In the following

days complete bacterial killing was achieved in rats treated with free GEN + LE-GEN, whereas the majority of rats treated with only free GEN died. Bacterial counts in blood on day 6 were approximately 100-fold lower for free GEN + LE-GEN treated animals compared to rats treated with only free GEN. Median bacterial blood counts stabilized in the following days in the rats that received free GEN + LE-GEN. Examination of bacterial counts in consecutive blood samples of individual rats revealed that the bacteremia was episodic in nature. The gentamicin-susceptibility of *K. pneumoniae* recovered from dead or surviving animals was not changed compared to the inoculated bacteria.

Table 1. Number of high gentamicin-susceptible *K. pneumoniae* in left lung and blood. Number (#) of bacteria (median and range) in left lung (LL) and blood in leukopenic rats infected with the high gentamicin-susceptible *K. pneumoniae*, receiving the indicated combination treatments, at different time-points after inoculation.

Day	Free GEN 5 mg/kg/d q12h for 5 days + rigid LE-GEN 5 mg/kg day 1				Free GEN 5 mg/kg/d q12h for 5 days + free GEN 5 mg/kg day 1			
	Log # bacteria/LL		Log # bacteria/ml blood		Log # bacteria/LL		Log # bacteria/ml blood	
	Median	Range	Median	Range	Median	Range	Median	Range
0	5.0		0.0		5.0		0.0	
1	8.9	8.6-9.3	1.7	1.0-2.2	8.9	8.6-9.3	1.7	1.0-2.2
6	5.7	3.0-8.2	1.8	0.0-2.0	6.7	3.9-7.7	3.5	3.1-4.3
10	3.2	0.0-5.0	2.2	0.0-3.4	✞		✞	
14	0.0	0.0-3.1	1.2	0.0-2.5	✞		✞	

✞ bacterial counts are not presented as the majority of rats had died. 6 animals were used per experimental group.

3.3 Leukopenic rats infected with the low gentamicin-susceptible K. pneumoniae

The third model combines the clinically encountered problems of an impaired host defense and low bacterial antibiotic-susceptibility. Leukopenic rats were infected with the low gentamicin-susceptible *K. pneumoniae* (MIC=4 µg/ml). Treatment consisted either of free GEN or a combination of free GEN and LE-GEN, and was started at 24 h after bacterial inoculation. The survival rates are shown in Figure 4.

Untreated control animals died, similarly as in the previous models, on day 3 and 4. Free GEN given at the MTD of 40 mg/kg/d q12h for 5 days, which resulted in complete survival

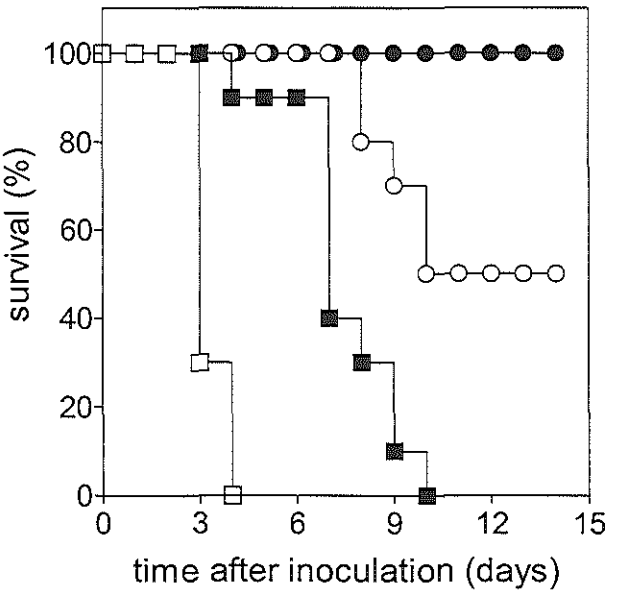

Figure 4. Effect of GEN and rigid LE-GEN or fluid LE-GEN on survival of leukopenic rats infected with a low gentamicin-susceptible *K. pneumoniae*. Treatments: control (no drug treatment) (□), free GEN 40 mg/kg/d q12h for 5 days (■), free GEN 40 mg/kg/d q12h for 5 days plus rigid LE-GEN 20 mg/kg/d q24h for 5 days (○), or free GEN 40 mg/kg/d q12h for 5 days plus fluid LE-GEN 20 mg/kg/d q24h for 5 days (●).

in the previous model, was unsuccessful. Higher doses of free GEN produced acute mortality. Addition of LE-GEN 20 mg/kg/d q24h for 5 days to 5-day treatment with free GEN at the MTD, increased survival up to 50% ($P<0.01$ compared to treatment with only free GEN), without producing acute mortality.

So far, only a rigid LE-GEN formulation has been used, as it proved highly effective. In the present model, the maximum dose of free GEN + rigid LE-GEN was only partially successful (50% survival). Therefore, a fluid LE-GEN formulation was investigated, as it has been shown that bilayer fluidity can influence the therapeutic efficacy of liposome-encapsulated aminoglycosides (7). A previous study already demonstrated that the fluid and rigid LE-GEN

localize with the same targeting efficiency at the target site (19). Addition of the fluid LE-GEN 20 mg/kg/d for 5 days instead of equivalent doses of rigid LE-GEN to the 5-day free GEN-treatment at the MTD resulted in complete survival ($P<0.05$, compared to free GEN + rigid LE-GEN).

The median number of bacteria (± range) recovered from the left lung and blood on day 0, 1, 6, 10, and 14 after bacterial inoculation of leukopenic rats infected with the low gentamicin-susceptible *K. pneumoniae* is shown in Table 2. Rats received 5-day treatment with free GEN 40 mg/kg/d q12h with addition of rigid LE-GEN or fluid LE-GEN 20 mg/kg/d q24h for 5 days. Median bacterial counts, 24 h after the last dose (day 6), in the left lung of rats treated with free GEN + fluid LE-GEN were 10-fold lower compared to rats treated with equivalent doses of free GEN + rigid LE-GEN. In the following days the median number of bacteria in the left lung of rats treated with free GEN + fluid LE-GEN was reduced further to zero. 50% of the rats treated with the rigid LE-GEN combination died in this period. Median number of *K. pneumoniae* in blood of rats treated with the GEN + fluid LE-GEN remained zero throughout the study period, whereas bacteria were present in the blood of rats treated with GEN + rigid LE-GEN. The gentamicin-susceptibility of *K. pneumoniae* recovered from dead or surviving animals was not changed compared to the inoculated bacteria.

Table 2. Number of low gentamicin-susceptible *K. pneumoniae* in left lung and blood. Number (#) of bacteria (median and range) in left lung (LL) and blood in leukopenic rats infected with the low gentamicin-susceptible *K. pneumoniae*, receiving the indicated combination treatments, at different time-points after inoculation.

Day	Free GEN 40 mg/kg/d q12h for 5 days + rigid LE-GEN 20 mg/kg/d q24h for 5 days				Free GEN 40 mg/kg/d q12h for 5 days + fluid LE- GEN 20 mg/kg/d q24h for 5 days			
	Log # bacteria/LL		Log # bacteria/ml blood		Log # bacteria/LL		Log # bacteria/ml blood	
	Median	Range	Median	Range	Median	Range	Median	Range
0	7.0		0.0		7.0		0.0	
1	10.0	8.6-10.3	1.7	1.0-2.2	10.0	8.6-10.3	1.7	1.0-2.2
6	7.3	3.3-8.3	0.7	0.0-2.7	6.4	4.6-7.8	0.0	0.0-1.2
10	✞		✞		5.4	4.1-6.8	0.0	0.0-1.5
14	✞		✞		1.0	0.0-4.9	0.0	0.0-2.4

✞ bacterial counts are not presented as the majority of rats had died. 6 animals were used per experimental group.

3.4 Pharmacokinetics

The time course of blood concentrations of therapeutically available gentamicin or total (therapeutically available plus liposome-encapsulated) gentamicin after injection of free GEN, fluid LE-GEN, or rigid LE-GEN are shown in figure 5 A and B. The pharmacokinetics of free GEN are independent of dose in the range of 2.5 mg/kg to 20 mg/kg, as the gentamicin concentrations in the circulation in time were approximately proportional to the injected dose. Disappearance of free GEN from the bloodstream was relatively rapid, with a half-life of approximately 20 min. At 8 h after injection of the highest dose (20 mg/kg) blood levels were already below 0.1 µg/ml.

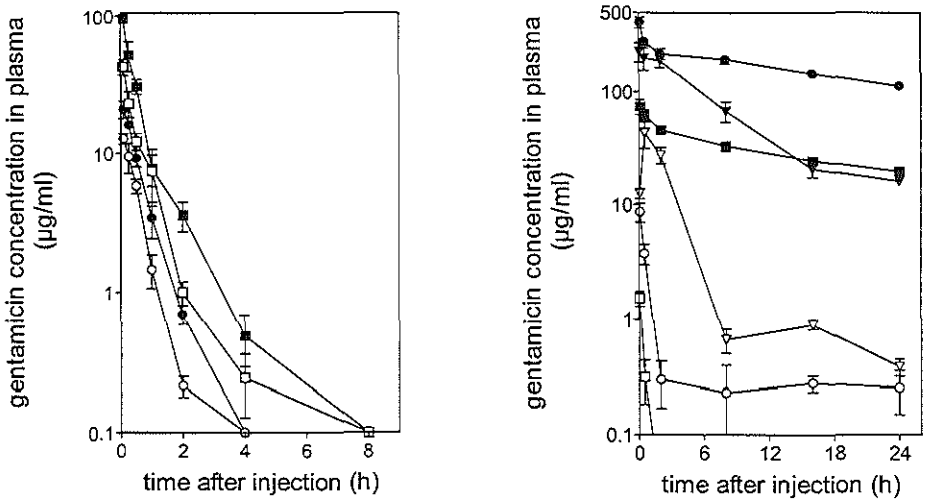

Figure 5. **Left: Time course of gentamicin concentrations in plasma of rats after injection of a single dose of free GEN** 2.5 mg/kg (○), 5 mg/kg (●), 10 mg/kg (□), or 20 mg/kg (■). 3 animals per experimental group (mean ± S.D.)

Right. Time course of gentamicin concentrations in plasma of rats after injection of a single dose of rigid LE-GEN or fluid LE-GEN. Open symbols indicate microbiologically active (i.e. not liposome-encapsulated) gentamicin concentrations after a single dose of rigid LE-GEN 5 mg/kg (□), rigid LE-GEN 20 mg/kg (○), fluid LE-GEN 20 mg/kg (▽). Filled symbols indicate total (i.e. microbiologically active and liposome-encapsulated) gentamicin concentrations after a single dose of rigid LE-GEN 5 mg/kg (■), rigid LE-GEN 20 mg/kg (●), fluid LE-GEN 20 mg/kg (▼). 3 animals per experimental group (mean ± S.D.)

Encapsulation of gentamicin in rigid liposomes resulted in dramatically increased total gentamicin concentrations in the circulation after injection. Even at 24 h after injection 15-20% of the injected dose was still present in the bloodstream. Fluid LE-GEN showed a completely different picture. Less than 5% of the injected dose was present in the circulation at 24 h after injection. As rigid and fluid liposomes exhibit similar blood clearance kinetics, as has been demonstrated previously (19), the difference between the two liposome types is the result of faster drug release from the fluid liposomes, as can also be deduced from the higher free gentamicin levels after injection of the fluid liposomes. It is not the result of renal impairment, as administration of the highest doses of free GEN, rigid LE-GEN, or fluid LE-GEN used in this experiment did not result in significantly different blood creatinin or blood urea nitrogen levels between these three experimental groups at 24 h after administration (data not shown).

4. Discussion

The preferential localization of liposomes at sites of infection offers an attractive way to selectively increase antibacterial drug concentrations at the target location, with the intention to increase therapeutic efficacy of antimicrobial treatment. Most studies in this field have, up to now, been performed in animal models with an intact host defense with high antibiotic-susceptible bacteria. However, in clinical practice, two important complicating factors should be taken into account. Particularly patients having an impaired host defense carry a high risk of treatment failure, which further increases when the infectious organism is moderate to low susceptible to the applied antimicrobial agent. Generally, the studies in animals with an intact host defense report enhanced therapeutic efficacy of the liposomal formulation compared to the free drug (3, 7, 16, 21). The same conclusion can be drawn from the present study in the intact host defense model of a high gentamicin-susceptible *K. pneumoniae* pneumonia. As a result of the local delivery of the antibiotic by LE-GEN, a single dose of the liposome-encapsulated drug dramatically improves survival compared to an equivalent dose of free GEN. Increasing the dose of LE-GEN may improve survival to 100%. Yet, the clinical relevance of this approach is limited as a relatively short course of

treatment for only 3 days with free GEN 5 mg/kg/d q12h already yields in complete survival. Therefore, the present study was undertaken to investigate the therapeutic potential of LE-GEN in clinically more relevant models of infection, that are difficult to treat with conventional antibiotics as a result of an impaired host defense and low antibiotic-susceptibility of the inoculated bacteria.

In leukopenic rats, inoculated with the high-susceptible *K. pneumoniae*, approximately 10 to 100 bacteria per ml blood were present already at 24 h after injection. Thus, in these leukopenic rats, antimicrobial therapy should not only be directed towards the bacteria at the infectious focus (the left lung) but also towards the rapidly occurring bacteremia. The survival experiments in the leukopenic model of a high gentamicin-susceptible *K. pneumoniae* pneumonia show that antimicrobial treatment is far less effective as a result of impaired host defense. Treatment with free GEN 5 mg/kg/d q12h for 3 days, that produced complete survival in the rats with an intact host defense, hardly prolongs survival in the leukopenic animals. Continuing treatment up to 5 days, prolonged survival during treatment. However, after termination of treatment only 10% survives up to 14 days. Doses of free GEN have to be increased up to 40 mg/kg/d for 5 days (MTD) to obtain complete survival. These data illustrate why in clinical practice aminoglycosides are used in combination with other classes of antibiotics to increase the therapeutic efficacy in these conditions.

Addition of a single dose of LE-GEN 5 mg/kg on day 1 to the 5-day treatment with free GEN 5 mg/kg/d q12h appeared to confer substantial therapeutic benefit. All rats survived, and the cumulative amount of gentamicin administered was 7-fold lower compared to the amount of free GEN (40 mg/kg/d for 5 days) needed to obtain complete survival. This reduction in gentamicin exposure may reduce the risk of the well-known toxicity of gentamicin on kidney and audiovestibular apparatus. On the other hand, the altered tissue distribution in general and increased gentamicin concentrations at the site of infection in particular as a result of the liposome-encapsulation might change the toxicity profile. Yet, previous studies in Beagle dogs with liposome-encapsulated amikacin suggest a favorable safety profile of liposome-encapsulated aminoglycosides as doses of 20 mg/kg/day for one

month did not result in adverse effects despite steady-state plasma concentrations of 750 µg/ml (14). Rats treated with only LE-GEN 5 mg/kg for 5 days already show mortality during treatment. The blood clearance kinetics of free GEN and LE-GEN offer an explanation for the superior efficacy of the combination of free GEN + LE-GEN. Free GEN is therapeutically active in the circulation against the bacteremia, but is rapidly cleared after injection with a half-life of approximately 20 min. Activity in the infected left lung is expected to be limited. When encapsulated in liposomes, on the other hand, the drug is only slowly leaving the circulation. LE-GEN hardly releases gentamicin in the bloodstream, and consequently shows limited activity against the bacteremia, but localizes substantially in the infected left lung (3). Release of gentamicin from the liposomes localizing in the infected lung leads to the efficient bacterial killing seen. As a result, combination of free GEN + LE-GEN reduces the numbers of bacteria in left lung tissue and blood 10-fold and 100-fold more efficiently compared to the treatment with free GEN alone, respectively. These numbers are further reduced to zero in the remaining study period. In contrast, the majority of rats treated with free GEN or LE-GEN alone die during this period. The third model addresses an additional factor complicating clinical antimicrobial therapy, i.e. low antibiotic-susceptibility of the bacteria. The 8-fold increase in MIC clearly had an effect on the efficacy of treatment, as free GEN at the MTD was no longer effective and all rats died. Addition of LE-GEN 20 mg/kg/d q24h for 5 days to the 5-day treatment with free GEN at the MTD resulted in 50% survival on day 14 without producing acute toxicity. Probably, as a result of the low gentamicin-susceptibility of the *K. pneumoniae*, the therapeutic availability of gentamicin released from this rigid liposome formulation in the infected lung is insufficient to obtain 100% survival. As fluid liposomes have been reported to release encapsulated aminoglycosides more easily than their rigid counterparts (7), a fluid LE-GEN formulation was investigated. Cholesterol was omitted from the liposome formulation as cholesterol has a major rigidifying effect on liposomal bilayers (10). Furthermore, the partially hydrogenated phospholipid (PHEPC) was replaced by EPC as bilayer rigidity increases with the degree of hydrogenation of the phospholipids in the bilayer. Addition of fluid LE-GEN instead of rigid LE-GEN to 5-day free GEN treatment at the MTD, resulted in an increase in therapeutic

effect: complete survival was obtained. The blood clearance kinetics of fluid LE-GEN show that gentamicin is released in the circulation to a larger extent from this liposome formulation compared to rigid LE-GEN. As a result, higher therapeutically active drug concentrations were measured in the bloodstream. Yet, a sufficient amount of gentamicin remained liposomally encapsulated to be delivered to the left lung to control the local infection, as is supported by the efficient bacterial killing in the left lung observed. These results show that lipid composition is an important determinant of therapeutic efficacy. A careful balance must be sought between release of antibiotic in the circulation to obtain sufficiently high drug levels in the circulation versus liposomal retention of the drug in order to achieve sufficiently high levels of (locally released) antibiotic at the infectious focus.

In conclusion, in rats with an intact host defense infected with high gentamicin-susceptible *K. pneumoniae* LE-GEN is clearly superior over free GEN treatment. Yet, the clinical relevance is limited as complete survival can also be obtained with multiple dosing of the free drug. In leukopenic rats infected with high gentamicin-susceptible *K. pneumoniae*, addition of LE-GEN to free GEN treatment shows substantial therapeutic benefit. Complete survival can be obtained using a 7-fold lower amount of gentamicin compared to administration of the free GEN alone. In leukopenic rats infected with low gentamicin-susceptible *K. pneumoniae*, free GEN at the MTD shows 0% survival. The use of LE-GEN is a strict requirement for achieving therapeutic success. It appears that the increased release of gentamicin by fluid LE-GEN compared to rigid LE-GEN is more favorable. These results warrant further clinical studies of liposomal formulations of aminoglycosides in immunocompromised patients with severe infections.

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7. In vivo synergistic interaction of liposome-co-encapsulated gentamicin and ceftazidime

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Abstract

Antimicrobial agents may act synergistically when both drugs are present at the site of infection at sufficiently high concentrations for an adequate period of time. Co-encapsulation of drugs in a drug carrier may ensure parallel tissue distributions and increase drug concentrations at the infectious focus. Therapeutic efficacy of liposome-co-encapsulated gentamicin and ceftazidime was examined by monitoring survival rate in rat models of an acute unilateral pneumonia caused by antibiotic-susceptible and antibiotic-resistant *Klebsiella pneumoniae*. It is shown in both models that combination of gentamicin with ceftazidime in the free form as single dose or as 5-day treatment twice daily failed to display synergy but resulted in an additive therapeutic effect. In contrast, targeted delivery of the liposome-co-encapsulated drugs resulted in a synergistic effect of the antibiotics in both models. Consequently, liposome co-encapsulated antibiotics allowed a shorter course of treatment and lower cumulative doses compared to administration of the unencapsulated antibiotics.

1. Introduction

Administration of combinations of antimicrobial agents is frequently employed in clinical practice to increase therapeutic efficacy. Efficacy may be increased by broadening the antimicrobial spectrum of the treatment, preventing the emergence of resistant strains, reducing toxicity, eliminating multiresistant micro-organisms, and/or enhancing bacterial killing by exploiting the synergistic interaction of a specific drug combination [1-3]. To ensure a synergistic drug interaction in vivo, the drugs should both be present at the site of infection at sufficiently high concentrations for an adequate period of time [4-7]. Due to the differences in physicochemical properties between the various antimicrobial agents, the pharmacokinetics and tissue distributions of these agents vary substantially. A significant interaction of antibiotics at the infectious focus resulting in a synergistic activity is therefore not guaranteed. The use of a drug carrier containing both antibiotics could enforce a parallel tissue distribution of both of the encapsulated agents. In addition, the use of a targeted drug carrier (including liposomes) may increase the concentrations of the drugs at the site of infection, which would further strengthen the synergistic drug interaction. In this respect, co-encapsulation of antibiotics in liposomes may open up new perspectives.

Liposomes have been widely investigated as targeted drug carriers in infectious diseases. Liposomes have been shown to localize selectively at the infected target site in a variety of experimental models of infection [8-13]. The selective localization appears to be the result of the locally increased capillary permeability allowing local liposome extravasation [14-15]. Up to now, only liposomes containing a single antimicrobial agent have been investigated. In the present study, we have co-encapsulated two antibiotics, gentamicin and ceftazidime, that have documented synergy in vitro [16] into liposomes. The therapeutic efficacy in vivo was examined by monitoring survival in a rat model of an acute unilateral *Klebsiella pneumoniae* pneumonia. Both an antibiotic-susceptible and an antibiotic-resistant *K. pneumoniae* strain was studied.

2. Material and Methods

2.1 Liposome preparation

Poly(ethylene) glycol (PEG)-coated LCL were used as previous studies have demonstrated that this liposome type shows substantial localization at the site of infection in the investigated model [8]. Liposomes were prepared as described previously [8]. Appropriate amounts of the indicated lipids partially hydrogenated egg phosphatidylcholine (PHEPC) (Asahi Chemical Industry Co. Ltd., Ibarakiken, Japan), cholesterol (Chol) (Sigma Chemical Co., St. Louis, Missouri), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethylene glycol-2000] (PEG-DSPE) (Avanti Polar Lipids, Alabaster, Alabama) in a molar ratio of 1.85:1.00:0.15, respectively, were dissolved in a mixture of chloroform and methanol. After evaporation of the solvent under constant rotation and reduced pressure, the lipid mixture was dried under nitrogen, dissolved in 2-methyl-2-propanol (Sigma Chemical Co., St. Louis, Missouri) frozen by immersing in ethanol (-40 °C), and freeze-dried overnight. The resulting lipid film was hydrated for 2 h in aqueous solutions of appropriate concentrations of ceftazidime (CZ) (Glaxo-Wellcome, Zeist, The Netherlands) or gentamicin (GEN) (Duchefa Biochemie, Haarlem, The Netherlands). For co-encapsulation of the drugs in liposomes, the CZ-solution was added first, followed by the GEN solution. Lipid concentration was diluted to a final concentration of 100 µmol total lipid/ml using Hepes/NaCl buffer pH 7.4 (10 mM N-[2-hydroxy ethyi] piperazine-N'-ethane sulfonic acid (Hepes) (Sigma Chemical Co., St. Louis, Missouri) and 135 mM NaCl (Merck, Darmstadt, Germany). The lipids were sonicated for 8 minutes with an amplitude of 8 µm using a 9.5 mm probe in an MSE Soniprep 150 (Sanyo Gallenkamp PLC, Leicester, UK) to obtain LCL with a mean particle size of 100 nm. Particle size distribution was measured using dynamic light scattering, detected at an angle of 90° to the laser beam on a Malvern 4700 System (Malvern Instruments Ltd., Malvern, UK). In addition to the mean particle size, the system reports a polydispersity index (a value between 0 and 1). A polydispersity index of 1 indicates large variations in particle size, a reported value of 0 means that size variation is apparently absent. All liposome preparations used had a polydispersity index below 0.3. Unencapsulated GEN and/or CZ was removed by ultracentrifugation of the liposomes in two changes of Hepes/NaCl buffer at 265,000 x g for 2 h at 4 °C. Phosphate concentration was determined spectrophotometrically according to Bartlett [17]. Total (liposome-encapsulated and free) and free (unencapsulated) GEN and/or CZ was measured using a diagnostic sensitivity test agar (Oxoid, Basingstoke, UK) diffusion test with *Staphylococcus aureus* Oxford strain (ATCC 9144) (CZ-resistant) and an *Escherichia coli* strain (clinical isolate, GEN-resistant) as the indicator organism for GEN and CZ, respectively, as described previously [18]. For total (unencapsulated and encapsulated) drug measurements, liposomes were disintegrated by 0.1% v/v (final concentration) Triton X-100 (Janssen Chimica, Geel, Belgium). Less than 10% of the GEN and/or CZ was shown to be unencapsulated after ultracentrifugation. The validity of the agar diffusion test for the determination GEN and CZ concentrations in the combination was ascertained in a separate experiment. Enzymatic inactivation of GEN using aminoglycoside acetylating enzyme [19] or of CZ using ß-lactamase (Koch-Light Ltd. Haverhill, UK) yielded similar inhibitory zones as without deactivation of either one of the antibiotics, thus showing the possibility to measure one drug at a time in the combination.

2.2 Bacterial strains

The susceptible *K. pneumoniae* (ATCC 43816, capsular serotype 2, MIC=0.5 µg/ml for both GEN and CZ) was used. The MIC was determined by plating an inoculum of 10^4 cfu per spot on Mueller-Hinton (MH) agar (Difco laboratories, Detroit, MI, USA) plates containing two-fold dilutions of GEN or CZ, according to [20]. The resistant *K. pneumoniae* (MIC=32 µg/ml for GEN and 16 µg/ml for CZ) was obtained by culturing the susceptible strain in Mueller-Hinton (MH) broth (Difco laboratories, Detroit, MI, USA) containing increasing concentrations of CZ. The MIC was determined according to the method described above. The resulting CZ-resistant strain was conjugated with an *Escherichia coli* R176 strain

(clinical isolate) that produced a plasmid encoding for an aminoglycoside-acetylating enzyme. In this way, a strain resistant to both GEN and CZ was obtained. The stability of the GEN/CZ resistant phenotype in vitro was checked by culturing the bacteria 5 times in succession in antibiotic-free medium, followed by determination of the MIC. All out of 100 tested bacterial colonies remained resistant to both antibiotics.

2.3 Checkerboard titrations

Checkerboard titrations were performed with GEN and/or CZ at the indicated concentrations in MH-broth of 37 °C in a total volume of 3 ml. An inoculum of 5 x 10^5 susceptible or resistant *K. pneumoniae* cfu/ml in the logarithmic phase of growth was used. Tubes were incubated for 24 h at 37 °C, and (the absence of) microbial growth was determined macroscopically. Each titration was performed in triplicate.

2.4 Time-kill curves

Time-kill curves were performed with GEN and/or CZ at the indicated concentrations in MH-broth of 37 °C in a total volume of 3 ml. An inoculum of 5 x 10^5 susceptible or resistant *K. pneumoniae* cfu/ml in the logarithmic phase of growth was used. Samples were taken at 0, 1, 2, 4, 6, and 24 h after addition of the inoculum. Number of bacteria in the samples was determined by making serial dilutions in phosphate buffered saline (PBS) 4 °C. 200 µl of each dilution was plated on tryptone soy agar (TSA)-plates and incubated overnight at 37 °C. Colonies were counted. Each curve was determined in triplicate.

2.5 Unilateral pneumonia

The animal experiments ethical committee of the Erasmus *university* Medical Center Rotterdam approved the experiments described in this study. Female albino RP/AEur/RijHsd strain albino rats, 18-25 weeks of age, body weight 185-225 g (Harlan, Horst, The Netherlands) with a specified pathogen free status were used. A left-sided unilateral pneumonia was induced as described previously [21]. In brief, rats were anesthetized and the left primary bronchus was intubated. Through the tube, 0.02 ml of a saline suspension containing 10^6 susceptible *K. pneumoniae* were inoculated. Inoculated bacteria were in the logarithmic phase of growth. For the resistant *K. pneumoniae* strain the inoculum was adjusted to 2 x10^8 to establish a median survival of untreated controls that was comparable between both models. Rats were housed individually. In vivo stability of the phenotype of the *K. pneumoniae* was checked by culturing dilutions of homogenized left lung tissue obtained at 24 h after bacterial inoculation (the starting point of therapy) on MH-plates. Colonies were isolated and MIC was determined as described above on MH-plates. All out of 100 tested colonies of both the susceptible and resistant strain had a stable phenotype , regarding GEN and CZ-susceptibility, after inoculation in vivo.
Treatment was started at 24 h after bacterial inoculation. Controls were left untreated. Gentamicin was administered at two-fold increasing doses ranging from 1.25 to 40 mg/kg. Ceftazidime was administered at two-fold increasing doses ranging from 0.4 to 400 mg/kg dose. In the model of the susceptible *K. pneumoniae*, gentamicin doses administered were 1.3, 1, 0.8, or 0.6 mg/kg/d because of the steep dose-response curve in this model. GEN and/or CZ were administered either as a single dose or as multiple doses every 12 h. In case of combination of GEN and CZ, the drugs were injected with an interval of 5 min. Liposome-encapsulated gentamicin or ceftazidime (LE-GEN or LE-CZ, respectively) or liposome-co-encapsulated gentamicin and ceftazidime (LE-GEN-CZ) was administered either as a single dose or as multiple doses every 24 h. The formulations were injected intravenously into the tail vein. Survival of rats was examined every day until 14 days after bacterial inoculation. The MIC of the *K. pneumoniae* bacteria recovered from deceased rats was determined as described above and similar to that of the inoculated bacteria.

2.6 Statistical analysis

To identify synergy, in vitro and in vivo, the effect of a drug combination was compared to the expected effect for each of the drugs alone. This method to identify synergy, also known as the isobole or iso-effect curve-method, has been validated by Berenbaum [22]. The method is based on the equation:

$d_a/D_a + d_b/D_b = 1$

where D_a and D_b are the doses of agent A alone and agent B alone, respectively, needed to produce a desired effect. The terms d_a and d_b are the doses in a combination of agent A and agent B, respectively, that produce the same effect (iso-effect). If no interaction between agent A and agent B is present, or in other words the effects of agent A and agent B are additive, $d_a/D_a + d_b/D_b = 1$. Deviations from the equation indicate synergy ($d_a/D_a + d_b/D_b < 1$) or antagonism ($d_a/D_a + d_b/D_b > 1$). Curves, resulting from the checkerboard titrations were compared to lines describing absence of drug interaction using the F-test. Survival between experimental groups was compared by the log-rank test. Area under the time-kill curve (AUKC) was calculated using the trapezoid rule. Analyses were performed using Graph Pad Prism 3.00 software (Graph Pad Software Inc., San Diego, CA, USA). AUKC's were compared by one-way analysis of variance (ANOVA) corrected for multiple comparisons using the Bonferroni method.

3. Results

3.1 Checkerboard titrations

The results of the checkerboard titrations with the susceptible strain and resistant strain are shown in figure 1 A and B, respectively. The shape of the best-fitted curve in figure 1 A as well as 1 B is concave up and describes the relationship significantly better than the line that would represent the relationship in absence of drug interactions (F-test, $p<0.0001$ for both curves). Thus, CZ and GEN act synergistically against both *K. pneumoniae* strains.

3.2 Time-kill curves

The time-kill curves of the susceptible strain and the resistant strain are shown in figure 2 A and B, respectively. Bacterial density for both strains rapidly increased to a plateau of 10^9 bacteria/ml in absence of antibiotics. For the susceptible strain, GEN alone, at a concentration of 0.3 µg/ml, initially reduced bacterial numbers. After 4 h incubation >99% of bacteria were killed. However, between 6 and 24 h incubation, bacterial outgrowth was observed to 10^7 bacteria/ml. Similar results were obtained with CZ alone at a concentration of 0.3 µg/ml. Incubation of 0.15 µg/ml GEN and 0.15 µg/ml CZ in combination reduced

bacterial numbers more efficiently. After 4 h of incubation >99.99% of bacteria were killed, whereas after 24 h

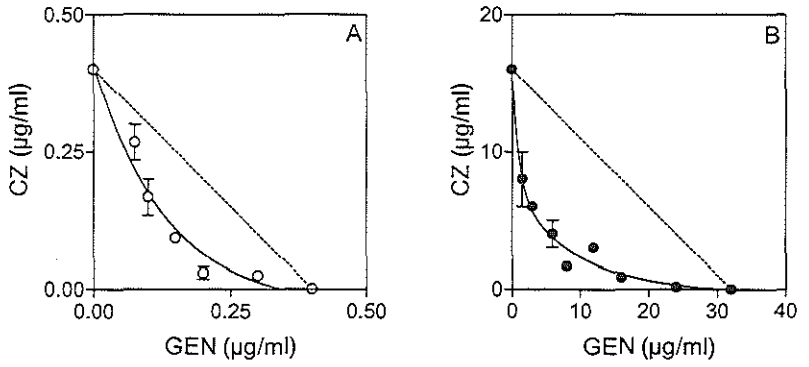


Figure 1. Checkerboard titrations of gentamicin (GEN) and ceftazidime (CZ) against the susceptible *K. pneumoniae* (A) and the resistant *K. pneumoniae* (B). The symbols represent the lowest concentrations of GEN and CZ that resulted in absence of bacterial growth. The curve shows the best fit through the symbols, whereas the dotted line represents the relationship that would be obtained in absence of drug interactions. Both experiments were performed in triplicate.

of incubation the bacterial density was 10^5-fold lower compared to the single agent incubations. The AUKC-values of the time-kill curves are shown in table 1. As the AUKC-value of the combination is significantly lower than the AUKC's of the single agent incubations (ANOVA, $p<0.05$ for both GEN and CZ), and thus $d_{GEN}/D_{GEN} + d_{CZ}/D_{CZ} < 1$, it can be concluded that GEN and CZ display a synergistic interaction against the susceptible *K. pneumoniae* [20].

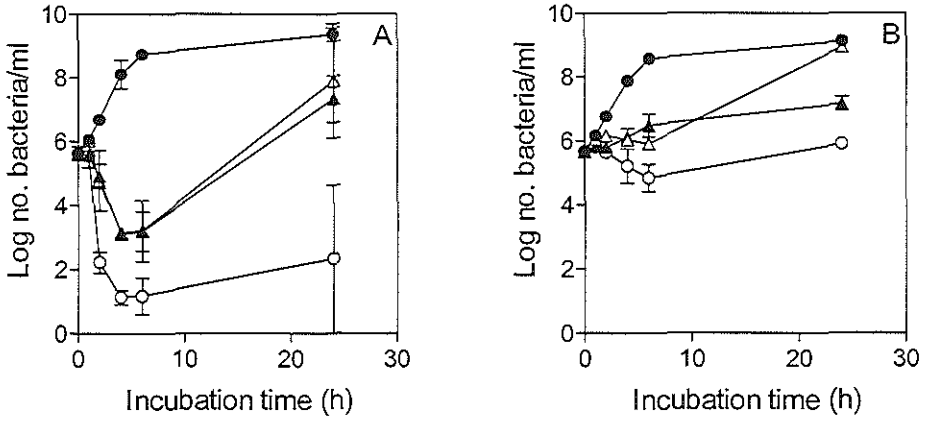


Figure 2.Time-kill curves of the susceptible *K. pneumoniae* (A) and the resistant *K. pneumoniae* (B). In figure A the susceptible bacteria were incubated without antibiotics (●), 0.3 µg/ml GEN (△), 0.3 µg/ml CZ (▲), or 0.15 µg/ml GEN in combination with 0.15 µg/ml CZ (○). In figure B the resistant bacteria were incubated without antibiotics (●), 16 µg/ml GEN (△), 8 µg/ml CZ (▲), or 8 µg/ml GEN in combination with 4 µg/ml CZ (○). Both experiments were performed in triplicate.

Table 1. Area under the time-kill curve (AUKC)-values for the susceptible and the resistant *K. pneumoniae* strain.

Susceptible		**Resistant**	
Drug (µg/ml)	AUKC[Φ] (log # bacteria x h/ml)	Drug (µg/ml)	AUKC[Φ] (log # bacteria x h/ml)
Control	10.4 ± 0.1***	Control	10.2 ±0.1***
GEN (0.3)	8.9 ± 1.7*	GEN (16)	9.9 ± 0.1***
CZ (0.3)	8.2 ± 0.6*	CZ (8)	8.3 ± 0.1***
GEN/CZ (0.15/0.15)	5.9 ± 0.2	GEN/CZ (8/4)	7.0 ± 0.2

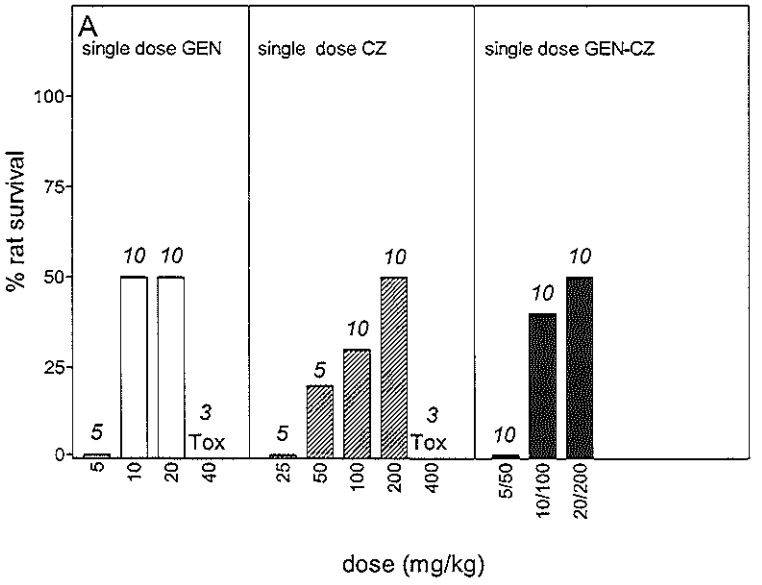
Bacteria were incubated with indicated concentrations of GEN and/or CZ. Experiment was performed in triplicate. [Φ]Results are expressed as mean ± S.D. Significant differences against the combination are noted as *** $p<0.001$, * $p<0.05$.

For the resistant strain, GEN alone, at a concentration of 16 µg/ml, initially stabilized bacterial numbers, but at 24 h after incubation bacterial density had increased to the control level. CZ alone, 8 µg/ml, also stabilized bacterial counts initially, but eventually bacterial outgrowth was observed to 10^7 bacteria/ml after 24 h incubation. The combination of GEN and CZ at concentrations of 8 and 4 µg/ml, respectively, initially killed and then stabilized bacterial counts throughout the study period of 24 h. As the AUKC-value for the combination was significantly lower compared to those of the single agent incubations (ANOVA, $p<0.001$), and thus $d_{GEN}/D_{GEN} + d_{CZ}/D_{CZ} < 1$, GEN and CZ showed synergistic interaction against the resistant *K. pneumoniae* (Table 1).

3.3 Rat survival in the susceptible K. pneumoniae pneumonia model

The results of the in vivo survival experiments with rats infected with the susceptible *K. pneumoniae* are shown in Figure 3. Maximum survival after a single dose of free GEN alone or free CZ alone was 50% (Figure 3A). The maximum dose for free GEN alone was 20 mg/kg and for free CZ alone 200 mg/kg, because 2-fold higher doses caused acute toxicity (local irritation at the site of injection for CZ and convulsions for GEN). As the combination of free GEN and free CZ did not increase survival compared to an equivalent dose of free GEN alone or free CZ alone, $d_{GEN}/D_{GEN} + d_{CZ}/D_{CZ} > 1$, which would suggest antagonism. However, as the single dose treatment never resulted in survival > 50%, these data seem more indicative for the conclusion that treatment is too short to have sufficiently prolonged concentrations at the site of infection for the drugs to exert their maximum effect on rat survival.

By prolonging treatment to 5 days and administration of the antibiotics every 12 h, survival is increased (Figure 3 B). Free GEN alone showed a steep dose response relation between


A
single dose GEN
single dose CZ
single dose GEN-CZ
% rat survival
100
75
50
25
0
5
10 10
3
Tox
5
5
10
10
3
Tox
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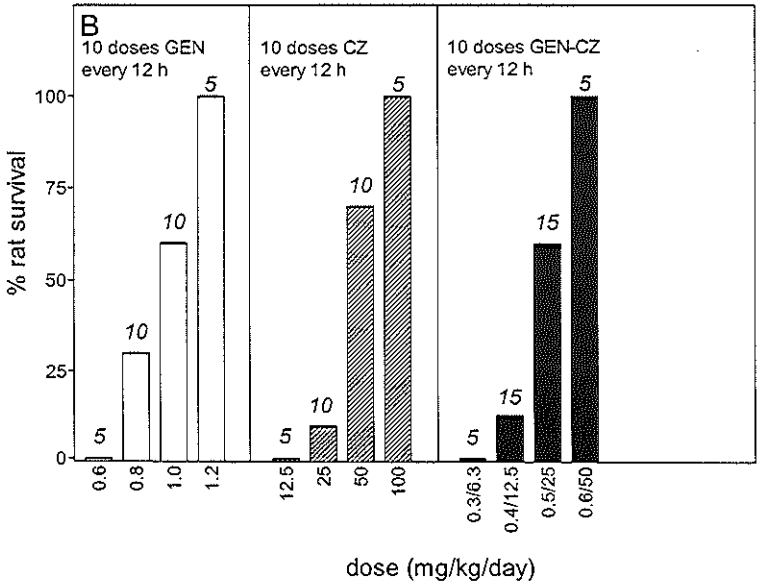
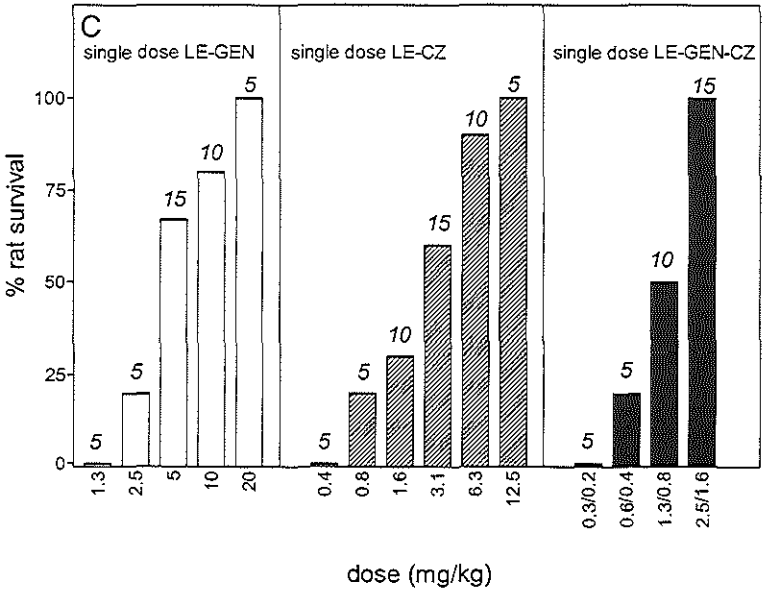
10/100
20/200
dose (mg/kg)


B
10 doses GEN
every 12 h
10 doses CZ
every 12 h
10 doses GEN-CZ
every 12 h
% rat survival
100
75
50
25
0
5
10
10
5
5
10
10
5
5
10
15
5
15
5
0.6
0.8
1.0
1.2
12.5
25
50
100
0.3/6.3
0.4/12.5
0.5/25
0.6/50
dose (mg/kg/day)



⇦⇧**Figure 3. Percentage of rat survival at 14 days after inoculation of the susceptible *K. pneumoniae* in the left lung.** Rats were treated at 24 h after bacterial inoculation with a single dose of free gentamicin (GEN) (open bars), or free ceftazidime (CZ) (hatched bars) or GEN and CZ (closed bars) (A), 10 doses every 12 h of GEN (open bars), or CZ (hatched bars) or GEN and CZ (closed bars) (B), single dose of liposome-encapsulated gentamicin (LE-GEN) (open bars), or liposome-encapsulated ceftazidime (LE-CZ) (hatched bars), or liposome-co-encapsulated gentamicin/ceftazidime (LE-GEN-CZ) (closed bars) (C). Number of animals per experimental group in italics.

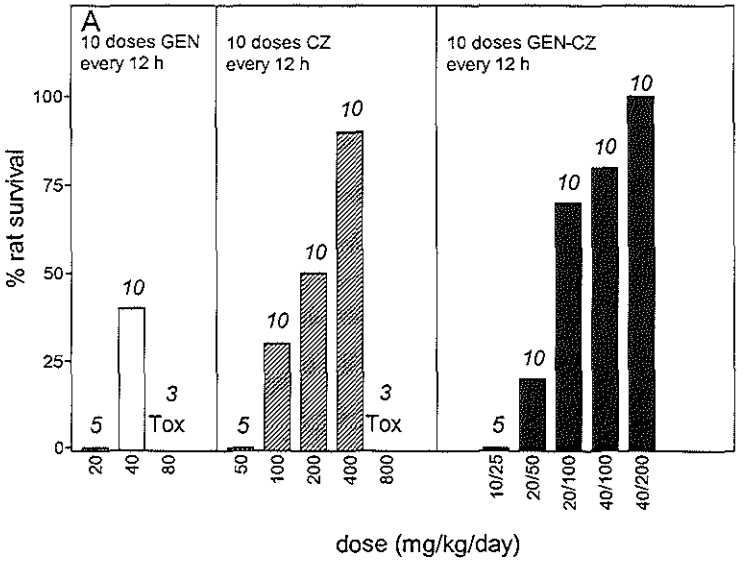
0.63 mg/kg/d (0% survival) and 1.25 mg/kg/d (100% survival), explaining why intermediate drug doses were also studied. Free CZ alone showed a response of 0% survival up to 100% at doses ranging from 12.5 mg/kg/d to 100 mg/kg/d dose. Looking at iso-effective doses of free GEN alone and free CZ alone (e.g. 1.05 mg GEN/kg/d and 50 mg CZ/kg/d) each resulting in 60-70% survival, shows that combination of half of these iso-effective doses for free GEN and free CZ (i.e. 0.53 mg GEN/kg/d combined with 25 mg CZ/kg/d) results in a similar survival percentage (60%). As a result, $d_{GEN}/D_{GEN} + d_{CZ}/D_{CZ} \approx 1$,

indicating that there is no interaction between free GEN and free CZ in vivo at the 5-day treatment schedule.

Using the liposome-encapsulated antibiotics, the survival data obtained with single doses of either LE-GEN alone, LE-CZ alone, or co-encapsulated LE-GEN-CZ were completely different (Figure 3 C). A single dose of LE-GEN alone produced a dose response with 0% survival at a dose of 1.25 mg/kg increasing to complete survival for the 20 mg/kg dose. Whereas, with LE-CZ 0% survival was obtained after administration of 0.38 mg/kg. Survival increased gradually to 100% for 12 mg/kg. Looking at iso-effective doses for LE-GEN and LE-CZ alone (e.g. 5 mg LE-GEN/kg or 3 mg LE-CZ/kg) each resulting in 60-67% survival, shows that co-encapsulation of half of these iso-effective doses (i.e. LE-GEN-CZ 2.5/1.5 mg/kg, respectively) showed a significantly better survival (100%) (Log-rank test, $p<0.05$). Consequently, $d_{LE\text{-}GEN}/D_{LE\text{-}GEN} + d_{LE\text{-}CZ}/D_{LE\text{-}CZ} < 1$, revealing a synergistic interaction of liposome-co-encapsulated GEN and CZ.

3.4 Rat survival in the resistant K. pneumoniae pneumonia model

The results of the in vivo survival experiments with rats infected with the resistant *K. pneumoniae* are shown in Figure 4. Administration of single doses of the free drugs, alone or in combination, at the maximum tolerated dose did not yield survival (data not shown). Prolongation of treatment to 5 days with the free drugs administered every 12 h, increased survival (Figure 4 A). Yet, free GEN alone at the maximum tolerated dose of 40 mg/kg/d showed only 40% survival. With free CZ alone a nearly complete dose-response relation could be obtained at doses ranging from 50 mg/kg/d (0% survival) to 400 mg/kg/d (90 % survival). Looking at iso-effective doses of free GEN alone and free CZ alone (e.g. 40 mg GEN/kg/d or 100-200 mg CZ/kg/d) each resulting in 30-50% survival, shows that combination of half of these iso-effective doses of GEN and CZ (i.e. 20 mg GEN/kg/d combined with 50 or 100 mg CZ/kg/d) did not increase the survival percentage significantly (20-70%). Consequently, $d_{GEN}/D_{GEN} + d_{CZ}/D_{CZ} \approx 1$, indicating that there is no interaction between free GEN and free CZ.


A
10 doses GEN every 12 h
10 doses CZ every 12 h
10 doses GEN-CZ every 12 h
% rat survival
100
75
50
25
0
5
10
3
Tox
5
10
10
10
3
Tox
5
10
10
10
10
20
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100
200
400
800
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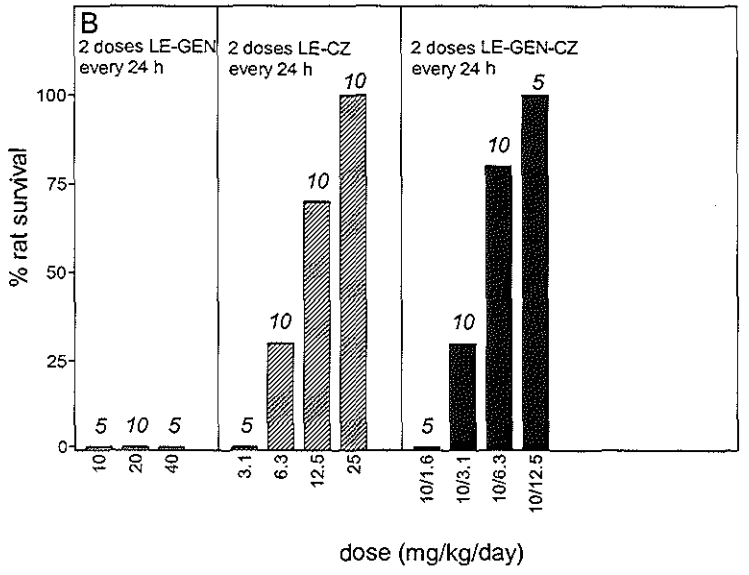
40/100
40/200
dose (mg/kg/day)


B
2 doses LE-GEN every 24 h
2 doses LE-CZ every 24 h
2 doses LE-GEN-CZ every 24 h
% rat survival
100
75
50
25
0
5
10
5
5
10
10
10
5
10
10
5
10
20
40
3.1
6.3
12.5
25
10/1.6
10/3.1
10/6.3
10/12.5
dose (mg/kg/day)

⇦ **Figure 4. Percentage of rat survival at 14 days after inoculation of the resistant *K. pneumoniae* in the left lung.** Rats were treated at 24 h after bacterial inoculation with 10 doses every 12 h of free gentamicin (GEN) (open bars), or free ceftazidime (CZ) (hatched bars) or GEN and CZ (closed bars) (A), two doses every 24 h of liposome-encapsulated gentamicin (LE-GEN) (open bars), or liposome-encapsulated ceftazidime (LE-CZ) (hatched bars), or liposome-co-encapsulated gentamicin/ceftazidime (LE-GEN-CZ) (closed bars) (B). Number of animals per experimental group in italics.

In contrast, treatment for only two days with LE-CZ alone showed 0% survival at a dose of 3 mg/kg/d, and complete survival was already obtained at a dose of 24 mg/kg/d (Figure 4 B). LE-GEN alone at the maximum administered dose of 40 mg/kg/d did not prevent death of the rats. However, at this dose of 40 mg/kg LE-GEN rats survived significantly longer compared to the controls ($p<0.01$). Liposomal co-encapsulation of GEN and CZ improved survival compared to LE-GEN alone or LE-CZ alone. LE-GEN-CZ, at a dose of 10 and 12 mg/kg/d, respectively, already produced complete survival, which was obtained for LE-CZ alone at 24 mg/kg/d and for LE-GEN alone at a dose that exceeded 40 mg/kg/d (probably by far). Consequently, $d_{LE\text{-}GEN}/D_{LE\text{-}GEN} + d_{LE\text{-}CZ}/D_{LE\text{-}CZ} < 1$, thus showing a synergistic interaction of liposome-co-encapsulated GEN and CZ. Similar reasoning shows a synergistic interaction for LE-GEN-CZ at doses of 10 combined with 6 mg/kg/d as well as 10 combined with 3 mg/kg/d CZ, respectively.

4. Discussion

Treatment with a combination of antimicrobial agents may improve therapeutic efficacy over single agent treatment as a result of synergistic drug interactions. Synergistic drug interaction in vitro has been clearly shown for various drug combinations. For a synergistic drug interaction to occur in vivo, the drugs in the combination should be present at the site of infection at sufficiently high concentrations for an adequate period of time. Theoretically, simultaneous drug delivery to the target site could strengthen synergistic interactions. Interestingly in this respect, targeted liposomal delivery of single antimicrobial agents has demonstrated superior therapeutic efficacy over conventional antimicrobial treatment in a number of experimental infection models, as has been reviewed previously [23-25]. The

superior efficacy is attributable to the increased concentration of the drug at the site of infection as a result of the targeted drug delivery. Up to now, only single agent liposome preparations have been investigated. The present study aimed to investigate the therapeutic efficacy of liposome-co-encapsulated antimicrobial agents in vivo in a rat model of pneumonia caused by an antibiotic-susceptible strain or antibiotic-resistant strain of *K. pneumoniae*. The results of the present study show that targeted liposomal delivery of GEN and CZ results in a synergistic interaction of these antibiotics in vivo. Importantly, the synergistic interaction was present in the animals infected with the susceptible strain as well as the animals infected with the resistant strain. In contrast, administration of the combination of the antibiotics in the free form, although showing synergy in vitro, displayed only an additive effect in both in vivo models. Synergy in vivo was not observed. As a result, by use of liposome-co-encapsulated GEN and CZ, 100% survival can be obtained using a shorter treatment schedule and lower cumulative doses compared to treatment with the free drugs.

The interaction between GEN and CZ against both the susceptible and resistant *K. pneumoniae* was first examined in vitro by performing checkerboard-titrations and time-kill experiments. Both in vitro assays show that GEN and CZ acted synergistically against both the susceptible strain and the resistant *K. pneumoniae* strain. The in vitro synergistic interaction of GEN and CZ, or more in general aminoglycosides and ß-lactam antibiotics, has been reported earlier. The interaction is suggested to be due to the limited penetration of aminoglycosides into bacteria to effect bacterial killing and the ability of beta-lactams to increase that penetration [26].

To investigate whether GEN and CZ can act synergistically in vivo, rats were infected with either the susceptible or the resistant *K. pneumoniae* strain, and survival was monitored for 14 days. At single doses of either free GEN alone or free CZ alone a maximum survival of 50% could be obtained in rats infected with the susceptible strain. Combination of single doses free GEN and free CZ did not improve survival compared to an equivalent single dose of either free GEN alone or free CZ alone. Likely, treatment at a single dose of GEN and CZ

is too short and thus adequate concentrations at the site of infection are too transient for synergistic interactions to have an effect on survival.

To increase therapeutic efficacy, treatment with the free drugs was prolonged to 5 days and both antibiotics were administered every 12 h. Using this dosing schedule, complete survival could be obtained with either free GEN alone or free CZ alone against the susceptible *K. pneumoniae* infection. The effects of free GEN combined with free CZ on rat survival in this 5-day treatment schedule, however, are merely additive. Synergism was not detected. This result was unexpected as, in vitro, GEN and CZ acted synergistically against both *K. pneumoniae* strains and synergism between aminoglycosides and ß-lactams in vivo has been reported [27-28]. The discrepancy between in vitro and in vivo data is possibly the result of the rapidly changing concentrations of the antibiotics at the site of infection in the rats as compared to the constant drug concentrations in the in vitro incubations [5-6]. Seemingly, the pharmacokinetics and tissue distributions of free GEN and free CZ in rats [29-32] do not provide adequate drug concentrations at the site of the *K. pneumoniae* infection in a timely fashion for synergistic drug interactions to occur. Consequently, the assessment of in vitro synergistic interactions does not guarantee in vivo synergy to occur predictably.

The results obtained with the liposome-encapsulated antibiotics contrast favorably with the results obtained with the free antimicrobial agents. Single doses of LE-GEN alone or LE-CZ alone were shown to be highly effective, as complete survival could be obtained in the susceptible *K. pneumoniae* infection. Apparently, the simultaneous targeted delivery of LE-GEN-CZ results in higher GEN and CZ concentrations at the target site for prolonged periods of time, enabling synergistic drug interactions to occur. A single dose of liposomal co-encapsulated agents produced complete survival at a comparable GEN-exposure and a 170-fold reduced CZ body exposure, compared to 10 injections of the free drug combination.

To investigate the strength of the synergistic drug interaction after administration in the co-encapsulated form, comparative studies were also performed in rats infected with the *K. pneumoniae* strain resistant to both antibiotics. In this model, survival in a 5 day treatment schedule could only be obtained with doses of free GEN alone or free CZ alone that were

well over the clinically recommended doses. Combinations of free GEN with free CZ were again just additive. In contrast, administration of two doses of LE-CZ alone of 24 mg/kg/d already resulted in complete survival. LE-GEN alone was less effective as two doses of 40 mg/kg/d failed to increase survival. Yet, liposome-co-encapsulation of GEN and CZ resulted in significantly improved survival compared to the expected efficacy based on the dose-response relations of LE-GEN alone and LE-CZ alone, demonstrating that the synergistic interaction was strong enough to overcome infection with a resistant *K. pneumoniae* infection. Two doses of liposome-co-encapsulated GEN and CZ produced complete survival at a 10-fold lower GEN-exposure and 40-fold lower CZ-exposure compared to 10 injections of the free GEN-CZ combination.

In conclusion, the present study demonstrates that targeted delivery of GEN and CZ by liposome-co-encapsulation results in synergistic drug interactions in a susceptible as well as resistant *K. pneumoniae* pneumonia model. In these models, synergistic interaction of a combination of free GEN and free CZ could not be demonstrated. Probably as a result of the synergistic interaction, administration of liposome-co-encapsulated gentamicin and ceftazidime allowed a shorter course of treatment as well as lower cumulative doses to obtain complete therapeutic efficacy compared to administration of the drugs in the free form. The reduced drug exposure may also reduce the risk of the development of microbial resistance. The observed synergism of liposome-co-encapsulated antibiotics may open up new perspectives for targeted drug delivery in serious infectious diseases.

Acknowledgements

Dr. R. Schifferstein is thanked for helpful comments on synergistic interactions.

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8. Summary

Failure of antimicrobial treatment is observed frequently in hospitalized patients resulting in morbidity and mortality. The long, invasive, and intensive hospital treatments lower the barriers to microbial invasion of host tissues and compromise the development of an adequate inflammatory/immune response. In addition, the host response may be weakened by administration of immunosuppressants or cytotoxic drugs or as a result of a pathology that affects the host defense. Under these circumstances antimicrobial treatment is often not successful. The possibility to increase therapeutic efficacy of antibiotics by targeted delivery of antimicrobial agents is discussed in **Chapter 1**. With regard to targeting of antibiotics to bacterial infections, Bakker-Woudenberg et al. demonstrated that intravenously injected sterically stabilized liposomes (SSL) localize preferentially at the site of infection in a rat model of a unilateral *Klebsiella pneumoniae* pneumonia in the left lung (Bakker-Woudenberg, 1993). An improved therapeutic effect of a single dose of SSL-encapsulated gentamicin or SSL-encapsulated ceftazidime was achieved compared to a single equivalent dose of the free drug in this model (Bakker-Woudenberg, 1995). For optimization of liposome targeting to the site of infection it is essential to:

- identify the critical factors (on the side of the liposomes as well as on the side of the host) that contribute to target localization of SSL in *K. pneumoniae* infected rat lung tissue (Chapter 2-4)

- evaluate the value of SSL-encapsulated antibiotics in clinically relevant infection models, addressing in particular the issue of impaired host defense and reduced bacterial susceptibility (Chapters 6-7).

These have been the aims of the present thesis.

Chapter 2 to 4 are devoted to the first aim of the project. In **Chapter 2** the effect of liposome characteristics (particle size, PEG-density, bilayer fluidity, and degree of negative surface charge) on liposome localization at the site of the *Klebsiella pneumoniae* pneumonia were investigated. It is shown that all liposome preparations studied, localize preferentially in the infected left lung compared to the contralateral non-infected right lung. However, the degree of left lung localization differed for the various liposome preparations. Decreasing circulation time, as observed by reducing PEG-density (from 5 to 1 mol%) and increasing particle size (from 100 nm to 280 nm or 360 nm) yielded a reduced degree of target localization. Interestingly, incorporation of a high amount of negatively charged phospholipids (30 mol% EPG) reduced target localization without a reduction in liposome circulatory half-life. Increase in PEG-density from 5 to 10 mol%, variation of liposome bilayer fluidity, or incorporation of 5 mol% EPG in the liposome bilayer did not affect liposome circulation time nor liposome target localization. These results show that SSL localize to a high degree at the target site, compared to conventional liposomes. The highest degree of target localization can be achieved using PEG-coated, small and neutral liposomes. It is concluded that the degree of liposome localization at the infected site is relatively independent of the physicochemical characteristics of the SSL. This is important for the tailoring of SSL to achieve an optimal therapeutic effect of the encapsulated drug. It will often be required to create a balance between the best properties with respect to target localization, drug incorporation efficiency, drug retention and drug release, which will probably differ for the various classes of antibiotics.

Chapter 3 is focused on the factors on the side of the host that are important for liposome target localization. Liposome localization appeared to be highest in the hemorrhagic zone of the infected lung, a zone characterized by markedly increased capillary permeability and high bacterial numbers. Both liposome localization and capillary permeability correlated positively with the severity of the infection, supporting the central role of increased capillary permeability in the selective liposome target localization. Lung instillation of inflammatory stimuli that increased capillary permeability other than viable bacteria (i.e. 0.1 M HCl or LPS)

also promoted liposome localization, showing that the nature of the inflammatory agent is not critical. This finding is in line with other reports that show enhanced liposome localization at the inflamed site as a result of various inflammatory and infectious agents (Dams, 1999a/b; Rousseau, 1999; Klimuk, 1999; Corvo, 1999; Awasthi, 1998a/b; Oyen, 1996; Morgan, 1981).

As liposomal target localization in leukopenic rats was similar to that in immunocompetent rats, contribution of circulating leukocytes seems of minor importance. This would suggest that liposomal drug targeting could be feasible in immunocompromised patients. These patients suffer a high risk of antibiotic treatment failure (Reynolds, 1999) and could therefore particularly benefit from targeted drug delivery.

Intrapulmonary distribution of liposomes shows that phagocytes at the target site take up extravasated liposomes. The extent of liposome uptake by phagocytes as well as the contribution of phagocytes to liposome processing and drug release is still unclear and deserves future investigation. The involvement of phagocytes in liposome uptake at the target site could, for example, be important information in the selection of appropriate (e.g. non-pH-sensitive) drugs, to prevent intracellular drug degradation.

In conclusion, increased capillary permeability plays a crucial role in liposome localization at the infected site, whereas contribution of infiltrating leukocytes is limited. The observation that liposomes are taken up by phagocytes at the target site could be important information in drug selection.

In **Chapter 4**, the results presented in the preceding chapters and additional experimental data are used to propose a general mechanism behind liposome localization at sites of infection or inflammation. The mechanism is based on an equation by Kedem and Katchalsky describing the natural accumulation of plasma proteins at sites of increased capillary permeability as a result of a local inflammatory response [Kedem and Katchalsky, 1967].

The simplified equation describing liposome flux into the target site is:

➢ $J_L = J_V C_s (1-\sigma)$

Or in words, the liposome flux into the target site (J_L) is determined by the fluid flux into the target site across the capillary endothelial barrier (J_v), the concentration of liposomes therein (C_s), and the reflection coefficient (σ) for that given liposome formulation.

Regarding the fluid flux term, Bakker-Woudenberg et al. already demonstrated that SSL localization in the infected left lung is linearly related to the intensity of the *K. pneumoniae* infection (as measured by the increase in left lung weight) (Bakker-Woudenberg, 1993). As the weight increase is the result of fluid flux into the lung as a result of increased capillary permeability (as confirmed by Evan's blue dye extravasation and lung wet-to-dry-weight ratio, described in **Chapter 3**) these results support the equation and the view that the nature of the inflammatory stimulus is not important as long as an adequate fluid flux is generated. Present investigations in our department together with Lorna Stearne and Inge Gyssens in a murine abscess model, also show a positive relationship between abscess weight and liposome target localization (unpublished observations), which supports the general validity of the equation.

The term C_s refers to the liposome concentration in the circulation. SSL showing prolonged circulation times have been demonstrated to show an increased localization at the site of infection compared to liposomes with shorter circulation times (Bakker-Woudenberg, 1993). This term in the equation explains the relationship between circulation time and degree of left lung localization obtained in **Chapter 2** and earlier studies (Bakker-Woudenberg, 1993/1992). In addition, it explains why long-circulating liposomes (LCL) without a PEG-coating but with similar circulatory half-lifes as SSL, show a similar degree of liposome target localization. It must be stressed that SSL have the advantage of allowing tailoring of liposome properties without compromising liposome circulation time and degree of liposome target localization (**Chapter 2**), which is not expected to be valid for LCL without a PEG-coating (Woodle, 1992). As a result, SSL properties can be manipulated to optimize the retention and release of the encapsulated drug. The equation states that the liposome concentration term (C_s) is proportional to the degree of target localization (J_L). As a result it

is suggested that liposome localization can be improved by designing liposomes with even longer circulation times. However, to our knowledge, these liposomes have not been designed as yet.

The term C_s cannot explain why incorporation of a high mole % of EPG does not affect blood circulation time but does affect liposome target localization. It can be explained by assuming an increased reflection coefficient (σ) for SSL containing a high mol% of EPG. Yet, the nature of this increase in reflection coefficient for this liposome type is not clearly understood. Experiments to determine the value of σ for liposomes should be carried out, as such experiments could indicate if liposome target localization can be improved by reduction of liposomal σ.

Chapter 5 provides an overview of the literature on therapeutic efficacy of liposome-encapsulated aminoglycosides. Liposome-encapsulation offers interesting prospects to increase the therapeutic index of this class of antibiotics. The local application of liposomes may provide a drug reservoir to prolong therapeutic drug concentrations at the site of infection. Specific liposome lipid compositions may enhance bacterial killing by interacting (e.g. fusing) with the infectious organism (Sachetelli, 2000).

The main role of intravenously administered conventional liposomes is established in the targeted delivery of drugs to cells of the MPS, primarily those in the liver and spleen. Treatment of intracellular infections in the MPS-cells may benefit from the high amounts of aminoglycosides that can be delivered intracellularly. Research is needed on the nephro- and ototoxicity of conventional liposomal aminoglycosides as liposomal aminoglycoside administration results in prolonged drug exposure. In addition, it should be investigated whether this leads to an increased risk of the development of microbial drug resistance (Hyatt, 2000; Baquero, 1997).

Intravenous administration of long-circulating liposomes potentially offers drug targeting to sites of infection not restricted to the MPS. Indeed, some reports have demonstrated enhanced therapeutic efficacy of long-circulating liposome-encapsulated aminoglycosides compared to free drugs or conventional liposomes. Up to now, most studies with liposome-

encapsulated aminoglycosides have been performed in animal models with an intact host defense infected with high antibiotic-susceptible bacteria. Treatment failure in clinical practice, however, particularly occurs in patients having an impaired host defense. In addition, low antibiotic-susceptibility of the infectious organism complicates effective treatment. These issues need to be addresses in appropriate animal models. Similarly as for the conventional liposome formulations, the effects that the prolonged tissue drug concentrations have on toxicity and development of resistance need to be addressed. Overall, the reviewed literature shows promising prospects for liposome-encapsulated aminoglycosides and warrants further investigations into the use of these formulations for the treatment of severe infections.

Chapter 6 presents experiments on the therapeutic potential of SSL-encapsulated gentamicin (LE-GEN) in clinically relevant rat models of bacterial infections, addressing the issues of impaired host defense and low bacterial antibiotic susceptibility. The results show that in immunocompetent rats infected with the high gentamicin-susceptible *K. pneumoniae*, a single dose of LE-GEN is clearly superior over free GEN. Yet, complete therapeutic efficacy can also be obtained with multiple administration of low doses of free GEN.
In leukopenic rats infected with the high gentamicin-susceptible *K. pneumoniae*, multiple administration of free GEN at low doses was no longer effective. Free GEN at the maximum tolerated dose (MTD) for 5 days was needed to obtain complete therapeutic efficacy. However, the addition of a single dose of LE-GEN to 5-day free GEN-treatment at low doses showed complete therapeutic efficacy using a 7-fold lower cumulative amount of gentamicin compared to free GEN-treatment alone. A single dose of LE-GEN alone is unsuccessful. This is probably the result of the low therapeutically active drug concentrations in the bloodstream after injection of LE-GEN, which are insufficient to control the rapidly occurring bacteremia in case of an impaired host defense. The bacteremia leads to mortality during treatment, in spite of substantial liposome localization in the infected left lung and subsequent local bacterial killing. Free GEN alone at low doses for 5 days is also unsuccessful. Probably, this dosing regimen produces sufficient drug concentrations to

control the rapid bacteremia, but after termination of treatment rats die as a result of insufficient bacterial killing in the infected left lung, leading to local bacterial outgrowth accompanied by septicemia.
Thereby, the combination is efficacious compared to treatment with either GEN alone or LE-GEN alone, since the combination acts complementary.
In leukopenic rats infected with low gentamicin-susceptible *K. pneumoniae*, free GEN at the MTD for 5 days did not result in therapeutic efficacy. The use of LE-GEN is needed for achieving therapeutic success. Addition of LE-GEN to free GEN treatment using liposomes with a more rigid lipid bilayer resulted in 50% survival of the infected rats. By increasing LE-GEN bilayer fluidity, the liposomes displayed a faster release of gentamicin in the circulation. Rat survival increased to 100%. These results show that liposome lipid composition is an important determinant of therapeutic efficacy. A careful balance must be sought between release of the antibiotic in the circulation to obtain sufficiently high drug levels in the bloodstream versus liposomal drug retention in order to achieve sufficiently high levels of antibiotic at the infectious focus. In conclusion, in clinically relevant leukopenic rat models of serious bacterial infection, addition of LE-GEN to GEN treatment enhances therapeutic efficacy considerably. These results support further studies on liposomal formulations of aminoglycosides for use in immunocompromised patients with severe infections.

In **Chapter 7** a new concept in liposomal drug delivery is introduced. It is based on the evidence that antimicrobial agents can interact synergistically in vitro. To ensure a synergistic interaction in vivo, both drugs should be present at the site of infection at sufficiently high concentrations for an adequate period of time. By co-encapsulation of the drugs in a targeted drug carrier (e.g. SSL), the tissue distribution of the drugs will be mediated largely by the carrier. As a result, the body distribution of co-encapsulated agents will be similar. Moreover, encapsulation of these antibiotics in a targeted drug carrier, may even increase drug concentrations at the site of infection.
The synergistically acting antibiotics gentamicin and ceftazidime were co-encapsulated into SSL. Therapeutic efficacy in vivo was examined by monitoring rat survival in a model of an

acute unilateral pneumonia caused by a high gentamicin/ceftazidime-susceptible or a gentamicin/ceftazidime-resistant *K. pneumoniae*. The results show that targeted liposomal delivery of gentamicin and ceftazidime results in a synergistic interaction of these antibiotics in vivo. Importantly, the synergistic interaction was present in the susceptible as well as the resistant *K. pneumoniae* model. In contrast, administration of the antibiotics in the free form failed to display synergy. Probably as a result of the synergistic interaction, administration of liposome-co-encapsulated gentamicin and ceftazidime allowed a shorter course of treatment as well as lower cumulative doses to obtain complete therapeutic efficacy compared to administration of the drugs in the free form. In future studies, it should be investigated whether the reduced drug exposure can also reduce the risk of the development of microbial resistance.

In conclusion, the observed synergism of liposome-co-encapsulated antibiotics may open up new perspectives for targeted drug delivery in serious infectious diseases. It could also have its impact on other pathologies, as SSL have been shown to localize preferentially in tumors and sites of rheumatoid arthritis, conditions for which synergistic drug combinations have been identified (Yokoyama, 2000; Burris 2000, Dijkmans, 1996; Isaacs, 1999; Monks, 2000).

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9. Samenvatting voor niet-ingewijden

Bij een snijwondje in de vinger is een druppeltje jodium en een pleister op de wond in het algemeen voldoende voor een spoedig herstel. Het aanbrengen van een druppeltje jodium is een eenvoudig voorbeeld van 'drug targeting' (medicijnmikken), oftewel het specifiek afleveren van een farmacon (geneeskrachtige stof) op de plaats waar het nodig is. Misschien onbewust toegepast, is het doel van deze specifieke aflevering:

- een hoge farmaconconcentratie op de juiste plaats
- bescherming van de rest van het lichaam tegen mogelijke bijwerkingen en het beschermen van het farmacon tegen inactivatie
- controle over de afgifte van het farmacon
- vermindering van de hoeveelheid farmacon

Wanneer de plaats waar het farmacon nodig is onbekend of niet van buitenaf bereikbaar is, wordt specifieke aflevering moeilijker en kan er een nieuw doel beoogd worden:

- het verbeteren van de aflevering van het farmacon

In dat laatste geval is in het algemeen een drager van het farmacon vereist, een zogenaamde 'drug carrier'. In de loop der tijd is een grote variëteit aan dragers ontwikkeld. Of een drager geschikt is voor een farmacon wordt bepaald door een aantal factoren: de kenmerken van de ziekte waarvoor het farmacon bedoeld is, het vermogen van de drager op de juiste plek aan te komen en de wijze waarop de drager het farmacon ter plaatse afgeeft. In het onderzoek beschreven in dit proefschrift is de bruikbaarheid van een 'drug carrier' in de behandeling van bacteriële infecties onderzocht.

Bacteriële infecties kunnen worden beschouwd als een interactie tussen een gastheer (de mens) en een bacterie, waar de gastheer schade van ondervindt. Infecties kunnen een ernstig beloop hebben.
De reden waarom het onderzoek juist op deze ziekten is gericht, is het feit dat de klinische praktijk uitwijst dat infectieziekten, met name in ziekenhuizen, niet altijd met succes behandeld kunnen worden. Enerzijds is dat te wijten aan een matige gevoeligheid van bacteriën voor antibiotica, anderzijds neemt het aantal patiënten met een verminderde weerstand toe, bijvoorbeeld als gevolg van behandeling met chemotherapie (zoals bij tumoren), vanwege een storing in het immuunsysteem zelf (zoals bij een HIV-infectie), of in geval van immuunsuppressieve behandeling (zoals bij transplantaties). Deze patiënten zijn niet alleen gevoeliger voor infecties, ze kennen ook een slechtere ondersteuning van de antibioticumbehandeling door de eigen afweer. Het gevolg is dat de antibioticum-behandeling kan falen.
Daarom is er gezocht naar dragers die antibiotica specifiek naar infectiehaarden zouden kunnen vervoeren, waardoor het mogelijk zou kunnen zijn om bacteriën te doden ondanks hun matige antibioticumgevoeligheid en/of gebrekkige ondersteuning van de behandeling door de afweer van de patiënt. Eén van de farmacondragers waarvoor aanwijzingen zijn dat ze lokaliseren op plaatsen van infectie zijn liposomen.

Liposomen zijn kleine bolletjes bestaande uit één of meer schillen van fosfolipiden die waterige ruimten omsluiten (Figuur 1, pagina 16). Hun grootte ligt tussen enkele tientallen nanometers en enkele micrometers. Stoffen die goed oplossen in water kunnen in de waterige ruimte(s) worden ingesloten, terwijl vetoplosbare stoffen in de fosfolipidenbilaag kunnen worden ingebouwd. Deze bolletjes worden, na injectie in de bloedbaan, in het algemeen snel herkend als lichaamsvreemd en opgenomen door cellen die het bloed ontdoen van lichaamsvreemde materialen, het mononucleair fagocyten systeem (MPS). Als de infectie zich in deze cellen bevindt kan dit een eenvoudige manier zijn om snel, grote hoeveelheden antibiotica aan dit celtype af te leveren. Bevindt de infectie zich elders dan hindert deze snelle opname door het MPS de aflevering van het antibioticum aan de andere

plaatsen waar het antibioticum nodig is. Voor een minder snelle herkenning van het liposoom als lichaamsvreemd materiaal zijn er aanpassingen gedaan aan het oppervlak van het liposoom om te zorgen dat ze langer onopgemerkt in de bloedbaan kunnen blijven. Door op het liposoomoppervlak een laag poly(ethyleen) glycol (PEG) aan te brengen (Figuur 2, pagina 18) is het mogelijk de circulatietijd in het bloed te verlengen en zo de blootstelling van de infectiehaard aan het liposoom te verhogen. Bakker-Woudenberg et al. hebben aangetoond dat deze lang-circulerende PEG-liposomen beter op de plaats van infectie lokaliseren dan kort-circulerende liposomen in een model van een enkelzijdige pneumonie veroorzaakt door de bacterie *Klebsiella pneumoniae* in ratten. Dit model is ook gebruikt in de experimenten beschreven in dit proefschrift. In dit model worden de bacteriën in de linkerlong van de rat gebracht, waardoor zich daar een pneumonie ontwikkelt. De rechterlong kan dienen als controleorgaan.
Voor het verder optimaliseren van de liposomen als dragers voor antibiotica is het van belang vast te stellen:

- welke factoren (aan de kant van het liposoom zowel als aan de kant van de gastheer) de lokalisatie van liposomen op de plaats van infectie beïnvloeden
- in hoeverre liposomale antibiotica een bijdrage kunnen leveren in de behandeling van ernstige infecties in het geval van een verminderde afweer of een matige antibioticumgevoeligheid van de bacterie

Deze inleiding is beschreven in het **eerste hoofdstuk**.

De volgende drie hoofdstukken gaan in op het eerste doel van het onderzoek. In **hoofdstuk 2** is de invloed van liposomale factoren onderzocht door de eigenschappen van het liposoom te veranderen. De hoeveelheid van het oppervlakte-modificerende PEG is verminderd (1 mol%) en vermeerderd (10 mol%) ten opzichte van de hoeveelheid die gebruikt was (5 mol%) in de experimenten van Bakker-Woudenberg et al. De grootte van het liposoom is gevarieerd (100, 280 en 360 nm), de vloeibaarheid van de fosfolipidenbilaag

(de vloeibaarheid beïnvloedt de farmaconafgifte) is veranderd en er zijn meer of minder negatief geladen fosfolipiden in het liposoom geïncorporeerd. Het gedrag van de verschillende liposoomformuleringen is bestudeerd in ons bacteriële longinfectie-model.
Geconcludeerd kan worden dat 1 mol% PEG-liposomen sneller uit het bloed verdwijnen dan de 5 of 10 mol% PEG-liposomen. Waarschijnlijk is 1 mol% PEG minder goed in staat het oppervlak van het liposoom te beschermen tegen herkenning als lichaamsvreemd materiaal. De lokalisatie in de infectiehaard was ook lager voor dit liposoomtype. 10 mol% en 5 mol% PEG-liposomen lieten geen verschillen zien.
Met betrekking tot de liposoomgrootte kon worden vastgesteld dat de grotere liposomen sneller uit het bloed verwijderd werden dan het 100 nm liposoom, en ook een slechtere lokalisatie in de geïnfecteerde linkerlong lieten zien. Aanpassingen aan de vloeibaarheid van de fosfolipidenbilaag maakte geen verschil in de verblijftijd van de liposomen in de bloedbaan of de lokalisatie op de plaats van infectie.
Bij de incorporatie van de grootste hoeveelheid negatief geladen fosfolipiden in het liposoom was de uitkomst verrassend. Zoals tot nu toe blijkt gaan een kortere circulatietijd van de liposomen en een verminderde lokalisatie in de geïnfecteerde linkerlong hand in hand. Voor de liposomen waarin een grote hoeveelheid negatieve lading was geïncorporeerd bleef de circulatietijd hetzelfde, maar was de lokalisatie in de infectiehaard lager. Het vermoeden bestaat dat de lading geen effect heeft op de circulatietijd (doordat de PEG-laag de lading afschermt), maar dat bij de lokalisatie op de plaats van infectie de negatieve lading de lokalisatie hindert.
De uiteindelijke conclusie is dat een groot gedeelte van de aanpassingen aan het liposoom weinig of geen invloed hebben op de lokalisatiegraad op de plaats van infectie. Dit is waarschijnlijk het gevolg van de beschermende PEG-laag rond het liposoom. Dit betekent dat, mits aan bepaalde eisen met betrekking tot deeltjesgrootte, PEG-dichtheid en hoeveelheid negatief geladen fosfolipiden wordt voldaan, er een uitgebreide variatie mogelijk is in liposoomeigenschappen zonder de lokalisatie op de plaats van infectie in gevaar te brengen.

In het **derde hoofdstuk** is onderzocht in hoeverre de liposoomlokalisatie wordt beïnvloed door de ernst van de infectie, in welke zones van de infectiehaard de liposomen vooral gevonden worden en of liposomen ook lokaliseren in de linkerlong wanneer een ontstekingsreactie wordt veroorzaakt met irriterende stoffen in plaats van met levende bacteriën. Daarnaast is de hoeveelheid witte bloedcellen in het bloed van de ratten verminderd om vast te stellen of deze cellen een bijdrage leveren aan de liposoomlokalisatie. Hierdoor kan worden bepaald of liposoomtherapie wel geschikt is voor patiënten met een verminderde weerstand, waarbij juist deze cellen kunnen zijn aangetast. Tenslotte is microscopisch vastgesteld waar de liposomen op de plaats van de infectie terechtkomen. Hiervoor zijn liposomen gevuld met colloïdaal goud en geïnjecteerd. Hierna zijn coupes van de linkerlong gesneden en, door zilver neer te laten slaan op het goud, is vast te stellen waar de colloïdaal-goud gevulde liposomen zijn gebleven. Gebleken is dat naarmate de ontstekingsreactie sterker was, de hoeveelheid vocht die vanuit de bloedvaten in de infectiehaard stroomde toenam en daarmee nam ook de hoeveelheid liposomen op de plaats van infectie toe. De vochtophoping is onderdeel van de ontstekingsreactie. Het vermoeden is dat liposomen door de vloeistofstroom worden meegevoerd en zo in verhoogde mate lokaliseren op plaatsen waar een ontstekingsreactie plaatsvindt. Of deze ontstekingsreactie werd veroorzaakt door levende bacteriën of irriterende stoffen, bleek daarbij niet uit te maken. Het verminderen van de hoeveelheid witte bloedcellen maakte geen verschil in de hoeveelheid liposomen die in de infectiehaard terechtkwam. Dit maakt liposoomtherapie in principe ook geschikt voor situaties waarin de afweer is aangetast doordat de witte bloedcellen in aantal of functie tekortschieten. De liposomen bleken in de linkerlong aanwezig te zijn rond de capillairen en opgenomen door fagocyterende cellen. Of deze cellen de liposomen afbreken waardoor het antibioticum zou kunnen vrijkomen of dat hierdoor juist antibioticum verloren gaat is nog onduidelijk.

In het **vierde hoofdstuk** is geprobeerd om, met alle verzamelde gegevens over de lokalisatie van liposomen in dit diermodel, een algemeen mechanisme te definiëren achter het lokalisatieproces. Omdat van eiwitten in het bloed al langer bekend was dat deze

lokaliseren op de plaats van infectie (en daar meehelpen de infectie te bestrijden) en dat dit proces beschreven kan worden met een formule opgesteld door Kedem en Katchalsky, is deze formule als uitgangspunt genomen voor het onderzoek. De formule van Kedem en Katchalsky luidt:

$$J_s = J_v C_s (1-\sigma) + P_c S_c \Delta C$$

Het gedeelte van de formule na het plusteken beschrijft lokalisatie onder invloed van diffusie en dat is alleen van belang voor kleine deeltjes (zoals eiwitten). Liposomen zijn daarvoor te groot. Het overgebleven gedeelte zegt dat de hoeveelheid van een stof die lokaliseert per tijdseenheid (J_s) gelijk is aan de hoeveelheid vocht die per tijdseenheid in de infectiehaard stroomt (J_v), vermenigvuldigt met de concentratie van deze stof (C_s) in dit vocht, vermenigvuldigt met een factor die aangeeft hoe goed deze stof uit het bloedvat in de infectiehaard kan stromen. De experimentele resultaten zijn in overeenstemming met deze formule. Hoe langer het verblijf in het bloed van een liposoom, dus hoe hoger C_s, hoe hoger de liposoomlokalisatie. En hoe meer vocht er naar de long stroomt, dus hoe hoger (J_v), hoe hoger de liposoomlokalisatie. Wat betreft de lage lokalisatie van de liposomen met de geïncorporeerde negatief geladen fosfolipiden, zoals beschreven in het tweede hoofdstuk, zou dit te verklaren moeten zijn met een bemoeilijkte passage door de bloedvatwand voor dit type liposomen.

Hoofdstuk 5 geeft een overzicht van klinische en pre-klinische studies met betrekking tot de therapeutische effectiviteit van liposomen gevuld met aminoglycosiden: een belangrijke klasse van antibiotica. Het vormt een inleiding op onze studies met liposomaal ingekapseld gentamicine (LE-GEN) (ook een aminoglycoside) in de volgende hoofdstukken.

Hoofdstuk 6 laat resultaten zien van een studie in verschillende pneumoniemodellen in ratten met een oplopende moeilijkheidsgraad in behandeling. Het eerste model is relatief eenvoudig te behandelen: de ratten hebben een intacte afweer en de bacteriën zijn goed

gevoelig voor het antibioticum gentamicine. In het tweede model is de afweer van de ratten verminderd en in het derde model is niet alleen de afweer aangetast maar is de bacterie ook laag gevoelig voor gentamicine.

In het eerste model is een goede effectiviteit van één enkele dosis LE-GEN te zien, waarschijnlijk is dit een gevolg van de specifieke aflevering van het geneesmiddel aan de infectiehaard. Maar ook met een behandeling van slechts 3 dagen met het vrije geneesmiddel (GEN) is de infectie prima te behandelen. De noodzaak om het liposomale gentamicine te gebruiken is er dus niet.

In het rattenmodel met de verminderde weerstand is het verloop van de longinfectie ernstiger. In de long zijn weinig witte bloedcellen aanwezig om de bacteriën te doden, waardoor de bacteriën snel en in hoge aantallen in de bloedbaan komen en daar een sterke ontstekingsreactie kunnen uitlokken die leidt tot het overlijden van de dieren. Toediening van GEN gedurende 5 dagen -mits gegeven in hoge doses- kan ervoor zorgen dat de ratten overleven. De hoge dosis is nodig om niet alleen een adequate concentratie van het antibioticum in de de bloedbaan te garanderen, maar ook een voldoende hoge concentratie in de infectiehaard te bewerkstelligen. Eenzelfde effect kan bereikt worden wanneer GEN in lage doses wordt gegeven en er op de eerste dag een extra dosis LE-GEN wordt toegevoegd om de infectiehaard aan te pakken. Met deze combinatie overleefden alle dieren.

In het model waarin zowel de afweer van de ratten was verminderd en de bacteriën laag gevoelig zijn voor gentamicine geven de maximale doses GEN en LE-GEN gecombineerd een overleving van 50%. Weliswaar bleek er bij analyse van de geïnfecteerde linkerlong een grote hoeveelheid gentamicine aanwezig te zijn in het geïnfecteerde weefsel. Waarschijnlijk was slechts een gedeelte van het gentamicine na toediening in de liposomale vorm actief. Daarom is de samenstelling van het liposoom gewijzigd. Zoals bekend was uit andere studies zorgt het verhogen van de vloeibaarheid van de liposomale bilaag gewoonlijk voor een snellere afgifte van ingesloten farmaca. Uit de experimenten in Hoofdstuk 2 bleek dat de vloeibaarheid van de fosfolipidenbilaag geen invloed heeft op de hoeveelheid liposomen die op de plaats van de infectie lokaliseert. Combinatie van deze vloeibare formulering van

LE-GEN en GEN resulteerde in het overleven van alle dieren. Liposomen kunnen dus een belangrijke bijdrage leveren in de therapie, met name in moeilijk behandelbare situaties.

Het **voorlaatste hoofdstuk** geeft een nieuw concept weer op het gebied van de anti-microbiële therapie met liposomen. Het is al langer bekend dat bepaalde antibiotica synergistisch (elkaar versterkend) kunnen werken, zo ook voor gentamicine en ceftazidime. Gentamicine verhindert de eiwitsynthese in de bacterie, maar slechts een klein gedeelte is in staat de celwand rond de bacterie te passeren. Ceftazidime verzwakt juist de celwand. Combinatie van ceftazidime dat de celwand verzwakt en gentamicine dat daardoor makkelijker deze barrière kan passeren leidt tot een snelle dood van de bacteriën bij lage concentraties van combinaties van beide middelen. In het geval van een infectie is het probleem bij het toedienen van de beide antibiotica in de vrije vorm de onzekerheid of beide middelen wel op het juiste moment in passende concentraties gedurende een voldoende lange periode op de plaats van infectie aanwezig zijn. Door middel van liposomale inkapseling van beide antibiotica tezamen zou het mogelijk kunnen zijn beide middelen op dezelfde plaats op dezelfde tijd in hoge concentratie aanwezig te laten zijn. Uit de experimenten in ons bacteriële longinfectiemodel waarin de bacterie goed gevoelig is voor beide antibiotica blijkt dat de combinatie van de vrije middelen geen synergie laat zien, terwijl dat wel het geval is wanneer beide zijn ingekapseld in de liposomen en als zodanig worden toegediend. Als gevolg hiervan kon met de liposomale formulering zowel korter als lager worden gedoseerd. Ook wanneer de bacterie, die de longinfectie veroorzaakt, resistent was voor beide antibiotica werd synergie na liposomale inkapseling van beide middelen gezien.

In het **laatste hoofdstuk** worden de resultaten samengevat en worden suggesties gedaan voor voortzetting van het onderzoek.

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Curriculum vitae

Raymond Schiffelers werd op 21 januari 1971 geboren te Den Haag. In 1989 werd het V.W.O. diploma behaald aan het Rijnlands Lyceum Oegstgeest. In datzelfde jaar begon hij aan de studie Biologie aan de Rijksuniversiteit Leiden. Na het behalen van zijn propaedeuse in 1990 stapte hij over naar de (toenmalige) bovenbouwstudie Bio-Farmaceutische Wetenschappen aan dezelfde universiteit. In 1993-1994 werd onder leiding van Dr. P.C.N. Rensen en Prof. dr. Th.J.C. van Berkel bij de Afdeling Biofarmacie van het Leiden Amsterdam Center for Drug Research een afstudeeronderzoek uitgevoerd gericht op de selectieve sturing van cytostatica naar tumoren met apolipoproteïne E-verrrijkte liposomen via de lage dichtheids lipoproteïne-receptor.

In 1994-1995 is een industriestage bij SmithKline&Beecham Pharmaceuticals, Department of Vascular Biology, in Welwyn (Engeland) gelopen onder leiding van Dr. P.H.E. Groot en Dr. G.M. Benson. Tijdens deze stage zijn muizenmodellen voor atherosclerose onderzocht. In september 1995 studeerde hij af.

Van 1996 tot 2000 was hij werkzaam als onderzoeker in opleiding, in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (N.W.O.), bij het instituut Medische Microbiologie & Infectieziekten van het Erasmus *university* Medical Center Rotterdam en de Disciplinegroep Biofarmacie en Farmaceutische Technologie van het Utrecht Institute for Pharmaceutical Sciences (U.I.P.S.). Het onderzoek is uitgevoerd onder leiding van Dr. I.A.J.M. Bakker-Woudenberg en Prof. dr. G. Storm en richtte zich op de mogelijkheden van selectieve sturing van antibiotica naar bacteriële infectiehaarden door inkapseling in lang-circulerende liposomen. In november 1999 ontving hij de 'Best Lecture Award' tijdens de Toogdag van de Nederlandse Vereniging voor Farmaceutische Wetenschappen.

Sinds 1 augustus 2000 is hij werkzaam als post-doctoraal onderzoeker bij de Disciplinegroep Biofarmacie en Farmaceutische Technologie van het U.I.P.S., in het kader van een project gefinancierd door het Koningin Wilhelmina Fonds, gericht op het afleveren van cytotoxische verbindingen aan angiogene endotheelcellen rond tumoren, met behulp van liposomen.

Nawoord

Dik vijf jaar geleden toen ik voor het eerst het Dr. Molewaterplein zag, kon ik er direct 2 uur van genieten. Ik had de reistijd Leiden - Rotterdam Ee 1751a, rekening houdend met de mij onbekende route in het algemeen, en geruchten over onneembare hindernissen als perron 0, de metro, de Maas en een laboratorium van 24 verdiepingen in het bijzonder, op 3 uur ingeschat. De zomers waren toen nog lang en heet en zo kon het zijn dat ik met zonder jas, zittend op het bankje voor de Erasmus, 120 minuten had om nogmaals te oefenen hoe ik glashard mijn geestdrift voor het onderzoek, werklust, zelfstandigheid en inventiviteit zou roemen, en gevraagd naar eventuele negatieve karaktereigenschappen mijn soms wat overtrokken perfectionisme en te grote collegialiteit met de juiste dosis bedremmeldheid zou brengen. Uit het feit dat ik dit nu in dit werkje op kan schrijven valt af te leiden dat ik:

- het best in staat was mijn ware aard te verhullen van het legioen der sollicitanten (dat was toen nog zo, je stond nog in de rij voor een baan. Maar ja wat wilde je toentertijd ook anders met bedragen van 4 cijfers voor de komma die maandelijks (!) werden uitbetaald. 1600 gulden brandde in je zak als je alleen al dacht wat je daar allemaal niet voor kon kopen)
- dat het uiteindelijk tot een tot tevredenheid stemmend eindproduct is gekomen.

Het is waar, maar houdt geen rekening met het water dat sindsdien door de Maas is gestroomd tussen bankje en boekje.

Mijn moeder heeft dit boekje nooit kunnen zien. Iets waar ik de eerste Rotterdamse jaren toch nog vurig op gehoopt had.

Dat het er uiteindelijk toch ligt is mede te danken aan een lange rij collega's, vrienden en familie.

Mijn (co)promotoren Irma, Gert en Henri. Henri, betrokken bij zoveel onderzoeken en lid van minimaal evenzovele raden, besturen en colleges, wist mij altijd te verbazen door op werkbesprekingen exact de juiste vragen te stellen en goede suggesties te doen voor het vervolg, alsof hij de data gisteren nog had doorgenomen. Ik wil hem graag bedanken voor zijn inzet.

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Marian, één van de steunpilaren van het lab. Vanaf mijn rattenbeet op de eerste dag van mijn aanstelling tot de laatste inoculatie voor de synergie was je betrokken bij al mijn in vivo werk. Praktisch en hard meewerkend, bereid tot een onvoorzien experimentje tussendoor en nog aardig bovendien kan ik alleen maar zeggen: "zulke goede mensen worden maar weinig gemaakt".
Samen met Wim ook nooit te beroerd om een avond of weekend op te offeren voor een gezellig potje injecteren, waarbij we voor een paar stellingen ratten en tegen de 1000 injecties per week onze hand niet meer omdraaiden.

"En wij dan?" hoor ik Susan, Liesbeth, Martin, Els, Marcel, Diana, kleine Liesbeth, Lorna, Marianne; mijn stagestudenten Debbie, Corine, Angela, Els, Coşkun, en Renate; Tannie, Renata, Jan, Hanneke, Harald, Timo, Roel, Marian H, Floor en alle andere stagestudenten en alle overige collega's van 'beneden' en 'boven' al zeggen. "Wij hebben toch ook wel eens een naaldje meegeprikt, een overnachtcultuurtje ingezet, een plaatje geteld, een WK ⇨ KK uitgevoerd, overleving gescoord en zijn aardig geweest?"

Jazeker, Susan, Liesbeth, Martin, Els, Marcel, Diana, kleine Liesbeth, Lorna, Marianne; mijn stagestudenten Debbie, Corine, Angela, Els, Coşkun, en Renate; Tannie, Renata, Jan, Hanneke, Harald, Timo, Roel, Marian H, Floor en alle andere stagestudenten en alle overige collega's van 'beneden' en 'boven'. En jullie zijn er ook de reden van dat ik geen dag met tegenzin naar mijn werk ben geweest.

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Bedankt allemaal,

Ray

Abbreviations

AIDS	aquired immunodeficiency syndrome
ATCC	American type culture collection
AUC	area under the curve
AUIC	are under the curve above the minimal inhibory concentration
AUKC	area under the killing curve
C	consolidate zone
C_s	concentration of solute
Chol	cholesterol
Cpm	counts per minute
CZ	(free) ceftazidime
d_a	dose of A in combination with B needed to produce a desired effect
D_a	dose of A alone needed to produce the desired effect
d_b	dose of B in combination with A needed to produce a desired effect
D_b	dose of B alone needed to produce the desired effect
DNA	deoxyribonucleic acid
DSPC	distearoyl phosphatidylcholine
DSPE	distearoyl phosphatidylethanolamine
EI	early infected zone
EPC	egg phosphatidylcholine
EPG	egg phosphatidylglycerol
GEN	(free) gentamicin
H	hemorrhagic zone
HEPES	N-[-2 hydroxy ethyl] piperazine-N'-ethane sulfonic acid
IgG	immunoglobulin G
i.p.	intraperitoneal(ly)
i.v.	intravenous(ly)
J_s	flux of solute

J_v	fluid flux
LCL	long-circulating liposomes
LE-CZ	liposome-encapsulated ceftazidime
LE-GEN	liposome-encapsulated gentamicin
LE-GEN-CZ	liposome-co-encapsulated gentamicin and ceftazidime
LL	(infected) left lung
LPS	lipopolysaccharide
MAC	*Mycobacterium avium* complex
MPS	mononuclear phagocyte system
MTD	maximum tolerated dose
PBS	phosphate buffered saline
P_cS_c	capillary permeability surface area product
PEG	poly(ethylene) glycol
PHEPC	partially hydrogenated egg phosphatidylcholine
PS	phosphatidyl serine
RL	right lung
spp.	species
SSL	sterically stabilized liposomes
TL	total lipid
ΔC	concentration difference across capillary wall
σ	reflection coefficient