Exogenous Surfactant as a Carrier for Intratracheally Instilled Antimicrobial Agents

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EXOGENOUS SURFACTANT AS A CARRIER FOR INTRATRACHEALLY INSTILLED ANTIMICROBIAL AGENTS

EXOGEEN SURFACTANT ALS VEHIKEL VOOR INTRATRACHEAAL GEÏNSTILLEERDE ANTIMICROBIËLE MIDDELEN

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Contents

Chapter 1

General introduction and scope of the thesis

Introduction

The pulmonary surfactant system has been the subject of extensive research during the last three decades, most often in association with the neonatal respiratory distress syndrome (RDS) which is caused primarily by a lack of surfactant. In the premature neonate, intratracheal instillation of exogenous surfactant preparations has become a life-saving therapy. World wide trials with different natural and artificial surfactant preparations have demonstrated improvement in lung function, reduction in the rate of pneumothorax and decreased mortality in neonates.

In addition to surfactant dysfunction in RDS, significant compositional and/or functional changes in surfactant have been observed in a number of other respiratory disorders, such as the adult respiratory distress syndrome (ARDS), pneumonia, sarcoidosis, idiopathic pulmonary fibrosis and in lung transplant. Especially surfactant dysfunction in ARDS patients and in animal models for acute respiratory failure has been well-studied and surfactant therapy in these patients is currently under investigation. Chapter 2 gives an overview on the surfactant abnormalities observed in experimental pneumonia models and in pneumonia patients. It furthermore discusses the rationale for surfactant therapy in pneumonia and presents the first experimental and clinical data in this field.

Based on the spreading properties of surfactant preparations in the lung after intratracheal deposition, it has, recently, been proposed to use exogenous surfactant preparations for delivering intratracheally instilled agents to the lung parenchyma. Although each agent with its mode of action in the alveolar space, the lung interstitium or the alveolar capillary bed, could be considered for this delivery mode, special interest went out to antimicrobial agents. It is expected that in pneumonia selective delivery of antibiotics to the lung parenchyma increases the local effectiveness and decreases the risk for systemic side effects of potentially toxic agents such as aminoglycosides, pentamidine and amphotericin B. Combination of exogenous surfactant with antimicrobial agents may, thus, exhibit a double effect in the treatment of pneumonia. First, the surfactant instillation itself is potentially therapeutic as it promotes re-expansion of atelectatic areas and can correct impaired lung function and gas exchange in pneumonia. Second, it is expected that together with the surfactant a high effective antibiotic dose can be delivered to the alveolar compartment.

Although theoretically an interesting approach, very few data was available on the concept of using surfactant as delivery agent, also named carrier or vehicle, for intratracheally administered antibiotics. Therefore, the scope of the present thesis was to evaluate different aspects of the usefulness of surfactant as carrier for antibiotics.

Scope of the thesis

The first objective was to investigate the possible existence of interactions between pulmonary surfactant and antibiotics which interfered with the activity of either substance. In Chapter 3 the results of *in vitro* studies on the activity of amoxicillin, ceftazidime and tobramycin in the presence of pulmonary surfactant are described. Vice versa, Chapter 4 contains the results of *in vivo* surfactant function after mixture with amoxicillin, amphotericin B, ceftazidime, pentamidine and tobramycin.

The second objective was to evaluate *in vivo* efficacy. In Chapter 5 the first results on the *in vivo* efficacy of surfactant as a carrier are described. This study investigated the efficacy of intratracheally instilled tobramycin with and without the use of surfactant as vehicle, as determined by survival rates of mice with a respiratory *Klebsiella pneumoniae* infection.

Supported by the positive results reported in Chapter 5, we continued the studies focussing on possible determining factors for the observed efficacy. Previous investigations have demonstrated that surfactant was superior to saline as a vehicle for intratracheally instilled agents, that is pulmonary distribution of the instilled agent is wider and more homogenous with the use of surfactant as vehicle. However, these data were obtained in healthy hamster lungs and lung injury is likely to influence pulmonary distribution patterns of intratracheally instilled agents. The third objective in our studies was therefore to investigate pulmonary distribution of an intratracheally instilled antibiotic with and without the use of pulmonary surfactant as delivery agent in infected lungs. For this, a method for labeling tobramycin with technetium (^{99m}Tc) was evaluated and pulmonary distribution patterns of intratracheally instilled ^{99m}Tc-tobramycin in *Klebsiella pneumoniae* infected rat lungs were investigated, using surfactant or saline as vehicle (Chapter 6).

The aim of the studies in Chapter 7 was to evaluate lung clearance of intratracheally instilled ^{99m}Tc-tobramycin with surfactant or saline as vehicle, studied in spontaneously breathing or ventilated rats. A few investigations reported a prolonged pulmonary retention of agents instilled intratracheally in combination with exogenous surfactant instillation. The studies in Chapter 7 investigated if this decrease in lung clearance was also found for tobramycin when instilled with surfactant as vehicle.

Finally, Chapter 8 contains the summary and conclusions, including clinical implications of the present results and indications for future studies.

Chapter 2

Rationale for surfactant therapy in pneumonia

Adapted from: A. van 't Veen, D. Gommers, B. Lachmann. Rationale for surfactant therapy in pneumonia. *In:* Yearbook of Intensive Care and Emergency Medicine. Ed. J.L. Vincent, Springer, Heidelberg, pp 638-653, 1997

Introduction

Pneumonia remains an important cause for morbidity and mortality, despite advances in antimicrobial therapy. Pneumonia causes injury to the terminal alveolar-capillary unit, which is followed by increased alveolar permeability, pulmonary edema and hemorrhage and may lead to respiratory failure. It has been demonstrated that the pulmonary surfactant system becomes impaired in pneumonia, causing decreased compliance, atelectasis, pulmonary edema, ventilation-perfusion mismatch, intrapulmonary shunting, and an impaired arterial oxygenation [1-3].

Pulmonary surfactant, produced by alveolar type II cells, is composed of phospholipids and four specific surfactant proteins and forms a lipid layer coating the alveolar and bronchial epithelium. Its primary function is to reduce surface tension at the air-liquid interface of the terminal airways, which promotes alveolar expansion during inspiration and prevents alveolar collapse at expiration [4]. This layer is the initial surface that invading microorganisms contact when entering the alveoli, and accruing evidence suggests a double role for surfactant in pneumonia: it is involved in primary host defence against inhaled pathogens and is simultaneously a target in pneumonia [2,3,5].

Human necropsy studies in the 1960s already demonstrated a decreased surfactant activity in lung involved in pneumonia [6]. Although to date the impaired surfactant function in pneumonia has been verified by a great number of studies, precise mechanisms are still unclear. Possible pathways include a direct interaction of pathogens with the surfactant film, damage to alveolar type II cells affecting surfactant synthesis, and surfactant inactivation by inflammatory mediators and/or protein-rich alveolar edema [2,3,7].

Because surfactant dysfunction plays a role in the pathogenesis of pneumonia, beneficial effects can be expected from exogenous surfactant therapy. A few reports have demonstrated an improvement in lung function after intratracheal instillation of surfactant in animals and humans suffering from acute respiratory failure caused by pneumonia [1,2]. It has, moreover, been postulated that the excellent spreading properties of exogenous surfactant in the lung and its capacity to re-expand atelectatic areas could be exploited for delivering antimicrobial agents directly to the sites most wanted in pneumonia [8]; that is, within the alveolar space and the lung

interstitium. Studies in this area are very limited but the first experimental results suggest that the use of pulmonary surfactant-antibiotic mixtures is a promising approach for treatment of severe pneumonia [9].

The current chapter will, after a brief description of the pulmonary surfactant system, outline the existing evidence for surfactant abnormalities in pneumonia. It will present the few experimental and clinical data on exogenous surfactant therapy in pneumonia and discuss the concept of using exogenous surfactant as a carrier for antimicrobial agents.

The pulmonary surfactant system

The pulmonary surfactant system has been the subject of extensive research during the past three decades. Many aspects on composition, metabolism and function of pulmonary surfactant have been elucidated and will only be discussed briefly here [for reviews see refs 1,4,5,10-12].

Composition and metabolism

The extracellular compound of pulmonary surfactant can easily be harvested by broncho-alveolar lavage (BAL) of the whole lung with saline and is essentially similar between several mammalian species. Surfactant consists predominantly of lipids (80-90%), proteins (10%), and small amounts of carbohydrates. Of the total lipid weight, the phospholipids (see Table 1) represent 80-90% of which dipalmitoylphosphatidylcholine (DPPC) is most abundantly present and the principal surface tension reducing compound [10].

Four surfactant specific proteins (SP) have been identified and named in the sequence of their discovery: surfactant proteins A, B, C and D [10]. SP-A and SP-D are hydrophylic proteins whereas SP-B and SP-C are highly hydrophobic proteins. The surfactant proteins have been accredited various roles in the intra-alveolar metabolism of surfactant and the dynamics of the surface film. In addition SP-A and SP-D belong to the so-called collectins and apparently play a role in the innate immunity of the lung [11].

Table 1. Percentual distribution of phospholipid fraction*

Phosphatidylcholine	75%
of which 50% DPPC	
Phosphatidylglycerol	10%
Phosphatidylethanolamine	5%
Sphingomyelin	5%
Phosphatidylinositol/ Phosphatidylserine	5%

* data from reference [10]

Surfactant lipids and proteins are synthesized in type II cells and secreted into the alveolar space. Synthesis and/or secretion of surfactant are known to be influenced by a number of different stimuli [10]. Once secreted the surfactant is transformed into specific structures called tubular myelin, from which the insertion of phospholipids into the air-liquid interface is thought to take place. The surface film changes dynamically during respiration, with phospholipids continuously incorporated in and removed from the surface film [10]. Clearance of surfactant material from the alveoli seems predominantly accomplished by re-uptake in the type II cells and uptake by alveolar macrophages [12].

Surfactant functions and properties

The lungs have a continual elastic tendency to collapse which is caused by the recoil tendency of the tissue and, most pronounced, by the surface tension at the air-liquid interface in the lung. By reducing surface tensions at the air-liquid interface, pulmonary surfactant promotes alveolar stability at end-expiration and reduces alveolar opening pressures, as explained by the law of Laplace. That is, the pressure that is required to keep the alveolus expanded (P) is directly proportional to the tension in the alveolar wall (γ) divided by the alveolar diameter (r): $P = 2\gamma/r$. Surfactant reduces the surface tension at the air-liquid interface and the degree of surface tension reduction is closely tuned to the alveolar radius. As alveolar size decreases during expiration the surface film becomes enriched in DPPC which accounts for the very low surface tension (close to 0 dynes/cm) needed to prevent alveolar collapse [1,4,10].

Surfactant further protects against the formation of lung edema by stabilizing the fluid

balance in the lung. In general, the forces that influence the circulation of liquid at the alveolar-capillary level in the lungs include: plasma colloid osmotic pressure on the one side and capillary hydrostatic pressure, interstitial colloid osmotic pressure and alveolar surface tension on the other. As surface tension increases, the combined forces for moving fluid into the alveoli increases, resulting in pulmonary edema [4,13].

Pulmonary surfactant not only lines the alveoli, but also the narrow conducting airways. Increasing evidence supports the concept that pulmonary surfactant maintains openness of those small airways. A lack of bronchial surfactant may thus cause airway obstruction or collapse of small bronchioli with air trapping [4,14,15].

Finally, there is accruing evidence that pulmonary surfactant plays a role in the primary host defence in the lung. Surfactant may impede pathogen adherence to epithelial surfaces and facilitates mucocilliary clearance. Furthermore, surfactant components stimulate alveolar macrophages, that is, phagocytosis, intracellular degradation and migration. SP-A and SP-D are members of a collectin class and may function as opsonins inhibiting infectivity of various organisms directly and by enhancing phagocytosis. In addition, an immunosuppressive effect may exist, as surfactant components decrease the stimulatory effects of antigens on pulmonary and blood monocytes. The immunosuppressive effects are predominantly in association with the surfactant lipids. Thus far, more questions than answers remain on the role of surfactant in pulmonary host defence, especially concerning its in vivo relevance [for review see refs 5,11].

Table 2. Reported changes in surfactant phospholipid composition in pneumonia

	Micro-organism	Animal	Sample source	Total PL	Quantitative							Percentual distribution							Ratio /other	
					PC	PG	PE	PS	PI	SPH	lyso PC	PC	PG	PE	PS	PI	SPH	lyso PC		
Viral																				
[22]	Influenza A	mice	chopped lung washing	-	↓ (DSPC)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[23]	Bovine Herpesvirus-1	calves	BAL	-	-	-	-	-	-	-	-	↓	ns	↑	ns	ns	ns	ns	-	-
[23]	Parainfluenza-3	calves	BAL	-	-	-	-	-	-	-	-	↓	ns	↑	ns	ns	ns	ns	-	-
Bacterial																				
[37]	Mycoplasma pulmonis	pig	tissue homogenates	↑	-	-	-	-	-	-	-	↑	-	ns	ns	ns	↓	-	-	-
[28]	Pasteurella multicoda	pig	BAL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓PC/PI	↓PG/PE
[30]	Endotoxin	guinea-pig	BAL	ns	↓	↓	ns	ns	ns	↑	↑	↓	↓	ns	ns	↑	↑	↑	-	-
[26]	Pseudomonas aeruginosa	baboons	BAL	-	↓ (DSPC)	-	-	-	-	-	-	-	-	-	-	-	-	-	↓PG/PI	-
Parasitic																				
[40]	Pneumocystis carinii	rat	BAL	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[39]	Pneumocystis carinii	rat	BAL	↓	↓	↓	↓	↓	↓	↓	ns	↓	ns	ns	ns	ns	↓	ns	-	-
Patient																				
[49]	Miscellaneous		BAL	↓	-	-	-	-	-	-	-	↓	↓	ns	ns	↑	↑	ns	-	-
[47]	Bacterial		BAL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓ Palmitic acid	
[50]	Bacterial		tracheal aspirates	-	ns	-	-	-	-	↑	-	-	-	-	-	-	-	-	↓ PC/SPH	
[50]	Viral		tracheal aspirates	-	ns	-	-	-	-	ns	-	-	-	-	-	-	-	-	↓PC/SPH	
[52]	HIV/PCP		BAL	ns	↓	↓	↓	ns	ns	↑	↑	↓	↓	↑	↑	↑	↑	↑	-	
[54]	HIV/PCP		BAL	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓ Diacylglycerol	
[56]	HIV/ miscellaneous		BAL	ns	ns	↑	-	-	-	-	-	-	-	-	-	-	-	-	↑ Cholesterol	

Number in brackets correspond to the references. Total PL= total phospholipid; PC=phophatidylcholine; PG=phophatidylglycerol; PE=phophatidylethanolamine; PI= phophatidylinositol; SPH=sphingomyelin; lysoPC = lysophophatidylcholine; DSPC= disaturatedphophatidylcholine; - = not reported; ns=not significantly changed

Surfactant impairment in pneumonia

Both human and animal studies have provided evidence that pneumonia is attended with significant changes in pulmonary surfactant composition and function (Table 2). The data is derived from extracts or lavage samples of lungs from patients or animals with pneumonia of, in most cases, known microbial etiology. In spite of the diversity between studies the changes in pulmonary surfactant in pneumonia can roughly be characterized as: 1) increased surface tension, 2) a decrease in the amount of phospholipids recovered, 3) a shift in the phospholipid or fatty acid profile of the samples, and 4) changes in the amount of surfactant proteins recovered (Table 2). Shifts in phospholipid profiles are often expressed as ratios, e.g. the concentration of phosphatidylcholine (lecithin) to sphingomyelin is referred to as the L/S ratio.

Possible pathways

There are several pathways along which an impairment of the surfactant system may develop in pneumonia (Figure 1). Pathogens can directly interact with the extracellular surfactant pool or cause a surfactant impairment through interactions with type II cells, through induction of an inflammatory response or by destroying the integrity of the alveolar-capillary membrane. Proteases [16], phospholipases [7,17], or oxygen radicals [18] released by microorganisms and/or inflammatory cells can directly affect the surfactant. Further, type II cell function may be affected by virus replication [19], bacterial cytotoxic agents, or oxygen radicals [20] and interleukins released by inflammatory cells leading to alterations in surfactant composition and/or a decreased surfactant synthesis. Type I and/or type II cell lysis and/or proteolytic activity derived from microorganisms [21] or inflammatory cells can, finally, damage the alveolar-capillary membrane leading to a protein-rich edema. It is well-established that plasma proteins are capable of inactivating pulmonary surfactant [7]. Dependent on the pathogen involved, one or more of the above mentioned mechanisms may contribute to a surfactant dysfunction. So far, however, ample data are available to support the proposed mechanisms and much awaits experimental proof.

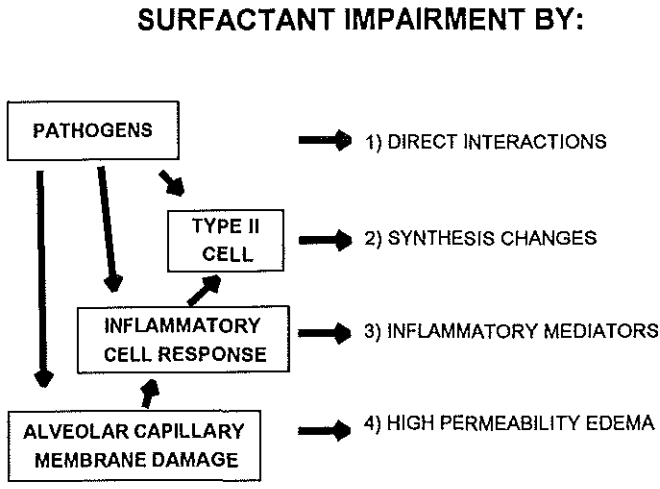

Figure 1. Pathways along which a surfactant impairment may develop in pneumonia. Pathogens can directly interact with the extracellular surfactant pool or cause a surfactant impairment through interactions with type II cells, through induction of an inflammatory response or by destroying the integrity of the alveolar capillary membrane.

Experimental viral pneumonia

In influenza A virus pneumonia in mice an increased surface tension together with a decreased disaturated phosphatidylcholine (DSPC) content was found as early as 2 days after infection, decraesing progressively to minimal values at 10 days after infection [22]. This was observed in uncollapsed and even more pronounced in collapsed infected lung tissue, and also in lung tissue that was not filled with edema. Morphological examination revealed swelling and degeneration of alveolar type II cells. It was suggested that injury and destruction of type II cells by the virus were the principal causes of the reduced surfactant activity [22]

Virus replication in type II cells may play a prominent role in surfactant disturbances in viral pneumonia. In mink kits, infection with aleutian mink disease parvovirus causes interstitial pneumonia resulting in a fatal respiratory failure [19]. Hyperplasia and hypertrophia of type II cells was observed and virus replication together with a decreased SP-C mRNA could be detected in type II cells by double in situ hybridization [19]. Finally, in calves exposed to bovine herpesvirus-1 or parainfluenza-3 virus, changes in the phospholipid profile of BAL fluid have

been reported [23].

Experimental bacterial pneumonia

Bacteria may directly interfere with surfactant function, as demonstrated in the studies by Rose and Lindberg [24]. Gram-negative rods, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, demonstrated a marked ability to destroy the surface tension reducing capacity of surfactant after in vitro incubation. *Streptococcus pneumoniae*, accordingly, had an adverse effect on surface tensions, but other Gram-positive bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes* did not exhibit this ability. A variety of bacteria secrete phospholipase C and this enzyme is capable of destroying surfactant and affecting its activity [7,17]. In vitro studies have demonstrated a complete degradation of DPPC in the presence of lipase and phospholipase C synthesized by *Pseudomonas aeruginosa* [25].

Two extensive studies, reported surfactant alterations in association with pathophysiological alterations in baboons ventilated with 80% oxygen for six days, followed by *Pseudomonas aeruginosa* infection and 50% oxygen ventilation for five days [26,27]. Pseudomonas infection caused a decrease in lung function, and surfactant analysis revealed a decrease in DSPC and a loss of surface activity in lavage samples [26]. The phosphatidylglycerol/ phosphatidylinositol (PG/PI) ratio was decreased compared with healthy non-ventilated control lungs; however, this decrease was also found in uninfected animals using a similar ventilatory protocol [26].

A decrease in the relative PG and increase in the relative PI levels was found in BAL fluid from pigs with a *Pasteurella multocida* pneumonia [28] (Table 2). PG and PI share a common precursor within the surfactant synthesis pathway and a compositional interchange between PG and PI has also been reported in other respiratory diseases such as idiopathic pulmonary fibrosis, alveolar proteinoses, the adult respiratory distress syndrome (ARDS), and during perinatal life [for review see ref 29].

In guinea pigs, endotracheal instillation of endotoxin caused impaired arterial oxygenation and decreased static compliance within 6 hours after instillation [30]. BAL fluid revealed increased surface tensions and decreased total phospholipids, and in the phospholipid profile significantly decreased amounts of DSPC and PG [30] (Table 2). Endotoxin is a

constituent of the cell walls of Gram-negative bacteria and is held responsible for many of the inflammatory phenomena associated with Gram-negative infections [31]. The major component lipopolysaccharide (LPS) of endotoxin can form complexes with whole pulmonary surfactant of sheep, and is capable of decreasing surface activity of surfactant in vitro [3]. SP-A [32,33] and SP-D [34] can specifically bind LPS in vitro. It has been speculated that this complex formation with LPS has a protective effect, restricting inflammation in the alveoli, rather than a destructive effect on surfactant function [33,34]. Future studies should clarify the in vivo importance of these observations. Lopez and colleagues [35] have demonstrated cytotoxicity of intratracheally instilled LPS to pulmonary epithelial cells causing epithelial desquamation. This may well affect pulmonary surfactant synthesis.

In *Mycoplasma pulmonis* infected rat lungs, an increased surface tension appeared to be the main cause for a decrease in lung compliance [36]. Increased surface tensions were also found in Mycoplasma pneumonia in pigs, in spite of an observed increase in DSPC [37]. This study, however, used whole lung homogenates which include cellular lipids and the increase in phospholipids should therefore be considered with caution.

Experimental Pneumocystis carinii pneumonia

During its life cycle in the alveolar space, *Pneumocystis carinii* can selectively adhere to alveolar type I cells leading to type I cell degeneration and causing increased alveolar-capillary permeability and pulmonary edema [38]. Studies with a *P. carinii* model in rats have demonstrated a significant decrease in the total amount of phospholipid recovered from BAL fluid [39,40], changes in phospholipid pattern [39] (Table 2) and an accumulation of SP-A and SP-D in the lung during *P.carinii* pneumonia [41,42]. Recently, it has been shown that SP-A and SP-D can bind to *P. carinii* organisms and augment attachment of *P. carinii* to alveolar macrophages [42-44]. It has been suggested that *P. carinii* has a pathogen specific effect on SP-A and SP-D homeostasis [41; for review see 45].

Patient studies

Sutnick and Soloff already reported in 1964 increased surface tensions in extracts from lungs involved in pneumonia [6]. Since then, several clinical studies have been published

demonstrating surfactant abnormalities in pneumonia (Table 2).

Hallman and colleagues studied BAL specimens from patients with respiratory failure and other respiratory diseases among which pneumonia patients [46]. Percentual changes in the phospholipid profile were observed in pneumonia patients in comparison to shifts previously reported in experimental pneumonia, that is decreased PC, PG and increased SPH [46].

Baughman and coworkers [47] studied fatty acid profiles of non-ventilated patients with bacterial pneumonia. Bacterial pneumonia was characterized by a decrease in palmitic acid which is the major fatty acid component of DPPC. This study clearly demonstrated surfactant abnormalities in bacterial pneumonia before the occurrence of severe respiratory failure. A more recent study from Baughman and coworkers [47] reported a decrease in SP-A levels in non-ventilated patients with bacterial pneumonia, more profound in Gram-positive than Gram-negative bacterial pneumonia [48].

Günther and colleagues [49] studied BAL fluid derived from intubated patients with severe pneumonia from various etiology, ARDS patients and patients with cardiogenic lung edema. They found in BAL fluid from pneumonia patients, a tendency towards decreased total phospholipid levels, changes in phospholipid profile (Table 2), a decrease in SP-A but not in SP-B levels, and an impaired surface activity. The same study showed that these surfactant abnormalities corresponded to those found in ARDS patients, but were absent in patients with cardiogenic lung edema [49]. LeVine and coworkers [50] studied tracheal aspirates from pediatric patients with viral pneumonia or bacterial pneumonia and found in both groups decreased L/S ratio and decreased SP-A levels but, no decrease in SP-B levels. The L/S ratio was increased by the time of extubation and correlated weakly with an increase in pulmonary compliance [50].

A number of studies have focused on surfactant abnormalities in *P.carinii* pneumonia in patients infected with the human immunodeficiency virus (HIV) and found decreased levels of total phospholipids [54], changes in phospholipid profile [51-53] and elevated levels of SP-A [54,55]. There is evidence that infection with HIV itself, without the presence of *P.carinii*, causes increased alveolar-capillary permeability [56] and surfactant abnormalities, i.e. decreased phospholipid [52] and SP-A levels [54].

Surfactant therapy in pneumonia

The increasing evidence for quantitative and qualitative changes in the surfactant system during pneumonia, has led to the hypothesis that surfactant instillation is beneficial in pneumonia. Exogenous surfactant may re-expand and stabilize alveoli and small airways, improve lung volumes, decrease required inspiratory pressures and enhances alveolar and interstitial fluid resorption in pneumonia. Although surfactant instillation does not directly treat the underlying lung infection, it may invert a progressive decay in lung function.

Instillation of exogenous surfactant preparations, derived from natural sources or artificially produced, is to date a well-established therapy in neonates suffering from infant respiratory distress syndrome, known to be triggered primarily by a lack of pulmonary surfactant [57]. Surfactant therapy is currently also under investigation for patients suffering from ARDS [1]. ARDS and pneumonia are closely associated: not only is pneumonia an important antecedent of ARDS, ARDS is also often complicated by secondary pulmonary infection [58]. A few experimental studies have focused on surfactant therapy in pneumonia and a profound number of the ARDS patients treated with surfactant so far, are primary pneumonia patients.

Experimental studies

In a Sendai virus model in rats, gas exchange and pulmonary compliance deteriorated after infection, with a lethal outcome within 4 days [59]. Increments of the ventilatory pressures could improve arterial oxygenation on day 2 after infection, but no longer at day 3 after infection (see Figure 2). At this time, keeping the same ventilator settings, replacement therapy with a natural surfactant (200 mg/kg) resulted in a significant improvement of arterial oxygenation [60] (Fig 2).

Similar improvements in gas exchange were observed in rats with a severe *P.carinii* pneumonia after instillation of 200 mg/kg of a natural surfactant [61]. Furthermore, studies in mice demonstrated that surfactant instillation could correct the decrease in lung compliance and lung volume during severe influenza A pneumonia [62]. Preliminary studies with the same virus in mice showed that surfactant instillation in spontaneously breathing animals at day 5 after infection, could improve survival outcome [63].

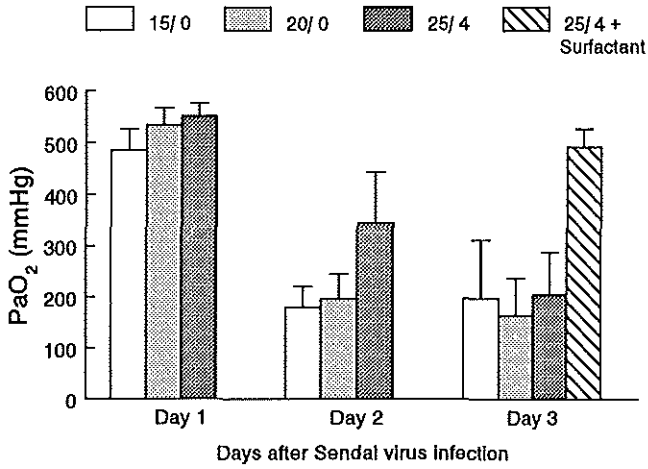

Figure 2. Effects of increased airway pressures (e.g. 15/ 0 meaning 15 cmH_2O peak inspiratory pressure and 0 cmH_2O positive end-expiratory pressure) or intratracheal surfactant instillation on arterial oxygenation (PaO_2) during artificial ventilation of rats infected with Sendai virus. (Data from refs 59 & 60). At day 3 after infection surfactant instillation significantly increased arterial oxygenation.

Recent studies have demonstrated the efficacy of surfactant instillation in models for severe respiratory failure induced by bacteria or bacterial products [64-66]. In ventilated rats suffering from severe respiratory failure after endotracheal injection of endotoxin, bolus instillation of a natural surfactant (100 mg/kg body weight) resulted in an improvement of the arterial oxygenation, stable for 3 hours after instillation, and an improvement in compliance [64,65]. Surfactant instillation (160 mg/kg body weight) could similarly improve arterial oxygnation in a rat model for severe respiratory failure induced by *Escherichia coli* pneumonia [66]. In this latter study, compliance was not improved by surfactant instillation [66].

Patient studies

An increasing number of reports have described the effects of surfactant instillation in patients suffering from acute respiratory failure due to pneumonia of different microbial etiology [4,67-71]. Lachmann described a four year old patient with bacterial pneumonia and acute

respiratory failure [4]. This patient received up to three doses of a natural surfactant of 150, 100 and 50 mg/kg body weight, respectively. Only after the last dose a dramatic improvement of gas exchange was observed. Four hours after instillation the chest X-rays appeared almost 'normal'. Accordingly, improvements in arterial oxygenation were observed after instillation of a natural surfactant in two patients with acute respiratory failure due to viral pneumonia by Buheitel and colleagues [67] and by Harms & Herting [68] in two neonates suffering from Chlamydial pneumonia. One recurrent observation in these reports is the often transient effect after surfactant instillation and the demand for multiple (2 to 3) and high surfactant doses (up to 550 mg/kg body weight) before a stable improvement in lung function is achieved [4,67,68]. This has been attributed to the large amount of surfactant inhibitors present in the lung [7].

A similar need for multiple doses was found in a study by Auten and coworkers [69] who studied the effects of surfactant instillation in seven full term newborns with pneumonia of different etiology. These patients received one to four doses of 90 mg/kg natural surfactant. Arterial oxygenation improved rapidly after a first and second dose but was less improved after the subsequent doses [69]. A recent study reported the results of surfactant therapy in acute hypoxemic respiratory failure in pediatric patients of which 13 out of 29 enrolled patients were diagnosed with pneumonia [70]. Patients received a natural surfactant as a bolus up to four doses. The overall results demonstrated, according to previous reports, a need for multiple doses in 17 out of 24 initially responding patients, and most profound responses in oxygenation index and ventilatory parameters after the first dose.

So far, only one report has described the effects of selective surfactant instillation in an adult man (aged 71 years, 78 kg) deteriorating from a right lower lobe pneumonia developed after abdominal surgery [71]. This patient received 240 mg of a natural surfactant suspended in 6 ml saline which was instilled into the affected lobe through a fiber bronchoscope. After instillation a gradual improvement of oxygenation was observed. This improvement was not as dramatic as usually observed after surfactant instillation, but this might be attributed to the low surfactant dose instilled. One might speculate that the improvement would be more pronounced if surfactant was instilled in a larger dose in the whole right lung.

Surfactant as a carrier for antimicrobial agents

It has been proposed to use the spreading properties and the inherent therapeutic potential of surfactant for delivering antimicrobial agents to the lung parenchyma [8]. Although each agent with its mode of action in the alveolar space and the lung interstitium could be considered for this administration mode, special interest has gone out to antimicrobial agents. Direct application of antibiotics to the airways offers many potential advantages in the treatment and prevention of pneumonia. Delivery directly to the airways, should increase the local effectiveness and reduce the risk for systemic toxicity caused by some antibiotics, e.g. aminoglycosides [72].

Locally administered antibiotics for prevention or treatment of lower respiratory tract infection has been studied extensively. However, despite the high antibiotic dose delivered to the lung, the question of efficacy remains controversial. One explanation includes failure of the antibiotic to reach the infected areas of the lung. When delivered as an aerosol, only a small amount of the nebulized antibiotic dose, around 10%, is actually deposited in the lung. Moreover, with increased airway obstruction and lung damage the amount of aerosol deposited in peripheral regions of the lung decreases [73,74]. Lung distribution of intratracheally instilled antibiotic solutions is poorly studied. However, it is known that distribution of intratracheally instilled saline is largely limited to the central regions of the lung [8,75]. Due to the small diameter of peripheral airways, fluids with a high surface tension, such as saline and water, require high pressures for passage through these airways [76].

It has been shown in experimental studies that pulmonary surfactant is superior to saline in distributing a radioactive colloid within healthy lungs: lung distribution is more peripheral and more homogeneous [8]. Furthermore, surfactant can re-expand non-ventilated atelectatic areas which are, most likely, the infected areas. It is, therefore, expected that intratracheally instilled antibiotics are more effective when the distribution within the lung is optimized by using pulmonary surfactant as a vehicle. Different aspects of this delivery method are further addressed to in the subsequent chapters.

Conclusion

Experimental and clinical studies have shown that pneumonia is indifferent of the

causative pathogen, attended with significant functional and compositional changes in pulmonary surfactant. This surfactant impairment attributes to the impaired lung function in pneumonia. Further studies have shown that impaired lung function in pneumonia can be corrected by instillation of exogenous surfactant. Moreover, exogenous surfactant preparations can be used as a carrier for antimicrobial agents thus improving the efficacy of local antimicrobial therapy in pneumonia. Surfactant therapy offers a potential approach in pneumonia. Many aspects of surfactant therapy, however, still need to be clarified. For instance, it is unclear which patients may benefit most from surfactant therapy, which doses should be used, and which type of surfactant preparation is preferred. Controlled clinical trials for surfactant therapy in severe pneumonia should clarify some of these questions. As for surfactant as a carrier for antibiotics, further experimental studies should establish the efficacy of this new delivery method.

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Chapter 3

Influence of pulmonary surfactant on in vitro bactericidal activity of amoxicillin, ceftazidime and tobramycin

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Summary

The influence of a natural pulmonary surfactant on antibiotic activity was investigated to assess the possible use of exogenous surfactant as a vehicle for antibiotic delivery to the lung. Influence of surfactant on the bactericidal activity of amoxicillin was tested against *Staphylococcus aureus* and *Streptococcus pneumoniae* and on the activity of ceftazidime and tobramycin against *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pneumoniae*. In vitro antibiotic activity was determined with killing curve studies in media with and without surfactant. Amoxicillin and ceftazidime activity were not changed in the presence of surfactant, except for a decreased killing rate of *S. pneumoniae* by ceftazidime in medium with additional rabbit serum. In contrast, killing curves with low concentrations of tobramycin (0.25 and 1 x MIC) showed a decreased activity of tobramycin against all pathogens tested in the presence of surfactant. With higher tobramycin concentrations (4 x MIC) killing rates were less decreased or unchanged in the presence of surfactant. Concluding from this study, both amoxicillin and ceftazidime can be combined with surfactant without loss of activity. For mixing surfactant with tobramycin, dosages should be adjusted to overcome the partial inactivation of tobramycin by surfactant.

Introduction

Lower respiratory tract infections remain an important cause of morbidity and mortality, despite the use of new potent antibiotics. Especially nosocomial pneumonia remains difficult to treat, with crude mortality rates of 10-30% and even higher rates in specific patient groups [1,2]. Efficient antimicrobial therapy is considered to be dependent on appropriate antibiotic concentrations at the site of infection [2,3]. For pneumonia this is within the alveolar space, together with the epithelial lining fluid and the lung interstium [4]. However, the high systemic doses needed for some antibiotics to reach therapeutic levels at these sites may be accompanied by adverse side effects, e.g. oto- and nephrotoxicity caused by aminoglycosides [5].

Local administration of antibiotics via the trachea offers the potential benefits of high concentrations at the site of infection and low systemic absorption. Previous studies have shown beneficial effects with both inhalation of antibiotics via aerosol [6-8] and direct endotracheal instillation of antibiotics [9-11]. However, with aerosol inhalation the amount of antibiotics deposited in the lung is small, only 10-20% even with the best nebulizers [9,12,13]. Moreover, pulmonary deposition is particularly high in the central airways and decreases towards the periphery in patients with decreased pulmonary function [12,14]. With direct endotracheal instillation, distribution is also largely limited to the central airways [15]. Thus, the therapeutic efficacy of these modes of administration is limited, especially since the location of infected areas is most often peripheral. The efficacy of locally administered antibiotics might, therefore, be improved by optimising the distribution within the lung.

Due to the small diameter of the airways in the periphery of the lung, fluids with a high surface tension, such as saline, will require high pressures for passage through these airways [16]. Surfactant, a mixture of phospholipids and specific surfactant proteins, has the capacity to lower surface tension [17]. Recently it is shown that exogenous pulmonary surfactant labelled with a radioactive colloid and mixed with pentamidine, results in a more peripheral and uniform distribution pattern in the lungs compared to a combination of pentamidine and saline [18]. It is, therefore, expected that the use of surfactant as carrier for antibiotics has great potential in treating patients with severe pneumonia.

However, it is unknown if surfactant affects the activity of antibiotics. Therefore, an in

vitro study was designed to investigate the influence of a bovine surfactant on the bactericidal activity of clinically relevant antibiotics against pathogens often involved in respiratory tract infection.

Materials and methods

Bacteria and preparation of inoculum

The following strains were used: *Klebsiella pneumoniae* (ATCC 43816), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (ATCC 6301) and *Pseudomonas aeruginosa* (ATCC 27853). For *S. aureus* and *K. pneumoniae*, stationary-phase cultures were prepared by incubation for 16 h at 37°C in Mueller Hinton broth (MHB) (Difco Laboratories, Detroit, USA) For *P. aeruginosa*, MHB was supplemented with magnesium (12.5 mg/l) and calcium (20 to 25 mg/l)(Merck, Darmstadt, Germany)[19]. After proper dilution and reincubation for 2 h at 37°C, suspensions of logarithmic growing bacteria were obtained.

For an *S. pneumoniae* inoculum, an overnight culture on 5% blood agar plate (Bactim, Breukelen, The Netherlands) was suspended in Todd Hewitt broth (THB) (Oxoid Ltd., Basingstoke, England) containing 10% normal rabbit serum (Dako A/S, Glostrup, Denmark) which was first inactivated for 30 min at 56°C. The culture was then incubated at 37°C and optical density (OD) was measured repeatedly. When OD remained constant for 30 min, the culture was diluted with THB to obtain the proper inoculum size of end log phase bacteria.

Antibiotics

Tobramycin (Eli Lilly, Amsterdam, The Netherlands), amoxicillin (SmithKline Beecham, Amstelveen, The Netherlands) and ceftazidime (Glaxo Pharmaceuticals Ltd., Greenford, England) were kindly provided by the manufacturers and prepared by diluting the standard powder or solution with the recommended dilutent to a stock solution of 2560 µg/ml which was stored at -80°C in small aliquots. For each experiment a fresh aliquot was used and diluted in broth to obtain the appropriate concentrations.

Surfactant

The surfactant used in these studies is a freeze-dried natural surfactant isolated from bovine lungs as previously described [20]. It consists of approximately 90-95% phospholipids, 1% hydrophobic proteins (surfactant proteins B and C) and 1% free fatty acids, the remainder being other lipids such as cholesterol and glyceride; there is no surfactant-protein A in this surfactant preparation. For every killing curve study or bacterial growth study, the surfactant was suspended in broth to a concentration of 25 mg/ml growth media.

Antimicrobial susceptibility tests

The Minimal Inhibitory Concentration (MIC) was determined with a microdilution technique. MIC of the drug was defined as the lowest concentration that suppressed visible growth after incubation at 5 x 10^5 colony-forming units (CFU) /ml for 18 h at 37°C in microtiter plates containing a total volume of 200 µl. For *K. pneumoniae* and *S. aureus* this was determined in MHB, for *P. aeruginosa* in MHB supplemented with Mg^{2+} and Ca^{2+} and for *S. pneumoniae* in THB. MICs did not differ more than one step from MICs earlier reported for these strains [21,22].

Timed killing curves were made at 0.25, 1 and 4 times the MIC of the tested antibiotic. Killing curves were calculated from growth in 10 ml glass tubes containing a total volume of 2 ml growth medium, with or without 25 mg/ml surfactant. For *S. pneumoniae*, influence of surfactant on bacterial killing by antibiotics was determined in THB with and without 10% rabbit serum. With each assay appropriate growth controls were studied. Starting inoculum (T=0) was adjusted to approximately 5 x 10^5 CFU/ml. Sampling was performed at 0, 1, 2, 4, 6 and 8 h after inoculation. Ten-fold dilution steps of 100 µl samples were made in PBS on ice (pH = 7.3) (Oxoid Ltd., Basingstoke, England). In order to determine the number of viable micro-organisms, 100 µl of the appropriate ten-fold dilution steps were plated on Iso-Sensitest agar (Oxoid Ltd., Basingstoke, England) or, in the case of *S. pneumoniae*, on blood agar plates. Colony counts were performed after 24 h incubation at 37°C. To minimize antibiotic carryover on subculture plates, only 50 µl of an undiluted sample was subcultured directly on agar plate. The lower limit of detection by this method was 20 CFU/ml, and the accurately countable number 600 CFU/ml. Killing curves were performed at least in duplicate.

Oher measurements

The pH of the incubation media was measured to assess a possible change in pH when surfactant and/or rabbit serum was added to the broth. For this, volumes of 2 ml broth with or without 25 mg/ml surfactant and with or without 10% rabbit serum were freshly made in duplo. The pH of these samples was measured with a glass electrode (model 920 A, Orion Research Inc, Boston, USA).

The non-bound tobramycin concentration in samples with and without surfactant and/or rabbit serum in THB was determined in duplo using the Amicron micropartition system (MPS-1, 4010, Amicron, Mass. USA). For this, 1 ml samples were made containing approximately 100 µg tobramycin and respectively: (i) 2 samples with no extra additions (controls), (ii) 2 samples with 25 mg/ml surfactant, (iii) 2 samples with 12.5 mg/ml surfactant and (iv) 2 samples with 10% rabbit serum. The samples were centrifuged at 2000 g for 30 minutes. Tobramycin concentrations were determined for filtration in the sample and after filtration in the filtrate, with a fluorescence analysis using the TDX (Abbott Diagnostic Division, Weesp, Nederland). Binding is expressed as 1 minus the ratio free tobramycin/total tobramycin * 100%.

Statistical analysis

For analysis of the data, a logarithmic transformation ($\log_{10}$) was performed on all data. With killing curves, the total area under the killing curve (AUKC) of transformed data was determined for presentation of the data. In this type of analysis the rate of killing is inversely related to the AUKC [23]. The effect of surfactant on antibiotic activity (with vs without surfactant) was tested with analysis of variance for repeated measures using the GLM procedure of the SAS® statistical package [24]. With these tests overall effect of surfactant on antibiotic activity was tested, as well as the effect of surfactant for each antibiotic concentration separately. Additionally, for *S. pneumoniae*, the effect of rabbit serum was tested. Growth rate was expressed as the slope (y/x) of the regression line from T0 - T8 and its units were the change in the $\log_{10}$ CFU/ml per hour [21]. Mean and standard deviation were calculated. Statistical significance between growth rate of bacteria in media with and without surfactant was tested using the t-test for two samples [24]. With all statistical tests, significance was accepted at p values ≤ 0.05 two-tailed.

Results

Bacterial growth rate (log_{10} CFU/ml per hour) was not altered for *P.aeruginosa, K. pneumoniae* or *S.aureus* in the presence of surfactant (Table 1). For *S. pneumoniae,* bacterial growth rate was not affected, except for a decreased growth rate in THB with rabbit serum in the presence of surfactant (Table 1). No statistical significant difference was found between growth rate of *S. pneumoniae* in THB with rabbit serum (0.509±0.03) and growth rate in THB with rabbit serum and surfactant (0.437±0.03) when compared to growth rate in THB alone ($p = 0.064$ and 0.606, respectively). Similarly, no statistical significant difference was found between growth rate of *S. pneumoniae* in THB with rabbit serum (0.509±0.03) and growth rate in THB with rabbit serum and surfactant (0.437±0.03) when compared to growth rate in THB with surfactant and without serum (0.481±0.007) ($p = 0.060$ and 0.306, respectively).

Table 1. Bacterial growth rates

Organism	Bacterial growth rate mean ± SD (log_{10} CFU/ml/ h)		
	without surfactant	with surfactant	*P* value*
K.pneumoniae	0.524 ± 0.084	0.576 ± 0.053	0.414
P.aeruginosa	0.349 ± 0.018	0.344 ± 0.010	0.652
S.aureus	0.474 ± 0.048	0.463 ± 0.046	0.740
S.pneumoniae			
without serum	0.403 ± 0.076	0.481 ± 0.007	0.131
with serum	0.509 ± 0.025	0.437 ± 0.033	0.015

* The *P* value for bacterial growth in medium with surfactant versus that for bacterial growth in medium without surfactant was determined by the *t*-test.

Bacterial killing of *S. pneumoniae* and *S. aureus* by amoxicillin and of *K. pneumoniae, P. aeruginosa* and *S. aureus* by ceftazidime, was not altered in the presence of surfactant (Tables 2 and 3). The killing of *S. pneumoniae* by ceftazidime in THB supplemented with rabbit serum was decreased in the presence of surfactant at 4 x MIC (Table 3). A reduction in bactericidal activity (increased AUKCs) of tobramycin against *K. pneumoniae, P. aeruginosa, S. aureus* and *S. pneumoniae* was observed when surfactant was added to the medium (Table 4). This was best demonstrated in killing curves at the MIC of the tested strain. The effect of surfactant on

Table 2. Inluence of surfactant on amoxicillin activity

Organism	Amoxicillin conc (μg/ml)	Area under killing curve mean ± SD ((log_{10} CFU/ml)xh)		*P* value	
		without surfactant	with surfactant	Be-tween[a]	Over-all[b]
S.aureus	0.03	47.99 ± 4.46	43.40 ± 0.17	0.303	0.774
	0.13	29.42 ± 1.15	29.29 ± 1.12	0.917	
	0.5	22.88 ± 0.17	22.40 ± 5.05	0.861	
S.pneumoniae					
without serum	0.03	52.32 ± 1.80	55.11 ± 0.38	0.174	0.393
	0.13	29.94 ± 0.26	17.28 ± 5.76	0.061	
	0.5	19.77 ± 4.53	15.51 ± 4.75	0.521	
with serum	0.03	53.57 ± 0.47	55.72 ± 0.36	0.346	0.394
	0.13	28.84 ± 1.98	26.70 ± 1.12	0.717	
	0.5	19.43 ± 4.33	17.39 ± 6.40	0.489	

[a]The *P* value for the effects of surfactant on antibiotic activity for each antibiotic concentration and [b] the *P* value for the effects of surfactant on antibiotic activity in all killing curves. Determined by analysis of variance for repeated time measurements

Table 3. Inluence of surfactant on ceftazidime activity

Organism	Ceftazidime conc (μg/ml)	Area under killing curve mean ± SD ((log_{10} CFU/ml)xh)		*P* value	
		without surfactant	with surfactant	Be-tween[a]	Over-all[b]
K.pneumoniae	0.13	19.90 ± 0.12	25.17 ± 3.08	0.346	0.378
	0.5	16.32 ± 2.62	17.88 ± 2.42	0.717	
	2	8.30 ± 8.20	13.11 ± 3.85	0.489	
P.aeruginosa	0.5	54.38 ± 2.25	51.70 ± 5.69	0.337	0.799
	2	38.59 ± 0.59	38.52 ± 0.10	0.409	
	8	35.23 ± 3.08	32.54 ± 0.96	0.348	
S.aureus	0.03	49.77 ± 0.75	43.78 ± 3.71	0.184	0.602
	0.13	27.74 ± 3.13	32.25 ± 0.39	0.250	
	0.5	24.84 ± 3.38	30.67 ± 0.50	0.158	
S.pneumoniae					
without serum	0.03	48.79 ± 2.46	52.54 ± 0.90	0.100	0.570
	0.13	24.51 ± 4.23	15.43 ± 3.51	0.076	
	0.5	16.48 ± 0.69	15.15 ± 0.90	0.198	
with serum	0.03	51.92 ± 1.49	52.99 ± 0.37	0.329	0.003
	0.13	22.32 ± 5.74	27.90 ± 1.41	0.120	
	0.5	18.41 ± 3.53	26.88 ± 1.24	0.050	

[a]The *P* value for the effects of surfactant on antibiotic activity for each antibiotic concentration and [b] the *P* value for the effects of surfactant on antibiotic activity in all killing curves. Determined by analysis of variance for repeated time measurements

bacterial killing by tobramycin was less (*S. aureus* and *S. pneumoniae*), or disappeared (*K. pneumoniae* and *P. aeruginosa*), when tobramycin concentrations were increased to 4 times the MIC for the tested pathogen (Table 4). Typical examples of decreased tobramycin activity in the presence of surfactant against *K. pneumoniae* and *S. pneumoniae* are shown in Figs. 1 and 2, respectively. Addition of rabbit serum to THB did not alter overall killing rates of *S. pneumoniae* foramoxicillin ($p = 0.647$) or ceftazidime ($p = 0.201$) compared to bacterial killing in THB alone. Overall killing by tobramycin in medium with rabbit serum was decreased compared to killing in THB alone ($p < 0.001$).

The pH of the incubation media was not affected by addition of surfactant and/or rabbit serum to the broth. A change in pH was < 0.05 in all experiments. Tobramycin binding was respectively: (i) 13% in THB alone (controls), (ii) 30% in THB + 25 mg/ml surfactant, (iii) 19% in THB + 12.5 mg/ml surfactant and (iv) 21% in THB + rabbit serum. Differences between duplo's was < 2% in all experiments.

Table 4. Inluence of surfactant on tobramycin activity

Organism	Tobramycin conc (μg/ml)	Area under killing curve mean ± SD (($\log_{10}$ CFU/ml)xh)		*P* value	
		without surfactant	with surfactant	Be-tween[a]	Over-all[b]
K.pneumoniae	0.13	64.48 ± 0.80	64.94 ± 0.01	0.222	0.001
	0.5	13.66 ± 0.27	61.92 ± 0.31	0.000	
	2	3.54 ± 0.92	4.04 ± 1.63	0.742	
P.aeruginosa	0.5	25.71 ± 4.53	41.22 ± 2.45	0.038	0.567
	2	5.34 ± 3.46	2.89 ± 0.00	0.423	
	8	2.89 ± 0.00	2.89 ± 0.00	1.000	
S.aureus	0.03	51.67 ± 4.18	53.13 ± 0.99	0.727	0.016
	0.13	12.56 ± 10.64	39.41 ± 15.17	0.169	
	0.5	6.15 ± 1.22	20.81 ± 1.57	0.011	
S.pneumoniae					
without serum	4	48.67 ± 5.03	55.74 ± 0.54	0.206	0.001
	16	15.23 ± 2.67	54.26 ± 0.18	0.002	
	64	4.35 ± 0.86	32.73 ± 1.19	0.001	
with serum	4	55.96 ± 2.17	54.86 ± 0.38	0.610	0.005
	16	54.63 ± 1.83	54.49 ± 0.14	0.651	
	64	21.90 ± 6.46	39.24 ± 0.27	0.001	

[a]The *P* value for the effects of surfactant on antibiotic activity for each antibiotic concentration and [b] the *P* value for the effects of surfactant on antibiotic activity in all killing curves. Determined by analysis of variance for repeated time measurements

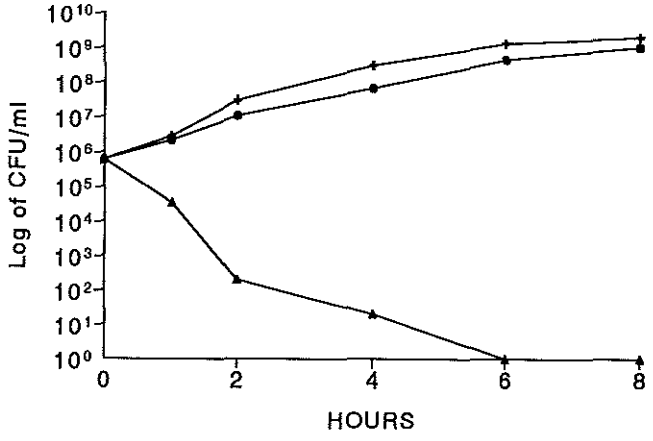

Figure1. Time-kill curves for *K.pneumoniae* with tobramycin (0.5 μg/ml = 1x MIC) in MHB (▲) and MHB with 25 mg of surfactant per ml (•). +, a growth control in MHB.

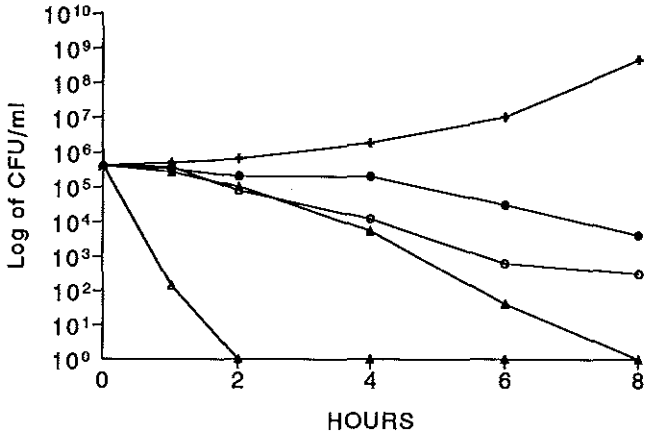

Figure 2. Time-kill curves for *S.pneumoniae* with tobramycin (64 μg/ml = 4x MIC) in THB (△), THB and rabbit serum (▲), THB and 25 mg of surfactant per ml (○), and THB with rabbit serum and surfactant (•). +, a growth control in THB.

Discussion

In this study, the influence of a natural pulmonary surfactant on antibiotic activity was investigated to assess the possible use of surfactant as a vehicle for antibiotic delivery to the lung. The results show that both amoxicillin and ceftazidime can be combined with pulmonary surfactant without loss of antibiotic activity. Tobramycin activity was reduced in the presence of surfactant, but this could be overcome by increasing the tobramycin concentration. It is furthermore concluded that pulmonary surfactant can alter antibiotic activity, therefore, antibiotic activity should be tested before using surfactant-antibiotic mixtures in severe pneumonia.

Several studies have reported inactivation of aminoglycosides, as found with tobramycin in this study. The pH strikingly alters the bioactivity of aminoglycosides. MIC increases, depending on the organism and the particular aminoglycoside involved, when pH of the culture medium falls below 7.0 [25,26]. Activity of aminoglycosides is also influenced by the amount of free unbound cations in the culture medium [27,28]. Other studies have reported inactivation of aminoglycosides in sputum which could be explained, in part, by binding of the antibiotic to subcellular components of sputum [29-31]. Aminoglycosides have been reported to interact with ribosomes, DNA and glycoproteins in bronchial secretions [32-34].

In the present study, the partial inactivation of tobramycin cannot be explained by alterations in pH or changes in free cation concentrations after addition of surfactant. The pH did not change after addition of surfactant to the medium. The free cation concentration in the surfactant was not measured. However, changes in free cation concentrations should in particular affect *P. aeruginosa* killing by tobramycin [35,36], whereas in the present study *P. aeruginosa* killing by tobramycin was least influenced by surfactant. The reduced killing by tobramycin is, therefore, unlikely to be due to a difference in free cation concentrations between the media.

To assess a possible binding of tobramycin with surfactant, we measured the amount of free drug by centrifugation-filtration. The amount of tobramycin recovered in the filtration fluid was most decreased in the presence of 25 mg/ml surfactant. Binding of aminoglycosides to negatively-charged phospholipids has previously been described as a mechanism for the nephrotoxic action of these antibiotics [37,38]. Pulmonary surfactant comprises 90% phospholipids, mostly phosphatidylcholine and phosphatidylglycerol [17]. The decreased activity

of tobramycin is, therefore, possibly induced by binding of tobramycin to phospholipids in the surfactant.

We have no explanation for the observed decrease in ceftazidime activity against *S. pneumoniae* in THB supplemented with rabbit serum. This decrease was only found at 4 times the MIC in medium with rabbit serum. In contrast to these findings, with *S. pneumoniae* in THB there was a tendency for increased killing in the presence of surfactant; this was, however, not statistically significant.

A few studies have investigated the direct influence of pulmonary surfactant on bacterial growth [39-42]. Coonrod and colleagues showed that a surfactant preparation from bonchoalveolar lavage fluid (BAL) of rats and, more specifically, the free fatty acids (FFA) present in the surfactant, caused lysis of *S. pneumoniae* [39]. Bactericidal activity of this surfactant preparation was also observed with several other gram-positive bacteria, including *Streptococcus viridans, Strepotcoccus pyogenes* and *Streptococcus bovis* [39]. Further studies from this group showed that there was a species variation in the amount of FFA recovered in the surfactant preparation of BAL, which could explain the difference in *S. pneumoniae* killing between this rat surfactant and surfactant prepared from BAL from guinea pigs [43].

Enhancement of growth has been found for *Escherichia coli* and *S. viridans* with a crude surfactant preparation from dogs [40] and for *S. aureus* with a surfactant from rabbits [42]. Studies with human alveolar lining material (ALM) showed that incubation of *S. pneumoniae* or *Haemophilus influenzae* with the ALM had no effect on the viability of these bacteria; in fact, ALM supported replication of *H. influenzae* [41]. In the present study, no effect of surfactant on bacterial growth of *K. pneumoniae, P. aeruginosa, S. aureus* was found.

Our results show no *S. pneumoniae* killing by surfactant. *S. pneumoniae* growth was not affected by surfactant, except for a decrease in growth rate in THB with both surfactant and rabbit serum compared to growth rates in THB with rabbit serum. The surfactant used in our study is a highly purified extraction from bovine lungs, which could explain the observed conflicting results on *S. pneumoniae* growth. It is comparable to the commercially available surfactants which are used for the treatment of infant respiratory distress syndrome. The concentration of 25 mg/ml is based on the current surfactant concentrations used for surfactant replacement therapy [44].

Preliminary results (this group, unpublished) on *S. pneumoniae* growth in the presence of surfactant showed a stimulation of growth compared to *S. pneumoniae* growth in unsupplemented THB. In the present study this could not be supported by the data. For optimal *S. pneumoniae* growth, THB is generally supplemented with serum. These preliminary results provided the rationale to study *S. pneumoniae* killing in both THB, as well as in THB supplemented with rabbit serum. Moreover, serum is known to inhibit the functional integrity of pulmonary surfactant [45] and this too could interfere in a surfactant-antibiotic interaction.

In two animal models of respiratory failure due to *Pneumocystus carinii* or influenza A virus, endotracheal instillation of exogenous surfactant restored lung function and gas exchange within 30 min [46,47]. These results, together with the histological findings in these studies show that surfactant can reopen atelectatic areas. With a surfactant-antibiotic mixture it is expected that a large antibiotic dosage can be delivered to the lung in non-aereated areas, which are often infected areas. The use of surfactant mixed with antibotics seems, therefore, a promising therapeutic approach in patients with severe pneumonia.

Whether the partial inactivation of tobramycin by surfactant can be compensated for by higher antibiotic dosages without an increased risk for toxic systemic levels needs further investigation in vivo. Future investigations should also focus on whether the function of surfactant remains unchanged after mixture with antibiotics, since this too could limit the use of a surfactant-antibiotic mixture.

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Chapter 4

Exogenous pulmonary surfactant as a drug delivering agent; influence of antibiotics on surfactant activity

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Summary

It has been proposed to use exogenous pulmonary surfactant as a drug delivery system for antibiotics to the alveolar compartment of the lung. Little, however, is known about interactions between pulmonary surfactant and antimicrobial agents. This study investigated the activity of a bovine pulmonary surfactant after mixture with amphotericin B, amoxicillin, ceftazidime, pentamidine or tobramycin. Surfactant (1 mg/ml *in vitro* and 40 mg/ml *in vivo*) was mixed with 0.375 mg/ml amphotericin B, 50 mg/ml amoxicillin, 37.5 mg/ml ceftazidime, 1 mg/ml pentamidine and 2.5 mg/ml tobramycin. Minimal surface tension of 50 μl of the mixtures was measured *in vitro* using the Wilhelmy balance. *In vivo* surfactant activity was evaluated by its capacity to restore gas exchange in an established rat model for surfactant deficiency. Surfactant deficiency was induced in ventilated rats by repeated lavage of the lung with warm saline untill PaO_2 dropped below 80 cm H_2O with 100% inspired oxygen at standard ventilation settings. Subsequently an antibiotic-surfactant mixture, saline, air, or surfactant alone was instilled intratracheally (4 ml/kg volume, n=6 per treatment) and blood gas values were measured 5', 30', 60' 90' and 120 minutes after instillation. The results showed that minimal surface tensions of the mixtures were comparable to that of surfactant alone. *In vivo* PaO_2 levels in the animals receiving ceftazidime-surfactant or pentamidine-surfactant were unchanged when compared to the surfactant group. PaO_2 levels in animals receiving amphotericin B-surfactant, amoxicillin-surfactant or tobramycin-surfactant were significantly decreased compared to the surfactant group. For tobramycin it was further found that PaO_2 levels were not affected when an 0.2 M $NaHCO_3$ (pH =8.3) buffer was used for suspending surfactant instead of saline. It is concluded that some antibiotics affect the *in vivo* activity of a bovine pulmonary surfactant. Therefore, before using surfactant-antibiotic mixtures in clinical trials, interactions between the two agents should be carefully evaluated.

Introduction

Efficient antimicrobial therapy is considered to be dependent on appropriate antibiotic concentrations at the site of infection [1]. For pneumonia this is within the alveolar space together with the epithelial lining fluid and the lung interstitium [2]. When administered systemically, it is difficult to ensure efficient concentrations of some antibiotics at the infection site without inducing severe adverse reactions, e.g. oto- and nephrotoxicity by aminoglycosides [3]. Methods for more selective delivery of antimicrobial agents to the lung and infected lung areas in particular seem, therefore, a potential way to increase therapeutic efficacy.

Application of antibiotics to the airways, either inhaled as an aerosol or injected directly into the trachea, has been studied almost since their discovery. Aerosols are however not deposited in non-ventilated lung areas [4]. Moreover, in patients with decreased pulmonary function, pulmonary deposition is particularly high in the central airways and decreases towards the periphery [4, 5]. With direct endotracheally instillation distribution is largely limited to the central airways [6]. Thus, the therapeutic efficacy of these administration modes seems limited, especially since the location of infection is more peripheral.

Tracheal instillation of exogenous pulmonary surfactant, a mixture of phospholipids and specific surfactant proteins, is an established therapy in neonates suffering from respiratory distress syndrome [7]. The excellent spreading properties of pulmonary surfactant within the lung suggest that exogenous surfactant could be exploited as a carrier for drug delivery to the alveolar compartment of the lung [8, 9, 10]. It is shown by Kharasch et al. [8] that tracheal instillation of a pentamidine-surfactant mixture marked with a radioactive colloid has a more uniform and wider distribution pattern in the lung then instillation of a pentamidine-saline solution.

Furthermore, it is shown that instillation of pulmonary surfactant in infected lungs can improve gas exchange, restore lung function and re-expand atelectatic areas [11-13]. It is expected that, mixed with the surfactant, efficient antibiotic dosages can be delivered even to the non-ventilated areas.

Little is known, however, about possible interactions between pulmonary surfactant and antimicrobial agents when mixed. A previous study [14] showed that the *in vitro* bactericidal activity of amoxicillin and ceftazidime was unaffected in the presence of pulmonary surfactant.

However, activity of tobramycin was significantly reduced in the presence of pulmonary surfactant. These results demonstrated the relevance of studying antibiotic activity and surfactant activity when they are mixed.

The present study investigated the surfactant activity after mixture with amphotericin B, amoxicillin, ceftazidime, pentamidine or tobramycin. These antibiotics, from different classes, are chosen upon their clinical relevance in the treatment of lower respiratory tract infections in the intensive care unit. Minimal surface tension of antibiotic-surfactant mixtures was measured *in vitro* using the Wilhelmy balance and compared to minimal surface tension of surfactant alone. Surfactant activity was evaluated *in vivo* by its capacity to restore gas exchange in a standardized model for acute respiratory insufficiency in adult rats.

Materials and Methods

Surfactant and antibiotics

A freeze-dried natural surfactant was used, isolated from bovine lungs as previously described [15]. It consists of approximately 90-95% phospholipids, 1% hydrophobic proteins (surfactant-proteins B and C) and 1% free fatty acids, the remainder being other lipids such as cholesterol and glyceride; there was no surfactant-protein A in this surfactant preparation.

The commercial formulations of the antibiotics for intravenous administration were used in all experiments: amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands), amoxicillin (SmithKline Beecham, Rijswijk, The Netherlands), ceftazidime (Glaxo, Nieuwegein, The Netherlands), pentamidine (Rhône-Poulenc Rorer, Amstelveen, The Netherlands), tobramycin (Eli Lilly, Nieuwegein, The Netherlands). The dosages used in these experiments were based upon maximal daily dosages for adults: amphotericin B 1.5 mg/kg, amoxicillin 200 mg/kg, ceftazidime 150 mg/kg, pentamidine 4 mg/kg and tobramycin 10 mg/kg.

Surfactant and antibiotic-surfactant suspensions were freshly made for each experiment. The surfactant powder was suspended in 0.9% NaCl solution, except in group 8 (see below) where 0.2M $NaHCO_3$ was used as solvent. Antibiotics were dissolved in 0.9% NaCl or in H_2O (amphotericin B). The antibiotic solutions were added to the surfactant suspension and

handshaken.

Minimal surface tension measurements

Minimal surface tension of each antibiotic with and without additional surfactant was measured and compared with the minimal surface tension of surfactant alone. Samples of surfactant, antibiotic and antibiotic-surfactant mixtures were freshly made in duplicate. A low surfactant concentration (1 mg total lipids/ml saline) was used to facilitate the detection of changes in the minimal surface tension when antibiotics were added to this surfactant solution. The antibiotic concentrations of the samples were similar to the antibiotic concentrations used in the *in vivo* experiments (Table 1).

Minimal surface tensions of the samples were measured using a modified Wilhelmy balance (E. Biegler GmbH, Mauerbach, Austria) which keeps the temperature constant at 37°C . The trough was filled with warm saline (37°C) and calibrated. After calibration, 50 μl of a sample (containing 50 μg total lipids) was placed upon the surface, using an eppendorf pipet. Two minutes were waited for spreading of the sample. Subsequently, the measurement was continued. Surface area was compressed and expanded with a cycling time of 3 min/ cycle and maximum and minimum surface areas of 64 and 12.8 cm^2, respectively, (100% and 20%). Minimal surface tension was measured after 3 cycles at 20% surface area, and is expressed as milli Newton/meter (mN/m) [16].

Animal studies

The study protocol was approved by the institutional Animal Care Committee. Male Sprague-Dawley rats (SPF, Iffa Credo, Belgium), mean bodyweight 275 ± 20 gram were used in all experiments.

Respiratory failure was induced by lung lavage as described previously [17]. Briefly; under inhalation anesthesia, O_2, N_2O and Isoflurane 2% (65:33:2), the trachea and the carotid artery were cannulated. Rats were connected to the ventilator. Anesthesia and muscle relaxation was maintained during the experiment with pentobarbital sodium (60 mg/kg intraperitoneally) and pancuronium bromide (0.5 mg/kg, intramuscularly) every hour. Lungs were lavaged 5-7 times with 30 ml/kg bodyweight of warm saline to achieve a $PaO_2 < 80$ mmHg at the following

ventilator settings using a Servo Ventilator 300 (Siemens-Elema, Solna, Sweden): pressure-controlled ventilation, frequency = 30 breaths/min, peak airway pressure = 26 cm H_2O, positive end expiratory pressure (PEEP) = 6 cm H_2O, I : E ratio = 1 : 2 and FiO_2=1. These ventilation settings were maintained throughout the study period. There were 9 different treated groups. Volume instilled intratracheally was 4 ml/kg bodyweight (b.wt.).

1 - n=17	surfactant, 160 mg/kg b.wt. (40 mg/ml)
2 - n=6	air
3 - n=6	saline
4 - n=6	surfactant + amphotericin B, 1.5 mg/kg b.wt. (0.375 mg/ml)
5 - n=6	surfactant + amoxicillin, 200 mg/kg b.wt. (50 mg/ml)
6 - n=6	surfactant + ceftazidime, 150 mg/kg b.wt. (37.5 mg/ml)
7 - n=6	surfactant + pentamidine, 4 mg/kg b.wt. (1 mg/ml)
8 - n=6	surfactant + tobramycin, 10 mg/kg b.wt. in saline (2.5 mg/ml)
9 - n=6	surfactant + tobramycin, 10 mg/kg b.wt. in $NaHCO_3$ (2.5 mg/ml)

Treatment with surfactant, air, saline or an antibiotic-surfactant mixture was started within 6-10 minutes after the last lavage. For this, rats were disconnected from the ventilator and the 4 ml/kg bolus of surfactant, air, saline or antibiotic-surfactant mixture was instilled intratracheally followed by insufflation of 24 ml/kg of air. After instillation, animals were immediately reconnected to the ventilator. Blood samples were taken from the carotid artery of each rat shortly before and 5 minutes after the lung lavage procedure (t=0') and then at t=5', 30', 60', 90' and 120 minutes post treatment. Blood gas values were measured with the ABL 505 Acid-Base Laboratory (Radiometer, Copenhagen, Denmark).

Each experiment consisted of six rats placed at one ventilatory unit. In each experiment one or two positive surfactant controls were included. The surfactant group consisted, therefore, of 17 animals. All other treatment groups consisted of 6 rats per group. At the end of the observation period animals were killed by an intraperitoneal overdose of pentobarbital.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). In the *in vivo* study statistical

significant differences were evaluated with an analysis of variance (ANOVA) for repeated measurements using the GLM procedure of the SAS statistical package (SAS, 1990). Tests performed were: (1) within group, the effect of time on changes in PaO_2 and $PaCO_2$ and (2) the difference in PaO_2 and $PaCO_2$ values between groups, using the surfactant-treated group as a positive control and the saline and air treated groups as negative controls. Tests were performed from t=0' to t=120' to evaluate overall differences between and within groups. To evaluate the acute effect of surfactant treatment (within 5 min), tests were performed from t=0' to t=5' and to evaluate the stability of PaO_2 increases tests were performed from t=30' to t=120'. Statistical significance was accepted at a *P*-value ≤ 0.05.

Results

In vitro

Table 1 shows the minimal surface tension (mean ± SD, n=2) of the samples. Addition of 1 mg/ml surfactant to the antibiotic solution decreased minimal surface tension to levels comparable to that of surfactant alone. When surfactant alone was applied to the surface in higher concentrations ($\geq$ 3 mg/ml) the minimal surface tension would decrease further to values below 5 mN/m.

Table 1. Mean minimal surface tension of the antibiotics with and without surfactant

	Concentration (mg/ml)	Minimal surface tension (mN/m)	
		without surfactant	with surfactant
Saline	-	72.8 ± 0.3	20.9 ± 0.4
Amphotericin B	0.375	53.4 ± 0.4	21.4 ± 0.6
Amoxicillin	50	65.3 ± 3.1	20.6 ± 0.8
Ceftazidime	37.5	61.2 ± 3.9	22.1 ± 0
Pentamidine	1	59.2 ± 2.4	18.1 ± 0.8
Tobramycin	2.5	68.1 ± 0.8	21.4 ± 0.4

Values are means ± SD

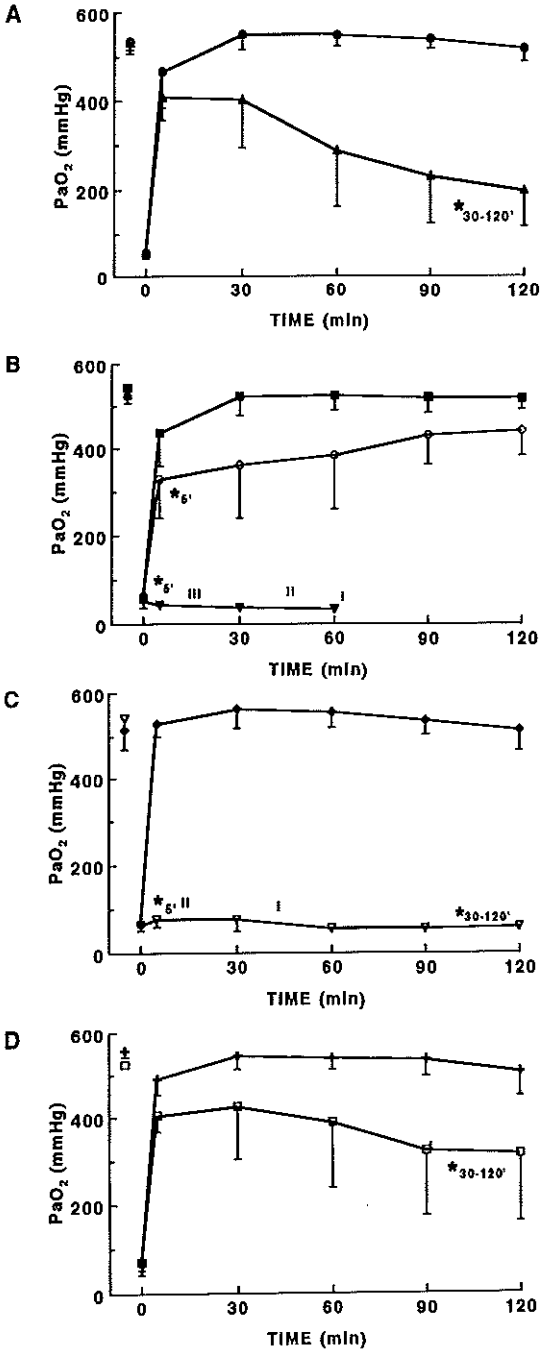

Figure 1 A-D. PaO_2 values (mean ± SD) over time for the nine treated groups. A: Surfactant marked (•) and amphotericin B-surfactant marked (▲); B: saline marked (▼), amoxicillin-surfactant marked (○) and ceftazidime-surfactant marked (■). C: pentamidine surfactant marked (♦) and air marked (▽). D: tobramycin-surfactant in saline marked (□) and tobramycin-surfactant in $NaHCO_3$ marked (+).

Animals who died during the study period are marked with an I.

*5 min $P \leq 0.05$, for the differences between groups over t=0' - t=5' when compared to the surfactant treated group (ANOVA repeated time measurements).

*30-120 min $P \leq 0.05$, for the differences between the groups over t=30' - t=120' when compared to the surfactant treated group (ANOVA, repeated time measurements).

In vivo

When comparing the antibiotic-surfactant treated groups with the surfactant treated group significant differences in blood gas values over time were found when surfactant was mixed with amphotericin B, amoxicillin or tobramycin. PaO_2 and $PaCO_2$ levels in the ceftazidime-surfactant treated group and the pentamidine-surfactant treated group were not significantly different at any time point from those in the surfactant treated group (Figure 1A-D and Table 2).

In the amphotericin B-surfactant treated group, PaO_2 levels initially increased comparable to the PaO_2 increase in the surfactant treated group. However, in time PaO_2 levels decreased significantly compared to the surfactant treated group (Fig 1A). $PaCO_2$ levels were increased compared to the surfactant treated group (Table 2).

In the amoxicillin-surfactant treated group the initial rise in PaO_2 (at 5 min) as well as the PaO_2 levels in the subsequent 120 minutes were significantly decreased compared to PaO_2 levels in the surfactant treated group (Fig. 1B). In time PaO_2 levels tended to rise in the amoxicillin-surfactant treated group; however, this increase was not statistically significant ($P = 0.055$, within subjects t5' - t120'). $PaCO_2$ levels were significantly increased from 30 - 120 minutes compared to the surfactant treated group.

After instillation of tobramycin-surfactant suspended in saline, PaO_2 levels varied between the animals and were on average lower than PaO_2 after surfactant instillation (Fig. 1D). PaO_2 levels tended to decrease in time (P =0.054, within subjects effect t5'-t120'). $PaCO_2$ levels were significantly higher in the tobramycin-surfactant group when compared to the surfactant treated group (Table 2).

Preparing the solutions for the *in vivo* tests showed that addition of tobramycin to surfactant suspended in saline resulted in a precipitation of the suspension. Since aminoglycosides are known to bind to negatively charged phospholipids [18] the effect of the pH of the solution on visible precipitation was studied. It was found that at a pH of 8.3 using 0.2 M $NaHCO_3$ as solvent, no visible precipitation occurred when tobramycin was added to the surfactant suspension. PaO_2 levels in the group receiving tobramycin-surfactant suspended in 0.2M $NaHCO_3$ were uniform and not significantly different from PaO_2 levels in the surfactant treated group (Fig. 1D). In all groups receiving either surfactant or an antibiotic-surfactant mixture PaO_2 levels were significantly higher than PaO_2 levels in the air or saline treated group (Fig. 1A-D).

Table 2. $PaCO_2$, mean ± SD (mmHg) of the nine groups

								P values (compared to surfactant group)		
	pre-lavage	t=0	t=5'	t=30'	t=60'	t=90'	t=120'	t0'-t120'	t0'-t5'	t30'-t120'
Surfactant	40 ±6.1	73 ±11	46 ±9.2	42 ±8.2	39 ±8	39 ±6.7	37 ±7			
Surfactant-amphotericin B	34 ±2.2	79 ±7	54 ±6.1	45 ±10.9	44 ±8.5	49 ±8.8	58 ±14.8	0.056	0.198	0.044
Surfactant - amoxicillin	36 ±2.5	71 ±7.1	52 ±5.8	51 ±9.8	49 ±10.2	46 ±7.7	47 ±9	0.082	0.645	0.026
Surfactant - ceftazidime	34 ±5.1	74 ±7.1	47 ±8.8	42 ±9.3	41 ±8.8	39 ±7.4	36 ±9.2	0.940	0.856	0.996
Surfactant - pentamidine	38 ±6.5	72 ±5.7	45 ±9.4	42 ±9.1	41 ±13.4	40 ±10.5	41 ±9.7	0.840	0.784	0.653
Surfactant tobramycin in saline	36 ±3.4	78 ±8.9	55 ±6.9	49 ±7.3	49 ±9.7	50 ±9.3	51 ±9.2	0.040	0.112	0.020
Surfactant - tobramycin in 0.2M $NaHCO_3$	35 ±1.2	70 ±9.4	50 ±6.1	45 ±6.9	43 ±7.2	42 ±5.5	41 ±7.1	0.363	0.912	0.191
Air	36 ±10.4	67 ±17.3	53 ±16.7	48** ±17.3	47*** ±17.1	47*** ±19.7	46*** ±18.3	0.001	0.255	0.001
Saline	37 ±3.4	73 ±15.5	89 ±18.7	132*** ±22.9	155***** ..	..	..	..	0.001	..

Values shown are means ± SD. P values for between group differences in the $PaCO_2$ values of the surfactant treated group vs the surfactant antibiotic treated group, determined with ANOVA for repeated time measurements. * Indicates the number of animals that died during the study period

Discussion

This study investigated the influence of five antimicrobial agents on pulmonary surfactant function to assess the possible use of surfactant as a pulmonal drug delivery system. It was found that surfactant function was unaffected when mixed with ceftazidime and pentamidine. Surfactant function was reduced when combined with amphotericin B and amoxicillin. With tobramycin-surfactant mixtures, surfactant activity was reduced when saline was used as solvent. Surfactant function was, however, unaffected in the presence of tobramycin when 0.2M $NaHCO_3$ was used as solvent.

It has been previously discussed that evaluation of surfactant function *in vitro* is valuable.The *in vitro* results will, however, not accurately predict surfactant function *in vivo* [19,20]. In the present study *in vitro* examination was, therefore, limited to the question whether minimal surface tension of the antibiotic-surfactant mixtures was comparable to the minimal surface tension of surfactant alone. The minimal surface tensions did not vary strongly between surfactant and antibiotic-surfactant mixtures which encouraged us to evaluate the mixtures *in vivo*.

In vivo surfactant function was evaluated in a standardized model of surfactant deficiency in adult animals induced by whole lung lavage with warm saline. This model has been used extensively for testing various aspects of exogenous surfactant therapy [15,21-25]. One of the advantages of this model is that the level of induced lung damage can be excellently standardized [26].

Two properties of the pulmonary surfactant can be evaluated in this model. First, its capacity to open up the atelectatic lung which is characterized by the immediate increase in PaO_2. Second its capacity to keep the lung open over a longer period without changing the ventilatory settings, which is characterized by the unchanged PaO_2 over time [15]. When exogenous surfactant is merely used for delivery of agents in the peripheral regions, one can assume that the first quality (to open up the lung) is most important. However, when surfactant-antibiotic mixtures are simultaneously used for treatment of respiratory failure, the second quality (to keep the lung open) is essential for proper surfactant therapy. Therefore, we evaluated the results from the first five minutes after instillation and the results from 30 to 120 minutes separately.

Instillation of amphotericin B-surfactant mixtures improved gas exchange within five

minutes. In time, however, the gas exchange deteriorated, which indicates an inhibition of the surfactant function. To our knowledge, interactions between surfactant function and amphotericin B have not been reported before and the results should be interpreted with care. Studies in patients receiving amphotericin B delivered as an aerosol or instilled endotracheally have reported minimal or no side effects [26]. A study on aerosolized amphotericin B in rats reported that it was well tolerated and produced no histopathologic changes in the lungs [27]. Although rare, lung injury has been reported when amphotericin B was instilled intravenously [28,29].

In the present study a high dose of amphotericin B was instilled directly into a severely damaged lung. Plausible mechanisms involved in the observed inhibition of surfactant by amphotericin B could be direct interaction of the agent with surfactant or an interaction of the agent with the alveolar capillary membrane resulting in an influx of plasma proteins. Plasma proteins are well-known inhibitors of pulmonary surfactant function [30].

The initial increase in gas exchange after instillation of the amoxicillin-surfactant mixture was decreased compared to the surfactant treated group but gradually improved in time. As with amphotericin B, the cause for the changed surfactant function is unknown. One explanation could be that amoxicillin binds to the specific surfactant proteins B and C present in this surfactant preparation. Both Sp-B and Sp-C are an important factor in the physical surfactant function (for review see Johansson et al, [31]).

A previous study from our group showed that tobramycin activity was decreased in the presence of surfactant [14]. Binding of aminoglycosides to negatively-charged phospholipids has been described as a mechanism for the nephrotoxic action of these antibiotics [18]. Therefore, it was speculated that the decreased activity of tobramycin found in the presence of surfactant was induced by tobramycin binding to phospholipids in the surfactant.

The present study showed that when surfactant and tobramycin are dissolved in saline (pH=6.3) a precipitation occurred. Instillation of this mixture in lavaged lungs resulted in a decreased surfactant function. Since the charge of the phospholipids and/or the tobramycin seemed to be relevant, the effect of the pH of the solvent was investigated. When tobramycin and surfactant were suspended in 0.2M $NaHCO_3$ (pH = 8.3) the suspension was homogeneous at sight. Restoration of gas exchange after instillation of this mixture in lavaged rats was uniform and not different from that in rats treated with surfactant only.

The purpose of this study was to investigate possible interactions between antimicrobial

agents and an exogenous pulmonary surfactant. This study together with a previous study from our group [14] demonstrated that interactions between antimicrobial agents and exogenous surfactant exist and may influence the activity of both substances.

Due to the differences in chemical composition between all currently available surfactant preparations [32] extrapolation of the present results to other surfactant preparations is not recommended. It has been shown in several studies that the compositional differences have a large impact on the *in vitro* and *in vivo* physical behaviour of the surfactant preparations [21, 32-34]. Accordingly it can be expected that possible interactions between exogenous surfactant and other agents differ between the various surfactant preparations. Therefore, before using surfactant-antibiotic mixtures in clinical trials alterations in activity of both substances should be considered and carefully examined.

This study further showed that simple changes, such as the use of a different solution for suspending the surfactant, can overcome changes in surfactant activity due to interactions between surfactant and antibiotics. Therefore, although the results with amphotericin B are poor in this study this should not definitely exclude the use of amphotericin B-surfactant mixtures. For example, as with tobramycin, the use of other solvents could be investigated. The use of surfactant as a delivering agent for antibiotics is expected to have great potential in selected patient groups. However, questions remain open on both distribution patterns in infected lungs and *in vivo* efficacy. Future studies should, therefore, focus on these issues.

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Chapter 5

Pulmonary surfactant as vehicle for intratracheally instilled tobramycin in mice infected with *Klebsiella pneumoniae*

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Summary

It has been proposed to use pulmonary surfactant as a vehicle for antibiotic delivery to the alveolar compartment of the lung. This study investigated survival rates of mice with a respiratory *Klebsiella pneumoniae* infection treated intratracheally with tobramycin using a natural exogenous surfactant preparation as vehicle. At day 1 after infection, animals were injected intratracheally with 20 μl of the following solutions: 1) a mixture of surfactant (500 μg) and tobramycin (250 μg); 2) tobramycin (250 μg) alone; 3) surfactant (500 μg) alone; and 4) $NaHCO_3$ buffer (control, sham-treatment). A fifth group received no treatment (control). Deaths were registered every 12 hours for 8 consecutive days. The results show an increased survival in the group receiving the surfactant-tobramycin mixture compared to the group receiving tobramycin alone ($p<0.05$), the group receiving surfactant alone ($p<0.01$) and the control groups ($p<0.01$). It is concluded that intratracheal instillation of surfactant-tobramycin is superior to tobramycin alone in protecting animals from death due to a respiratory *Klebsiella pneumoniae* infection.

Introduction

The efficacy of locally administered antibiotics for prevention or treatment of lower respiratory tract infection has been studied extensively [1-4]. For some antibiotics, e.g. aminoglycosides, it is expected that delivery directly to the airways increases the local effectiveness and reduces the risk of toxicity. However, despite the high antibiotic dose delivered to the lung, the question of efficacy remains controversial.

An explanation for a disappointing efficacy is likely to include failure of the antibiotic to reach the infected areas of the lung. When delivered as an aerosol, only a small amount of the nebulized antibiotic dose, around 10%, is actually deposited in the lung [2, 3]. Moreover, with increased airway obstruction and lung damage the amount of aerosol deposited in peripheral regions of the lung decreases [2, 3]. Lung distribution of intratracheally instilled antibiotic solutions is poorly studied. However, it is known that distribution of intratracheally instilled saline is largely limited to the central regions of the lung [5, 6].

Due to the small diameter of peripheral airways, fluids with a high surface tension, such as saline and water, require high pressures for passage through these airways [7]. Pulmonary surfactant, a mixture of phospholipids and specific surfactant proteins, has the capacity to lower surface tension. Kharasch and colleagues [6] investigated lung distribution patterns of intratracheally instilled surfactant or saline, both mixed with pentamidine and a radioactive colloid, and demonstrated a wider distribution pattern of the colloid when mixed with surfactant compared to saline. Since infection is most often localized in the peripheral lung regions, it is expected that intratracheally instilled antibiotics are more effective when the distribution within the lung is optimized by using pulmonary surfactant as a vehicle.

Moreover, it has been shown that intratracheal instillation of exogenous surfactant itself is beneficial in pneumonia. Pneumonia is an important cause of acute respiratory failure and is associated with a decreased surfactant function [8, 9]. Both experimental and clinical reports have shown that instillation of exogenous surfactant in infected lungs restores gas exchange and lung function by re-expanding atelectatic lung areas [10-15]. It is expected, therefore, that use of a surfactant-antibiotic mixture has great potential in treatment of patients with severe pneumonia.

However, to date, no data on in vivo efficacy of surfactant-antibiotic mixtures are available. Therefore, in the present study, the efficacy of intratracheally instilled tobramycin was studied with and without the use of a natural exogenous surfactant as vehicle. Tobramycin is an antimicrobial agent which can cause severe oto- and nephrotoxicity and is frequently studied for local antimicrobial therapy against severe gram-negative infections [1, 4]. Efficacy of intratracheally instilled tobramycin with and without the use of surfactant, was determined by investigating survival curves of mice with a severe pulmonary infection with *Klebsiella pneumoniae.*

Materials and Methods

Male NMRI mice (n=90; SPF, Iffa Credo, Brussels, Belgium) weighing 18-22 g, age 6-8 weeks at arrival, were kept under conventional conditions; food and water were given ad libitum. Mean weight (± sd) at day 1 of the experiment was 24 ± 1.8 g. Animals were randomly divided in five groups: 3 groups of n=20 (treatment groups) and 2 groups of n=15 (control groups). During the study 7 animals died or were excluded for various reasons, none related to the study protocol.

A $1x10^7$ colony forming units (CFU)/ml *Klebsiella pneumoniae* (ATCC 43816) inoculum was prepared from an overnight culture in Mueller Hinton Broth (MHB; Difco Laboratories, Detroit, Michigan, USA) as follows: 100 μl of the overnight culture was added to 10 ml MHB and incubated at 37°C for 1.5 hours, then the culture was washed twice with saline. The inoculum was stored on ice until usage. To verify the number of viable bacteria in the inoculum, 100 μl of tenfold dilution steps in saline were plated on isosensitest agar plates (Oxoid Ltd., Basingstoke, England). Agar plates were incubated overnight and CFU were counted the following day.

For infection, mice were anesthetized by placing them in a container through which a mixture of O_2 : N_2O (1:2) and 3% ethrane was led. Anesthetized mice, held in vertical position, were injected with 50 μl of the inoculum intranasally using a catheter (OD 0.62 mm) connected to a syringe (0.25 ml, Hamilton, Bonaduz, Switzerland). All animals recovered within 2 minutes

after the infection procedure.

At day one after infection, solutions for intratracheal instillation were freshly prepared. A freeze-dried natural surfactant preparation (MSE 110, provided by MSE-Pharmazeutika GmbH, Bad Homburg, Germany) was used, isolated from pig lungs as previously described [16]. This preparation consists of approximately 90-95% phospholipids, 1% hydrophobic proteins (surfactant-proteins B and C) and 1% free fatty acids, the remainder being other lipids such as cholesterol and glyceride; there was no surfactant-protein A in this surfactant preparation. Tobramycin (Obracin, Eli Lilly, Amsterdam, The Netherlands, 40 mg/ml) was diluted with 0.2 M $NaHCO_3$ to a concentration of 12.5 mg/ml. For a surfactant-tobramycin preparation, the surfactant was suspended in a 12.5 mg/ml tobramycin solution to a concentration of 25 mg total lipids/ml and hand shaken. Surfactant only, was suspended in 0.2 M $NaHCO_3$ in a concentration of 25 mg/ml.

Animals, anesthetized by inhalation (see before), were hung vertically from an intubating block. A cold lamp (KL1500, Schott, Wiesbaden, Germany) was placed at the throat to visualize the larynx and vocal cords. A blunt needle connected to a hamilton constant flow syringe (CR200, Hamilton, Reno, USA) was placed 0.5 cm in the trachea and 20 μl of one of the following preparations was injected; 1) surfactant-tobramycin, dose 500 μg surfactant and 250 μg tobramycin; 2) tobramycin, dose 250 μg; 3) surfactant, dose 500 μg; 4) $NaHCO_3$ (sham-treatment). An additional control group received no treatment. All animals recovered within 2 minutes after the procedure. Death from infection was registered every 12 hours for 8 consecutive days. Of diseased animals, spot-check samples were taken with a cotton wool stick from the lungs. Swabs on blood agar plates (Bactim, Breukelen, The Netherlands) showed abundant presence of *Klebsiella pneumoniae*.

Statistical analysis

Statistical significance between differences in survival rates in the groups was evaluated with a product limited survival estimates using the SAS statistical package (SAS Inc, Cary, N.C., USA). Significance was accepted at $P \leq 0.05$, two tailed.

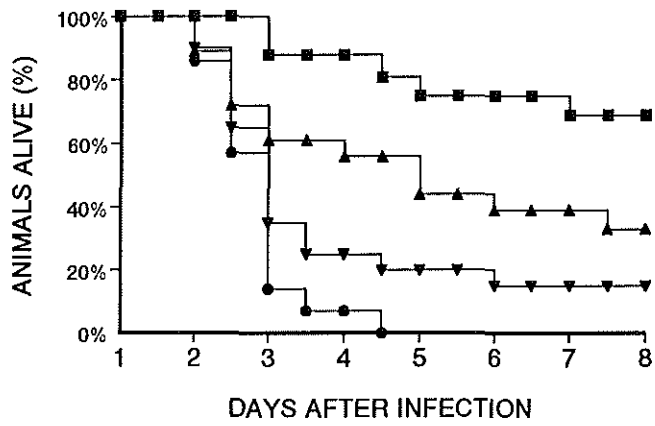

Figure 1. Survival rates of the treatment groups: surfactant-tobramycin mixture (■, n=16), tobramycin alone (▲, n=18), surfactant alone (▼, n=20), sham-treatment (•, n=14). Survival in the surfactant-tobramycin group was significantly increased compared to the tobramycin group, $P < 0.05$ (Log-rank test)

Results

Figure 1 shows the survival curves of the intratracheally treated groups. At day 8 after infection, 0% of the sham-treated animals was alive versus 15% in the surfactant-treated group, 33% in the tobramycin-treated group and 69% in the surfactant-tobramycin treated group. Survival in the group receiving surfactant-tobramycin was significantly increased compared to the group receiving tobramycin alone ($p<0.05$, Log-rank test), the group receiving surfactant alone ($p<0.01$, Log-rank test) and the group receiving sham-treatment ($p<0.01$, Log-rank test). Survival in the group receiving tobramycin alone was significantly increased compared to the group receiving sham-treatment ($p<0.01$, Log-rank test) but not compared to the group receiving surfactant alone. Differences in survival were not significant between the group receiving surfactant alone and the group receiving sham-treatment.

All animals in the non-treated group died within 6 days, mean ± sd survival time was 3.3

± 0.27 days vs 2.9 ± 0.17 days in the sham-treated group. Survival rate in the group receiving no treatment was not significantly different from that in the sham-treatment group.

Discussion

The results of the present study show that intratracheal instillation of a surfactant-tobramycin mixture is more effective in protecting mice from death of a respiratory *Klebsiella pneumoniae* infection than intratracheal instillation of tobramycin alone. It is concluded that the therapeutic efficacy of intratracheally injected tobramycin can be improved when pulmonary surfactant is used as a vehicle.

Previous studies from our group showed that mixture of pulmonary surfactant with antibiotics can influence the activity of both substances [17, 18]. In vitro studies on the bactericidal activity of tobramycin against *Klebsiella pneumoniae* showed a decreased tobramycin activity in the presence of pulmonary surfactant. It was speculated that the partial inactivation of tobramycin resulted from binding with surfactant [17]. Recent data further showed that the in vivo surfactant activity was decreased after mixture with tobramycin. However, when 0.2 M $NaHCO_3$ (pH 8.3) was used for suspending the surfactant instead of the usual saline, the surfactant function was unaffected [18]. In accord with these results, 0.2 M $NaHCO_3$ was also chosen for suspending surfactant in the present study.

Considering the results of the present study, in which surfactant-tobramycin proves to be more effective than tobramycin alone, it seems that the previously reported interactions between surfactant and tobramycin play little role in the in vivo model. Explanations for this remain speculative. A decreased tobramycin activity in the presence of pulmonary surfactant may be compensated for by an improved lung distribution of tobramycin when instilled as a surfactant-tobramycin mixture and/or a therapeutic effect of the surfactant instillation itself. In the present study, survival in the group receiving surfactant was, though higher, not significantly improved compared to survival in the sham-treated group.

In the present study, the lack of significant effects on survival of surfactant instillation alone may be due to the low surfactant doses used compared to surfactant doses used for

treatment of acute respiratory failure as a result of severe pneumonia. The surfactant dose instilled corresponds to 20 mg/kg BW in mice weighing 25 gram. The reported surfactant doses for treatment of repiratory failure in subjects with pneumonia are generally several times higher, ranging from 50-300 mg/kg or more [10-13, 15, 19, 20]. However, these subjects are mechanically ventilated and receive the surfactant dose in a larger instillation volume, 2-4 ml/kg. In the present study, the animals were breathing spontaneously. Therefore, to minimize the work of breathing directly after instillation, the instillation volume was limited to 20 μl, (0.8 ml/kg). Accordingly, the surfactant concentration was limited to 25 mg/ml as higher surfactant concentrations would become more viscous.

The tobramycin dose instilled in the present study corresponds to 10 mg/kg body weight in mice weighing 25 gram. This high dose was chosen based on the clinical daily intravenous dose used for treatment of serious infection. Little experimental data is available on intratracheally instilled aminoglycosides and most is focused on pharmacokinetics [21-23]. After intratracheal instillation of 1.5 mg/kg tobramycin, in healthy rats, high levels of tobramycin were found in the bronchoalveolar lavage fluid up to 6 hours after instillation [23]. Other studies showed that encapsulation of gentamicin [21] or tobramycin [22] in liposomes can result in a significant increase of the antibiotic residence time in the lungs. However, in the study by Omri *et al.* [22] no difference was observed in bacterial counts in *Pseudomonas aeruginosa* infected rat lungs, between the group receiving free tobramycin and the group receiving liposomal encapsulated tobramycin. It would be interesting to compare the efficacy of intratracheally instilled liposomal antibiotics and antibiotics instilled as a surfactant-antibiotic mixture, as both methods pursue a similar goal. Only one double-blinded, prospective placebo-controlled study has assessed the efficacy of intratracheally administered tobramycin in combination with systemic antibiotics[1]. In this study in patients with gram-negative pneumonia, causative pathogens were eradicated from the sputum significantly more frequently in patients receiving intratracheal tobramycin. However, no improvement in clinical outcome was observed between the two treatment groups.

In conclusion, the present study showed that the efficacy of an intratracheally instilled tobramycin-surfactant mixture against *Klebsiella pneumoniae* infection in vivo is superior to tobramycin alone. It is speculated that this results from both a more peripheral intrapulmonary

distribution pattern of the tobramycin and a therapeutic effect of the surfactant itself. These are the first indications to show exogenous surfactant to be effective in vivo as a vehicle and warrant further investigation. Future studies should focus on antibiotic and lung injury distribution patterns in infected lungs, as well as, the pharmacokinetic parameters: results from such investigations may provide explanations for the effects observed in the present study.

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Chapter 6

Lung distribution of intratracheally instilled ^{99m}Tc-tobramycin-surfactant mixture in rats with a *Klebsiella pneumoniae* lung infection

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Summary

It has been proposed to use pulmonary surfactant as vehicle for intratracheally instilled antibiotics to achieve a more peripheral and homogeneous antibiotic distribution within the lungs. The present study describes a method for labeling tobramycin with technetium (^{99m}Tc-tobramycin) and investigates lung distribution of intratracheally instilled ^{99m}Tc-tobramycin with and without the use of surfactant as vehicle in rats infected with *Klebsiella pneumoniae*. Twenty-four hours after infection animals received intratracheally 1 ml/kg of ^{99m}Tc-tobramycin-surfactant mixture (n=6), or ^{99m}Tc-tobramycin alone (n=6). Five minutes after instillation animals were killed and lungs were excised, frozen and cut in 1 mm thick slices. Computer assisted autoradiographs were analyzed to evaluate ^{99m}Tc-tobramycin distribution. Spatial distribution of radioactivity represented as the mean fractional area with radioactivity above threshold x 100%, was 47% in the ^{99m}Tc-tobramycin-surfactant group vs 36% in the ^{99m}Tc-tobramycin group ($p \leq 0.05$). It is concluded that spreading of intratracheally instilled ^{99m}Tc-tobramycin through infected lungs is increased when surfactant is used as vehicle.

Introduction

It is expected that, in pneumonia, selective delivery of antibiotics to the lower respiratory tract increases the therapeutic efficacy and decreases the risk for systemic side effects of potentially toxic agents such as aminoglycosides, pentamidine and amphotericine B [1-5]. Currently used delivery methods include inhalation of aerosolized antibiotics [1-4] or, more experimental, direct intratracheal injection of antibiotic solutions [4,5]. However, studies on intrapulmonary deposition patterns suggest that both methods are inefficient in delivering appropriate antibiotic doses to the infected, often peripheral and non-ventilated, lung regions [1,2,6]. The efficacy of local antibiotic therapy might, therefore, improve when antibiotic distribution within the lung is optimized.

Recent studies have investigated the possibility of using pulmonary surfactant preparations as vehicle for intratracheally deposited antibiotics [7-10]. The expected advantages are twofold. First, intratracheal instillation of exogenous surfactant has shown to be therapeutic in pneumonia, improving lung function and arterial oxygenation by promoting re-expansion of atelectatic areas (for review see reference 11&12). Second, due to the small diameter of peripheral airways, fluids with a high surface tension, such as water or saline, require high pressures for passage through these airways [13, 14]. Pulmonary surfactant has the capacity to lower surface tension and should, therefore, spread more easily to the alveolar compartment of the lung. The intrapulmonary spreading properties of pulmonary surfactant suggest that, together with exogenous surfactant, an appropriate antibiotic dosage can be delivered to the peripheral regions of the lung including atelectatic, non-ventilated regions.

In healthy hamster lungs it has been demonstrated that pulmonary surfactant as vehicle is indeed superior to saline in distributing an intratracheally instilled radioactive colloid to the periphery of the lung [7]. However, as lung infection is likely to influence distribution patterns it can be questioned whether surfactant is equally effective in improving lung distribution of intratracheally instilled antibiotics in infected lungs. Furthermore, in the study of Kharasch *et al.* [7] a technetium labeled sulfur colloid (TcSC) was used as marker for lung distribution; whether this was truly representative for antibiotic distribution remains inconclusive. Therefore, in the present study a method for labeling tobramycin with technetium was developed, and evaluated

by means of thin-layer chromotography and biodistribution of the labeled complex in rats. Subsequently, lung distribution of intratracheally instilled ^{99m}Tc-tobramycin was investigated, with and without use of surfactant as vehicle in *Klebsiella pneumoniae* infected rat lungs.

Materials and Methods

All animal experiments were approved by the local institutional board for animal care. Care and handling were in accordance with the European Community guidelines for animal experimentation (86/609/EEG). Female Wag/Rij rats (Charles River, Germany), body weight (b.wt.) 180-220 gram, were used in all animal experiments.

Labeling of Tobramycin and quality controls

The labeling of tobramycin with technetium followed methods previously described for labeling gentamicin (15). Ten mg tobramycin sulfate (Sigma Chemical Co, St. Louis, USA) was dissolved in 100 μl H_2O while flushing with nitrogen. Stannous chloride dihydrate (Merck Darmstadt, Germany) was dissolved in 0.5 M HCl solution (flushed beforehand with nitrogen) to a solution of 0.1 mg/ml. The following substances were added to the tobramycin solution under continuous nitrogen flushing:

100 µl 0.1 mg/ml $SnCl_2 . 2H_2O$ solution in 0.5 M HCl

250 µl 0.2 M NaOH solution

450 µl 0.2 M $NaHCO_3$ solution, pH = 8.3

100 µl $Na^{99m}TcO_4$, activity 100 Mbq

Sixty minutes was taken for reaction time.

To estimate the amount of free, unbound $^{99m}TcO_4^-$, a sample was taken at the end of the reaction time and placed on a silica gel thin layer chromotagraphy (Silica gel $60F_{254}$, Merck, Darmstadt, Germany) and run in an 85% methanol/water system. Distribution of activity over the TLC-strip was visualized on X-ray film and counted with a gamma counter (Wallac 1480, Wallac oy, Finland).

In order to differentiate between ^{99m}Tc-tobramycin and $^{99m}TcO_2$, biodistribution of the

labelled complex after intravenous instillation was studied in four rats. For this, animals were anesthetized with 0.5 ml/kg b.wt. intramuscular Hypnorm® (Janssen pharmaceuticals, Tilburg, The Netherlands) and 5 mg/kg b.wt. subcutaneous Dormicum® (Roche, Mijdrecht, The Netherlands) injected every 1.5 h. Animals were placed in abdominal position on a gamma camera (ROTA II, Siemens, USA) equipped with a LEAP collimator and using a 20% window around the ^{99m}Tc peak. Data were stored in a dedicated computer (Hermes, Nuclear Diagnostics, Stockholm, Sweden). Dynamic acquisition took place during 60 min; 12 frames of 5 min 256x256 matrix. In order to investigate organ distribution of radioactivity, animals were killed with an overdose of pentabarbitone sodium, 6 hours after injection of the radiolabel. Radioactivity in various organs and standards of the labeled complex were measured with a gamma counter.

Lung distribution studies

Surfactant

A freeze-dried natural surfactant preparation was used, isolated from porcine lungs as previously described [16]. It consisted of approximately 90-95% phospholipids, 1% hydrophobic proteins (surfactant-proteins B and C) and 1% free fatty acids, the remainder being other lipids such as cholesterol and glyceride; there was no surfactant-protein A in this surfactant preparation. Surfactant powder was added to the labelled complex and handshaken to a final concentration of 25 mg/ml.

Induction of infection

Induction of a lung infection with *Klebsiella pneumoniae* in rats followed methods previously described [17]. A $1x10^7$ colony forming unit (CFU) /ml *Klebsiella pneumoniae* (ATCC 43816) inoculum was prepared from an overnight culture in Mueller Hinton broth (MHB; Difco Laboratories, Detroit, Michigan, USA) as follows: 100 μl of the overnight culture was added to 10 ml MHB and incubated at 37°C for 1.5 hours, then the culture was washed twice with saline. The inoculum was stored on ice until usage. To verify the number of viable bacteria

in the inoculum, 100 μl of tenfold dilution steps in saline were plated on isosensitest agar plates (Oxoid Ltd., Basingstoke, England). Agar plates were incubated overnight and CFUs were counted the following day.

For infection, animals were anaesthetized with pentobarbitone sodium intraperitoneally (i.p.) 10 mg/kg b.wt. and 0.5 ml/kg b.wt. Hypnorm® intramuscularly. Animals were hung vertically from an intubating block, and larynx and vocal cords were visualized by means of an otoscope. A metal tube was inserted into the trachea and placed above the carina. A cannula, connected with a replicator syringe (P600.1, Hamilton Bonaduz AG, Switzerland), was inserted through the tube and 40 μl of the *K. pneumoniae* suspension was injected into the left main stem bronchus. Animals recovered within 30 min from this procedure.

Tracheal instillation of ^{99m}Tc-tobramycin(-surfactant) and preparation of the lungs

Twenty-four hours after infection rats were randomly divided in two groups. A trachea tube was inserted 2 cm into the trachea of anaesthetized, spontaneously breathing animals as described above. Each animal received in the trachea, as a bolus, a volume of 1 ml/kg of 1) ^{99m}Tc-tobramycin-surfactant suspension: 25 mg surfactant + 10 mg tobramycin/ml, n=6; or 2) ^{99m}Tc-tobramycin alone, 10 mg/ml, n=6.

Thirty seconds after instillation the rats were placed in supine position and received i.p. an overdose of pentobarbitone sodium. The abdominal aorta was cut, the trachea prepared and clamped 5 minutes after instillation. The thorax was opened and the lungs were removed leaving the trachea clamp in place. Lungs were immediately frozen in a mixture of iso-hexane and dry-ice and subsequently stored on dry ice. Frozen lungs were fixed with tissue tek OCT compound (Histolab products AB, Frölunda, Sweden) on a metal holder and 1 mm thick sagittal sections were cut with a meat slicer (Graef E-2000 Germany) from left lung to right lung.

Computed autoradiographic analysis of ^{99m}Tc-tobramycin distribution

Storage-phosphor autoradiography was used for analyzing distribution of ^{99m}Tc-tobramycin within the lungs [18]. Lung slices were placed in a precooled cassette with a 2 x 2 cm grid (Molecular Dynamics Inc, Krefeld, Germany). Digital photographs were made of the slices using a digital camera (Canonion RC260, Canon Europe, Amstelveen, The Netherlands).

The image plate (phosphor storagescreen, Molecular Dynamics Inc, Krefeld, Germany) was placed on top of the slices and the complete cassette was stored at -20°C. After 30 minutes exposure the image plate was removed from the slices and scanned using a phosphorimager (Molecular Dynamics Inc, Krefeld, Germany); spatial resolution was 100 μm. iPhoto plus (vs 1.1, U-lead system Inc, Farper, Taiwan) was used for resizing the digital images of each slice to the size and resolution equivalent to that of the scan of the slice. ImageQuant vs 1.0 for Macintosh (Molecular Dynamics Inc, Krefeld, Germany) was used for analyzing both digital pictures and scan images stored in the computer.

The distribution of ^{99m}Tc-tobramycin in the lungs was assessed following methods described by Kharasch *et al* [7]. Lungs were analyzed for: 1) the number of slices with radioactivity above threshold intensity and 2) the fractional area of each slice with an intensity above threshold intensity.

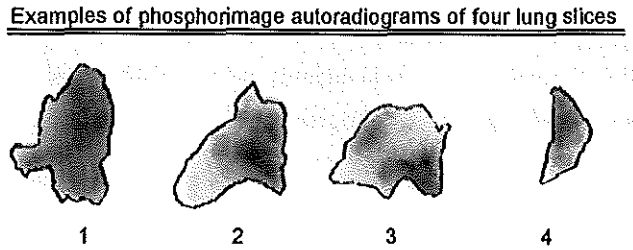

Figure 1 Real size examples of storage-phosphor autoradiograms of four different lung slices including the outline of the original slice as generated with the ImageQuant program (computer print-outs). Slices no 1 and 4 were taken from ^{99m}Tc-tobramycin-surfactant treated animals and slices no2 and 3 from ^{99m}Tc-tobramycin treated animals.

On each lung slice an outline was traced manually in the digital image and copied upon the corresponding scan of the phosphor autoradiogram. Examples of black-white print outs of computer generated scans of four lung slices are shown in figure 1. Threshold intensity was

calculated using the following equation; Threshold=MB + 25%(Max. int - MB). Where MB is the mean background, equal to the mean intensity in an oval drawn clearly outside the exposed areas on the scan; Max. int is the maximum pixel intensity registered within the exposed areas of the slices of one lung; 25% was chosen to correct for the interaction between exposed and unexposed areas, making the areas above threshold intensity visually within the actually exposed areas of the autoradiographs.

Using the spot-finder function of the ImageQuant program with kernel at 5 x 5 and minimal area size of 200 pixels, the areas with intensity above the threshold intensity were identified. The fractional area in each slice was calculated by dividing the number of pixels above threshold by the number of pixels in the slice outline times 100%. A weighted fractional area of each lung was defined as the ratio of the sum of the number of pixels above threshold intensity in all slices divided by the sum of the number of pixels in all slice outlines.

Statistical analysis

Data were analysed using the SAS statistical package (SAS Inc, Cary, USA). Two sample T-test with equal variances were used to compare differences between the group receiving ^{99m}Tc-tobramycin-surfactant and the group receiving ^{99m}Tc-tobramycin alone for the number of slices per lung with radioactivity above threshold intensity and the weighted area ratio for each lung. Significance was accepted at p values ≤ 0.05, two tailed.

Results

Tobramycin labeling with technetium

The mean percentage free $^{99m}TcO_4^-$ of five label procedures was 1.5 ± 0.7% (mean ± sd) sampled after 60 minutes. The amount of free $^{99m}TcO_4^-$ in the labeled complex, as tested with TLC, increased with time up 88.9% after 30 hours of incubation (Fig. 2).

Figure 3 shows twelve images taken with a gamma-camera during the first 60 minutes after intravenous injection of the labelled complex in one rat. The scans show a preferential localization of the labelled complex in the kidneys and bladder within the first 15 minutes (Fig

3, no3). No accumulation of activity was observed other than in the kidneys and bladder. Figure 4 shows the mean percentage of total injected radioactivity, accumulating in the uropoetic tract (kidneys and bladder) during one hour after intravenous injection of the labelled complex, as measured with the gamma-camera in four rats. Measurement of the organ distribution of radioactivity (Table 1) in four rats showed that after 6 hours both kidneys contain 40 ± 3.3% of the total injected dose (mean ± sd) versus 6 ± 2.0% in the liver.

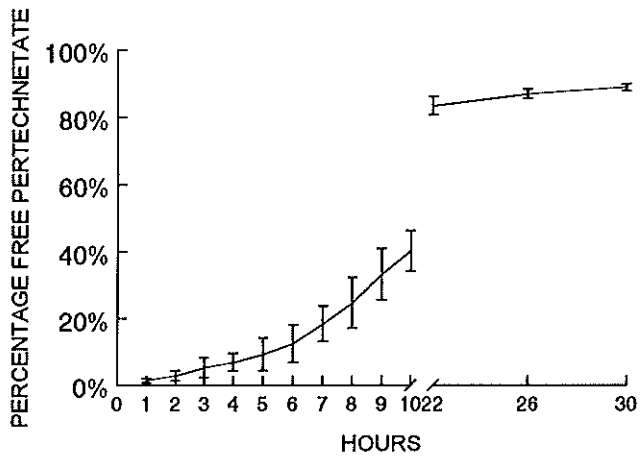

Figure 2. Percentage of free pertechnetate ($^{99m}TcO_4^-$) present in the labelings solution at different time points after the incubation at t=0h; mean ± SD of five labelingsprocedures. Percentages calculated from the distribution of radioactivity over a thin layer chromotagraphy silica gel run in 85% methanol/water.

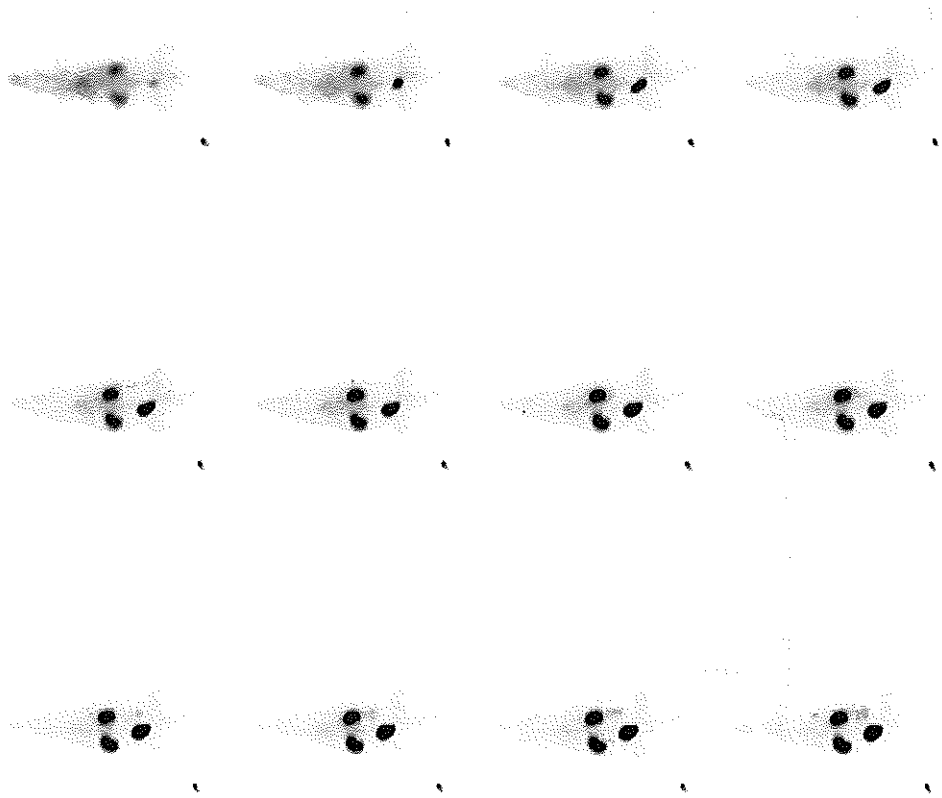

Figure 3. Gamma scans of one rat after intravenous injection of the labeled complex. Each figure represents the static image of 5 minutes, starting at the top from left to right. The left upper image (no 1), represents the first 5 minutes after injection and nr.12 (right lower image) from 55 to 60 minutes after injection. Accumulation of radioactivity is, within 10 minutes, seen in kidney regions and in the bladder; the spot of activity in the lower left leg corresponds with the intravenous catheter through which the complex was injected.

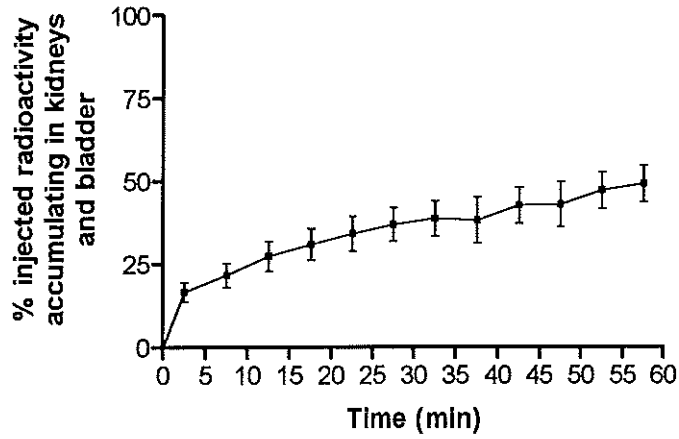

Figure 4. Accumulation of radioactivity in kidneys and bladder during one hour after intravenous injection of the labelled complex in rats. Accumulation of activity is expressed as a percentage of the amount of activity injected at t=0; mean ± sd, n=4. Data derived from static images acquired with a gamma camera.

Table 1. Organ distribution of radioactivity after IV injection of the labeled complex (n=4)

	% total injected dose (mean ± SD)	ratio weight corrected * (mean ± SD)
lung	0.4 ± 0.1	0.8 ± 0.1
left kidney	20 ± 3.3	39.1 ± 9.9
right kidney	20 ± 3.3	39.8 ± 11.1
liver	6 ± 2.0	1.1 ± 0.3
stomach	2 ± 1.4	0.5 ± 0.4
muscle	-	0.2 ± 0.1

* Equals the amount of radioactivity measureed in an organ divided by the weight of the organ/ total injected radioactivity divided by the total weight of the animal

Lung distribution studies

All lungs were cut into 8-10 slices: ^{99m}Tc-tobramycin-surfactant group a total of 57 slices, mean 9.5 slices per lung; ^{99m}Tc-tobramycin group a total of 51 slices, mean 8.5 slices per lung. All slices were analysed for activity above threshold and fractional area above threshold. In the group receiving ^{99m}Tc-tobramycin-surfactant 54 slices contained activity above threshold versus 42 in the group receiving ^{99m}Tc-tobramycin only. The mean percentage (± sd) of the number of slices with activity above threshold per lung was 95% (± 5.7%) in the ^{99m}Tc-tobramycin-surfactant group vs 83% (±11.4%) in the ^{99m}Tc-tobramycin group, p=0.04.

Figure 5 shows the weighted fractional area of each lung in the ^{99m}Tc-tobramycin-surfactant and ^{99m}Tc-tobramycin group as a percentage of the total lung area. Addition of surfactant to the ^{99m}Tc-tobramycin solution resulted in a 31% increase of the weighted fractional area, from 0.36 in the ^{99m}Tc-tobramycin group to 0.47 in the ^{99m}Tc-tobramycin-surfactant group, p=0.04.

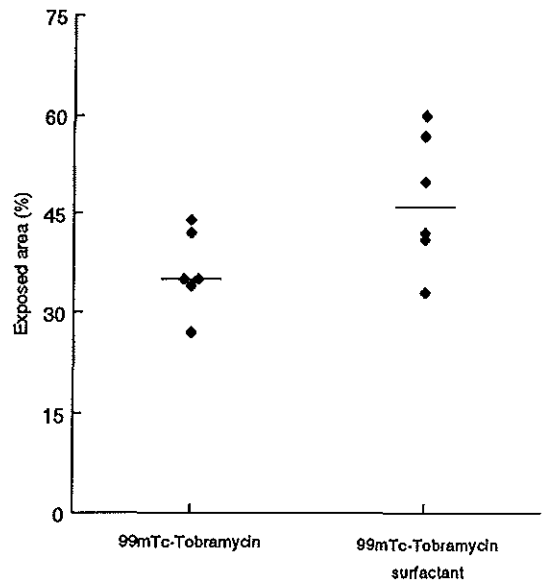

Figure 5. Effect of surfactant on lungdistribution of intratracheally instilled ^{99m}Tc-tobramycin. Each point represents the weighted fractional area x 100% (= exposed area) per lung in the ^{99m}Tc-tobramycin group and the ^{99m}Tc-tobramycin-surfactant group (n=5 per group). Horizontal line represents the median. $P = 0.04$, t-test for equal variances.

Discussion

In this study a method for labeling tobramycin with technetium was evaluated. Using the radiolabelled tobramycin pulmonary distribution of intratracheally instilled ^{99m}Tc-tobramycin was investigated with and without the addition of surfactant to the vehicle in *K. pneumoniae* infected rat lungs. The results show increased spreading of ^{99m}Tc-tobramycin within infected lungs when instilled as a surfactant-antibiotic mixture. The present results on pulmonary distribution are in comparison with those reported by Kharasch *et al.* [7] who studied lung distribution of intratracheally instilled technetium sulfur colloid using surfactant or saline as vehicle, in healthy hamster lungs.

Tobramycin labeling

Impurity of the tobramycin labeling with technetium could consist of ^{99m}Tc-pertechnetate ($^{99m}TcO_4^-$) and hydrolysed-reduced technetium ($^{99m}TcO_2$). As previously described for labeling gentamicin [15], large amounts of radioactivity remained at the application spot in the silica gel TLC system. This system was, therefore, only qualified for detection of the amount of $^{99m}TcO_4^-$ and could not differentiate between ^{99m}Tc-tobramycin and $^{99m}TcO_2$. However, in the present study, both the in vitro studies and the biodistribution studies of the labeled complex gave convincing evidence for binding of ^{99m}Tc to tobramycin. The in vitro studies showed that 30 hours after incubation the amount of $^{99m}TcO_4^-$ was increased to 89% of the total activity present. This indicates the formation of a complex with tobramycin which falls apart in time. $^{99m}TcO_2$, $^{99m}TcO_4^-$ and ^{99m}Tc-tobramycin have different clearance characteristics after intravenous administration: $^{99m}TcO_2$ is colloidal and accumulates in the liver; $^{99m}TcO_4^-$ accumulates in the thyroid gland, the gastric mucosa, and the salivary glands [19]; tobramycin is entirely cleared by renal filtration and would therefore accumulate in the kidneys and bladder [20]. The distribution of the radioactivity as measured with the gamma-camera showed a preferential renal clearance of the labeled complex. Similar results were obtained when measuring the organ distribution of the radioactivity with 40% of the injected dose was present in the kidneys after 6 hours versus 6% in the liver. It was concluded from these studies that technetium formed a complex with tobramycin.

Lung distribution

Factors reported to influence lung distribution of tracheally instilled solutions including surfactant, are: the instillation volume [6,7,21,22], the properties of the carrier fluid [6,7,23], the instillation technique [24,25] and the presence of lung injury [21,26].

Studies on surfactant distribution after intratracheal instillation in healthy rabbits have demonstrated that distribution is more homogeneous when the instillation volume is increased [21,22]. This finding was observed by Gilliard *et al.* [21] who studied surfactant distribution after instillation of 1.4 ml/kg and 14 ml/kg, and by Van der Bleek *et al.* [22] who studied volumes of 2.4 ml/kg b.wt. and 16 ml/kg b.wt. in rabbits. Comparable effects of volume have been reported after saline instillation marked with TcSC in healthy hamster lungs [6,7]. Brain *et al.* [6] reported that instillation of 0.1 ml/kg b.wt. saline-TcSC failed to run deep into the airways in contrast to 1.5 ml/kg b.wt. Kharasch *et al.* [7] reported that instillation of 5 ml/kg b.wt. and 15 ml/kg b.wt. saline-TcSC distributed throughout the entire lung in contrast to 0.25 ml/kg b.wt. Unfortunately, both studies failed to report the actual data. In the present study the instillation volume was limited to 1 ml/kg b.wt. Preliminary results in our laboratory have shown that this is still a safe instillation dose in anaesthetized, spontaneously breathing animals.

Other studies have shown that the properties of the carrier fluid influence pulmonary distribution of intratracheally instilled suspension. That is, surface tension, density and fluid absorption rate [6,7,23]. Davis *et al.*, [23] demonstrated in preterm pigs by means of dynamic scintography that a surfactant bolus of 3 ml/kg b.wt. was rapidly distributed throughout the lung in a relatively symmetrical fashion. This in contrast to piglets receiving ^{99m}Tc in saline, in which non-uniform distribution patterns were observed with multiple filling defects. In comparison, Kharasch *et al.* [7] demonstrated in healthy hamster lungs increased spreading of TcSC throughout the lung when instilled as a surfactant suspension compared to saline instillation. The present study extends these results to infected lungs and to spreading of an antibiotic. These results all support the concept that the surface active properties of surfactant help facilitate intrapulmonary spreading. In addition, Brain *et al.* [6] demonstrated that particle distribution preferentially goes to the dependent parts of the lung after intratracheal instillation and it was speculated that the density and absorption rate of the carrier fluid would influence pulmonary distribution. However, although these assumptions seem logical, no data are available to confirm

these speculations.

It has been shown that lung distribution of surfactant is influenced by the instillation technique. In comparison to bolus instillation, slow infusion (over 30-45 minutes) of a 4 ml/kg b.wt. surfactant dose into the trachea in ventilated surfactant deficient rabbits [24] or preterm lambs [25] resulted in an uneven distribution pattern. Ueda *et al.* [25] further reported a close match between the localization of a first surfactant dose with a second surfactant dose both after infusion and after bolus instillation and it was suggested that surfactant preferentially spreads to open and ventilated lung areas. To our knowledge the influence of the instillation technique, in particular infusion versus bolus instillation, has not been studied for saline.

It is well known that lung injury patterns strongly affect intrapulmonary distribution patterns of aerosols. Non-ventilated regions are not penetrated by aerosols and deposition is more central when lung function decreases [1,2]. The results of Ueda *et al.* [25] suggest a similar preferential distribution of surfactant to open and ventilated areas. This may lead to the assumption that surfactant has no advantage over aerosol inhalation regarding pulmonary distribution. However, Lewis *et al.* [26] demonstrated in a model of non-uniform lung injury in rabbits that pulmonary distribution of surfactant to the most injured lung areas was far superior after bolus instillation compared with aerosol administration. Studies by Gilliard *et al.* [21] further demonstrated that the presence of pulmonary oedema favours surfactant distribution throughout the lung. The presence of pulmonary oedema probably mimicks the effect of a large instillation volume.

Recent studies have demonstrated that tracheal instillation of a surfactant-tobramycin mixture is superior to instillation of tobramycin alone in protecting mice from death of a respiratory infection with *Klebsiella pneumoniae* [10]. It was speculated that this could result from an improved distribution pattern of the antibiotic within the lung and/or a therapeutic effect of the surfactant itself. The present study confirms that antibiotic distribution after tracheal instillation is enhanced when surfactant is used as vehicle. The current methods did not allow an accurate quantification of the infected area in the images. It was, therefore, not possible to assess the amount of ^{99m}Tc-tobramycin actually deposited in the affected areas which is a shortcoming of this study. Future studies should address this important issue.

In summary, the present study described a method for studying intrapulmonary

distribution of intratracheally instilled tobramycin in *Klebsiella pneumonia* infected rat lungs. For this, the tobramycin was labeled with technetium and subsequently ^{99m}Tc-tobramycin distribution in the lung after intratracheal instillation was quantified with computer assisted autoradiography. Using this method, it was found that intrapulmonary distribution of ^{99m}Tc-tobramycin was increased when pulmonary surfactant was added to the ^{99m}Tc-tobramycin solution. It is speculated that this increased intrapulmonary distribution of ^{99m}Tc-tobramycin enhances local antimicrobial therapy. The clinical relevance of these findings needs further investigation.

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Chapter 7

Lung clearance of intratracheally instilled ^{99m}Tc-tobramycin using pulmonary surfactant as vehicle

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Summary

It has been proposed to use pulmonary exogenous surfactant as a vehicle for intratracheally administered antibiotics to improve local antimicrobial therapy. The present study investigated in rats, lung clearance rates of intratracheally instilled technetium labelled tobramycin with and without the addition of surfactant to the antibiotic solution. The influence of surfactant on ^{99m}Tc-tobramycin lung clearance rates was studied dynamically with a gamma-camera in spontaneously breathing animals and in mechanically ventilated animals (n=4 per group, 16 studied rats in total). The results show that instillation of ^{99m}Tc-tobramycin with use of surfactant as vehicle significantly increases ^{99m}Tc-tobramycin lung clearance compared to instillation of ^{99m}Tc-tobramycin solution alone: P=0.006 between the two spontaneously breathing groups of animals and P = 0.02 between the two ventilated groups of animals (ANOVA for repeated time measurements). T½ of composite clearance curves in spontaneous breathing animals was 147 min for animals receiving ^{99m}Tc-tobramycin versus 61 min for animals receiving ^{99m}Tc-tobramycin with surfactant. In mechanically ventilated animals this was 163 min vs 51 min, respectively. It is concluded that exogenous surfactant used as vehicle for intratracheally instilled ^{99m}Tc-tobramycin increases lung clearance rate of ^{99m}Tc-tobramycin in rats.

Introduction

It has been proposed to use exogenous pulmonary surfactant as a carrier or vehicle for intratracheally administered antibiotics to enhance the efficacy of local antimicrobial therapy in pneumonia [1-6]. The expected advantages of this new delivery method are twofold. First, the surfactant instillation itself is potentially therapeutic in pneumonia: it promotes re-expansion of atelectatic areas and can correct impaired lung function and gas exchange in pneumonia (for review see ref. [7,8]). Second, it is expected that together with the pulmonary surfactant a high appropriate antibiotic dose can be delivered to the peripheral lung regions and even into atelectatic lung regions [1]. It is believed that in pneumonia, selective delivery of antibiotics to the lung parenchyma increases the local effectiveness and decreases the risk for systemic side effects of potentially toxic agents such as aminoglycosides [9,10].

We recently demonstrated the *in vivo* efficacy of pulmonary surfactant as a vehicle for intratracheally administered tobramycin in mice with a respiratory infection with *Klebsiella pneumonia*. Survival rates of infected animals were significantly improved after tracheal instillation of a surfactant-tobramycin mixture compared with instillation of tobramycin alone [5]. Explanations for the observed difference in survival remained speculative and included an inherent effect of the exogenous surfactant instillation and/ or an increased intrapulmonary spreading of the tobramycin when instilled with use of surfactant as vehicle. Preliminary results have shown that spreading of intratracheally instilled technetium labeled tobramycin (^{99m}Tc-tobramycin) within infected rat lungs was increased when pulmonary surfactant was added to the ^{99m}Tc-tobramycin solution [11].

To further explore the use of surfactant as a vehicle for antibiotics and explain the earlier observed *in vivo* efficacy [5], the present study investigated lung clearance of intratracheally instilled ^{99m}Tc-tobramycin with and without the use of surfactant as vehicle. Lung clearance of ^{99m}Tc-tobramycin was dynamically studied in healthy rats using sequential gamma-camera images. To study the effect of mechanical ventilation on lung clearance rates, lung clearance was studied in both spontaneously breathing animals and in artificially ventilated animals.

Materials and Methods

Antibiotic labeling

Tobramycin was labeled with technetium using a method previously described for gentamicin [12]. Briefly, 10 mg tobramycin sulfate (Sigma Chemical Co, St. Louis, USA) was dissolved in 100 μl H_2O while flushing with nitrogen. Stannous chloride dihydrate (Merck, Darmstadt, Germany) was dissolved in 0.5 M HCl solution (beforehand flushed with nitrogen) to a solution of 0.1 mg/ml. The following substances were added to the tobramycin solution under continuous nitrogen flushing:

100 µl 0.1 mg/ml $SnCl_2 . 2H_2O$ solution in 0.5M HCl,

250 µl 0.2 M NaOH solution,

450 µl 0.2 M $NaHCO_3$ solution (pH = 8.3),

100 µl $Na^{99m}TcO_4$, activity 100 Mbq.

Sixty minutes was taken for reaction time.

To estimate the amount of free, unbound $^{99m}TcO_4^-$, a sample was taken at the end of the reaction time and placed on a silica gel thin layer chromotagraphy (TLC, Silica gel $60F_{254}$, Merck, Darmstadt, Germany) and run in an 85% methanol/water system. Distribution of activity over a TLC-strip was counted with a gamma counter (Wallac 1480, Wallac oy, Finland).

Surfactant

A freeze-dried natural surfactant preparation was used, isolated from porcine lungs as previously described [13]. It consisted of approximately 90-95% phospholipids, 1% hydrophobic proteins (surfactant-proteins B and C) and 1% free fatty acids, the remainder being other lipids such as cholesterol and glyceride; there was no surfactant-protein A in this surfactant preparation. Surfactant powder was added to the antibiotic solution and handshaken to a final concentration of 25 mg/ml.

Animals

All animal experiments were approved by the institutional board for animal care. Care and handling were in accordance with the European Community guidelines for animal

experimentation (86/609/EEG). In all experiments male Sprague Dawley rats (Iffa Credo, Brussel, Belgium), body weight (b.wt.) 180-220 gram were used, kept under conventional conditions; food and water were given *ad libitum*.

Animal preparation

During the total study period, anesthesia was maintained in each animal with 0.5 ml/kg b.wt. intramuscularly Hypnorm® (Janssen pharmaceuticals, Tilburg, The Netherlands) and 5 mg/kg b.wt. subcutane Dormicum® (Roche, Mijdrecht, The Netherlands) injected every 1.5 h. Each animal received an intravenous line inserted in the arcus pedis, through which saline was administered (5 ml/kg/h) to maintain fluid balance. In addition, animals subjected to mechanical ventilation were tracheotomized after which the trachea was cannulated with a metal tube and received pancuronium bromide (0.5 mg/kg, intramuscularly) every hour to maintain muscle relaxation. Animals were mechanically ventilated using a Servo Ventilator 900C (Siemens-Elema, Solna, Sweden) at the following settings: pressure-controlled ventilation, frequency = 30 breaths per min, peak airway pressure = 14 cm H_2O, positive end expiratory pressure (PEEP) = 3 cm H_2O, inspiratory/expiratory ratio = 1 : 2 and 21% oxygen. These ventilatory settings were maintained throughout the study period. At the end of the study animals were killed with an overdose of Dormicum® injected intravenously.

Lung clearance studies

Lung clearance of intratracheally instilled ^{99m}Tc-tobramycin or ^{99m}Tc-tobramycin-surfactant was studied in 16 rats, randomly assigned to four groups of 4 animals each:

group 1 spontaneously breathing, intratracheal ^{99m}Tc-tobramycin: volume = 1 ml/kg, concentration = 10 mg tobramycin/ml

group 2 spontaneously breathing, intratracheal ^{99m}Tc-tobramycin - surfactant: volume = 1 ml/kg, concentrations = 10 mg tobramycin + 25 mg surfactant/ml

group 3 artificially ventilated, intratracheal ^{99m}Tc-tobramycin: volume = 1 ml/kg, concentration = 10 mg tobramycin/ml

group 4 artificially ventilated, intratracheal ^{99m}Tc-tobramycin - surfactant: volume = 1 ml/kg, concentration = 10 mg tobramycin + 25 mg surfactant/ml

Intratracheal instillation in spontaneously breathing animals was achieved by placing a metal tube per oral 2 cm into the trachea. A catheter, connected to a 1 ml syringe was inserted through the tube and extended 1 cm beyond the tip of the tube. Subsequently, 1 ml./kg b.wt. ^{99m}Tc-tobramycin or ^{99m}Tc-tobramycin-surfactant could be instilled. Cannula and tube were immediately removed after instillation.

For intratracheal instillation in mechanically ventilated animals, animals were disconnected from the ventilator and a catheter connected to a 1 ml syringe was inserted into the trachea cannula extending 1 cm beyond the tip of the canula for instillation of 1 ml/kg b.wt. ^{99m}Tc-tobramycin or ^{99m}Tc-tobramycin-surfactant.

Before instillation of the solution animals were placed in position on a gamma camera (ROTA II, Siemens) equipped with a LEAP collimator and using a 20% window around the ^{99m}Tc peak. ^{99m}Tc-tobramycin or ^{99m}Tc-tobramycin-surfactant was injected intratracheally at t=0 and acquisition was started immediately thereafter. Data were stored in a dedicated computer (Hermes, Nuclear Diagnostics, Stockholm, Sweden). Dynamic acquisition took place in groups 1 and 2 for a total of 300 min; 30 frames of 10 min, 256 x 256 matrix and in groups 3 and 4 for a total of 180 min; 10 frames of 2 min, 8 frames of 5 min and 12 frames of 10 min, 256 x 256 matrix.

Data analyses

The ^{99m}Tc-tobramycin clearance measurements were analysed by drawing a region of interest over both lungs and generating a time-activity curve, corrected for radioactive decay over time. No background corrections were made. Differences between animals in injected activity at t=0 were corrected for by dividing counts per minute (CPM) at t=x with CPM at t=0 for each rat.

Corrected data of the activity per lung region over time were log-transformed for equal distribution and analysed for statistical significance with an ANOVA for repeated time measurements using the SAS statistical package (SAS Inc, Cary, USA).

A mean composite time activity curve was generated for each group on which mono-exponential and bi-exponential functions were fitted, using the curve-fit function of GraphPad Prism version 2.00 (GraphPad Software Incorporated, CA, USA).Wit this program, equations

were compared for statistically significant best fit using the F-test, $P \leq 0.05$, two tailed. The amount of activity present in the lung as a percentage of the total injected dose was calculated at t=0, 30, 180 and 300 min. Differences between groups were analysed using a one-way ANOVA with a Student-Newman-Keuls post-hoc test. Differences were accepted significant when $P \leq 0.05$, two-tailed.

Results

Figure 1 shows representative images made with the gamma-camera at several time points after injection of the radioactive tobramycin label. After endotracheal instillation, radioactivity was instantly distributed within both lungs resulting in a gamma-camera image anatomically corresponding with lungs and trachea. Shortly after endotracheal instillation radioactivity appeared outside the lung region and accumulated, in particular, in regions corresponding to kidneys, bladder and to a lesser degree in stomach, liver an gut. Increased radioactivity was not observed in the thyroid or salivary glands.

The composite time activity data for ^{99m}Tc-tobramycin instilled with or without surfactant as vehicle are shown in Figure 2A for spontaneously breathing animals and in Figure 2B for artificially ventilated animals. The curves represent the mono-exponential function, best fitted on the composite data using the following equation:

$$y = Plateau + Span * \exp^{-kt}$$

Where t = time (min), k = the clearance rate (min^{-1})and *Plateau* and *Span* are constants. The constants *Plateau*, *Span* and k for the best fitted curve of the respective groups are shown in Table 1, including the calculated half-life (T½) of the radioactive tracer in the lung and the coefficient of determination (r^2) of the curve. T½ (min) of the fraction of the radioactive tracer

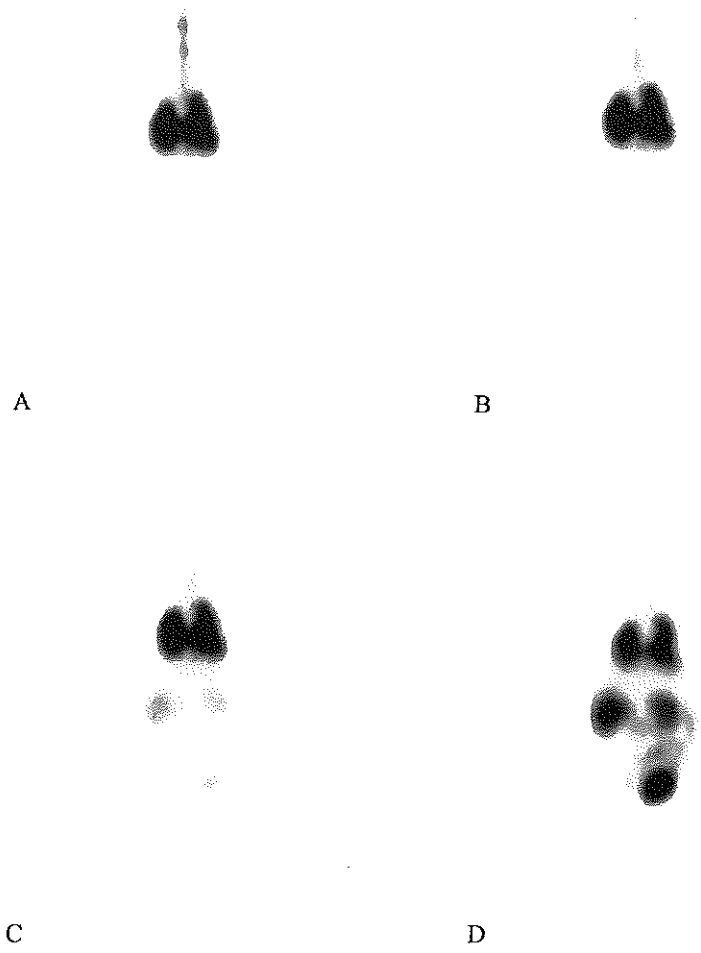

Figure 1. Gamma-camera scans from one representative animal at t=5' (A), 20' (B), 60' (C) and 300' (D) after endotracheal instillation of ^{99m}Tc-tobramycin - surfactant.

that is mono-exponentially cleared from the lung is calculated using the following equation:

$$T½ = \frac{\ln 2}{k}$$

Lung clearance of the ^{99m}Tc-tobramycin was significantly increased when surfactant was used as vehicle, both in spontaneously breathing animals (P =0.006, ANOVA repeated time measurements) and artificially ventilated animals; (P = 0.02, ANOVA repeated time measurements). No significant differences in lung clearance were found between spontaneously breathing animals and ventilated animals receiving ^{99m}Tc-tobramycin with surfactant as vehicle, or similarly receiving ^{99m}Tc-tobramycin only (P>0.05, ANOVA repeated time measurements).

Table 2 gives the percentage of the total injected dose present in the lungs at t=0, 30, 180 and 300 min after intratracheal instillation as measured with the gamma-camera. Percentage of the injected dose present in the lungs was significantly decreased in the tobramycin-surfactant group compared to the tobramycin group from t=0.5 h onwards both in the spontaneously breathing animals and the ventilated animals (P≤0.05, one-way ANOVA with Students-Newman-Keuls post-hoc test). No significant differences were found at t=0 h.

Table 1. Characteristics of the curves fitted on the composite time activity data *

	Plateau	Span	k (min^{-1})	r^2	T½
Spontaneously breathing					
Tobramycin	0.33	0.70	0.0047	0.93	147
Tobramycin-surfactant	0.33	0.62	0.0114	0.79	61
Mechanically ventilated					
Tobramycin	0.19	0.83	0.0042	0.84	163
Tobramycin- surfactant	0.29	0.68	0.0140	0.91	51

* Parameters of the mono-exponential function fitted to the data of the four studied groups according to the equation: $y = Plateau + Span * \exp^{-kt}$

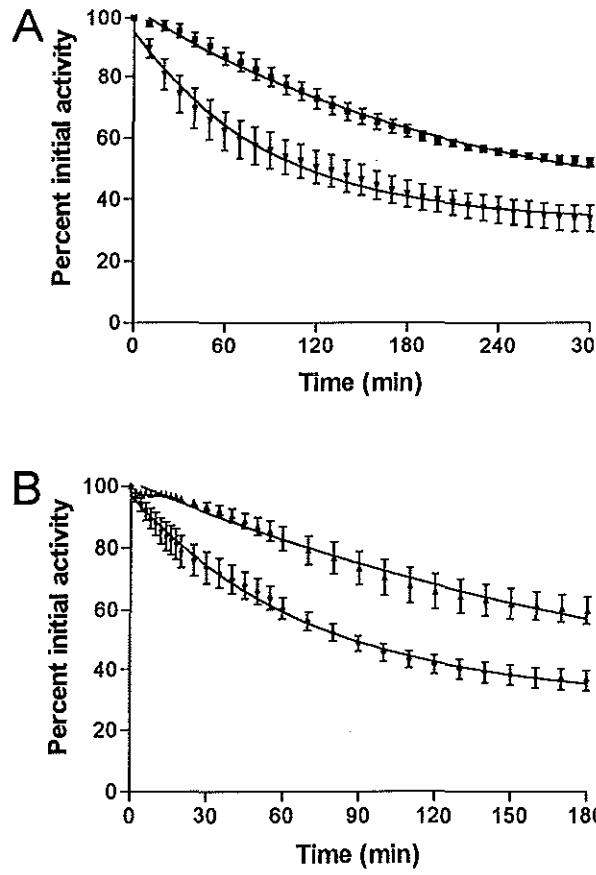

Figure 2 A&B. Average activity (mean ± SEM) present over the lung region as a percentage of the activity present over the lung region at t=0 plotted against time for the four studied groups. Figure 2A shows spontaneously breathing animals of which the ■ data points represent the group receiving ^{99m}Tc-tobramycin and the ▼ data points represent the group receiving ^{99m}Tc-tobramycin-surfactant. Figure 2B shows mechanically ventilated animals of which the ▲ data points represent the group receiving ^{99m}Tc-tobramycin and the ♦ data points represent the group receiving ^{99m}Tc-tobramycin-surfactant. Curve represents the mono-exponential function fitted to the data of which the parameters are shown in Table 1.

Table 2. Percentage injected dose (mean ± SD) present in the lung regions at different time points*

	t = 0' (%)	t = 30' (%)	t = 180' (%)	t = 300' (%)
Spontaneously breathing				
Tobramycin	94 ± 1.4	89 ± 4.9	58 ± 3.5	49 ± 2.2
Tobramycin-surfactant	91 ± 3.5	68 ± 13.1	38 ± 8.3	30 ± 7.1
Mechanically ventilated				
Tobramycin	90 ± 1.7	84 ± 2.9	54 ± 8.5	-
Tobramycin-surfactant	88 ± 5.7	66 ± 11.4	32 ± 7.8	-

* calculated as the amount of activity present in the lungs at t=x divided by the amount of activity present at t=0' in the whole body of the animal times 100%.

Discussion

The aim of the present study was to quantify the influence of pulmonary surfactant as vehicle on lung clearance of endotracheally instilled tobramycin in healthy rats. The results show that after endotracheal instillation of ^{99m}Tc-tobramycin, lung clearance rate of the radiotracer was significantly increased when surfactant was added to the ^{99m}Tc-tobramycin solution.

The use of technetium labelled tobramycin enabled an accurate quantification of the amount of tobramycin present in the lung and sequential analysis within one subject. The labeling method was similar to that described for gentamicin [12]. Impurity of the labeling could consist of ^{99m}Tc-pertechnetate ($^{99m}TcO_4^-$) and hydrolysed-reduced technetium ($^{99m}TcO_2$), both of which have different clearance characteristics than tobramycin and could, thus, result in false interpretations of the tobramycin lung clearance. $^{99m}TcO_2$ is colloidal and unable to pass the alveolar-capillary barrier [14]. In contrast $^{99m}TcO_4^-$ clears quickly from the lung; in patients, T½ of aerosolized pertechnetate approximated 7 min [15]. $^{99m}TcO_4^-$ accumulates in the thyroid gland, the gastric mucosa, and the salivary glands [14]. Tobramycin is primarily cleared from the circulation by renal filtration and would, therefore, accumulate in the kidneys and bladder after entering the systemic circulation [16]. Al-Kouraishi [12] estimated in his labelings procedure the percentage $^{99m}TcO_2$ to be 6-10% and the percentage $^{99m}TcO_4^-$ to be 1%. Control studies (data not shown) in our laboratory by means of chromatographic assays *in vitro* and biodistribution after

intravenous injection in rats *in vivo* revealed comparable quality of the tobramycin label as that reported for gentamicin.

According to the monoexponential curves best fitted on the composite time activity data, in each group a percentage of the intratracheally instilled activity was not cleared from the lung region; that is, equal to the value for plateau's in Table 1 x 100%. Although speculative, this percentage not cleared according to this mono-exponential curve may represent intracellular ^{99m}Tc-tobramycin, macromolecular bounded ^{99m}Tc-tobramycin, ^{99m}Tc-tobramycin present in the trachea tube and/or $^{99m}TcO_2$. Most likely a longer study period would reveal a bi-exponential clearance curve.

After intratracheal instillation, ^{99m}Tc-tobramycin can potentially be cleared from the lungs via the upper airways, lymphatics or vascular compartment, or it may remain within the lung parenchym. However, based on its molecular size it can generally be expected that once ^{99m}Tc-tobramycin has reached the distal airways after intratracheal instillation systemic absorption is the primary route for lung clearance. In the current study, although not actually measured, systemic absorption can readily be assumed based on the high lung clearance rates and the accumulation of radioactivity in primarily kidneys and bladder. A recent study demonstrated in six patients with cystic fibrosis that the systemic availibility of inhaled tobramycin ranged between 6.0% -27.4% [17]. These percentages are in close comparison with reported percentages for the dose that is actually deposited in the lungs after inhalation of an aerosol [18,19], suggesting that systemic absorption is the main clearance route of tobramycin after distal pulmonary deposition.

In the current study T½ of ^{99m}Tc-tobramycin in the lung ranged between 51 and 163 min. Valcke *et al.* reported a T½ of 126 min of tobramycin concentrations in alveolar lining fluid after aerosol inhalation by spontaneoulsy breathing healthy rats [20]. This seems comparable to the 147 min found in our study in the spontaneously breathing animals receiving ^{99m}Tc-tobramycin only. Also in range are the results by Cooney *et al.*, who investigated absorption characteristics of aerosolized tobramycin in adults with cystic fibrosis and found a mean absorption time across the alveoli to vary widely from 15-150 min [21].

The results of the current study demonstrate that clearance rates are more than twofold increased when the ^{99m}Tc-tobramycin solution is instilled with surfactant as vehicle. No

significant difference was found between mechanically ventilated animals and spontaneously breathing animals receiving the same intratracheal instillation: ^{99m}Tc-tobramycin or ^{99m}Tc-tobramycin-surfactant, respectively. Clearance rate of a solute from airways is dependent on a great number of factors related to the properties of both the solute and the lung. For instance, molecular size and charge of the solute, the epithelial integrity, regional surface area available and intra-alveolar pressures (for review see ref. [22]). As clearance rate is influenced by so many factors it seems impossible to define with certainty the mechanism that causes the current findings.

One very plausible explanation for the observed increase in lung clearance rate of ^{99m}Tc-tobramycin after instillation with surfactant compared to instillation without surfactant, would be an increased alveolar deposition of the tracer and an increased exposed surface area within the lung. It has been shown that radioaerosol clearance rates from the larger airways, bronchi, trachea and nasal epithelia are significantly slower than that of the alveolar-capillary membrane [23,24]. In addition, transfer rate of hydrophylic solution across the alveolar-capillary barrier is dependent on passive diffusion through intracellular junctions; increased exposed surface area can so increase clearance rates [22].

These explanations are in accordance with previous studies that demonstrated a more peripheral and more homogenous pulmonary distribution of an intratracheally instilled radiotracer [1,11] or adenoviral vectors [25] when surfactant was used as vehicle in comparison to saline. Unfortunately, a more peripheral and homogenous distribution could not be quantified in the current study due to a too low resolution of the images for this purpose.

The current findings are in contrast with two earlier studies [26,27]. Davis *et al.* found that surfactant instillation in healthy adult rats 30 minutes prior to intratracheal instillation of recombinant super oxide dismutase (rhSOD) resulted in significantly increased rhSOD lung concentrations 24 hours later [26]. It was speculated that rhSOD became entrapped in the lipid bilayer of surfactant which could cause a decrease in lung clearance. However, if so, this association appeared to be weak as subsequent *in vitro* studies showed that rhSOD could easily be separated from the surfactant by sedimentation [26]. Smith *et al.* reported a delayed absorption of furosemide from the lungs when surfactant, as compared to saline, was used as vehicle for intratracheal delivery in healthy guinea pigs[27]. It was speculated that the delayed absorption

of furosemide when instilled with surfactant as vehicle resulted from binding of furosemide to surfactant proteins or to liposome formation [27].

Explanations for the discripancy between these two studies [26,27] and the present results are speculative and must be sought in methodological differences between the studies and differences in the agents used. For instance, in the study by Davis *et al.* [26] no difference in pulmonary distribution of rhSOD is to be expected when instilled as a saline solution 30 minutes after surfactant instillation. The varying results demonstrate the complexity of pulmonary clearance rates of intratracheally instilled solutes and necessitates the need for further investigations.

In conclusion, the present study has demonstrated an increased clearance rate of intratracheally instilled technetium labelled tobramycin solution when surfactant was added to the solution. This increase in clearance might well be explained by a more peripheral distribution of the ^{99m}Tc-tobramycin within the lung when surfactant is added to the solution as has been observed in earlier studies on surfactant as a vehicle. More studies are needed to further clarify the pharmacokinetic interactions and to determine the clinical relevance of these findings.

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Chapter 8

Summary and conclusions

Summary and conclusions

This work comprises five studies on the use of exogenous pulmonary surfactant as a delivery agent, also called carrier or vehicle, for intratracheally instilled antimicrobial agents. These studies elaborate on the overall theme of surfactant dysfunction and exogenous surfactant therapy in pneumonia, as reviewed in **Chapter 2**. This chapter further contains the rationale for combining surfactant with antimicrobial agents. At the start of the present studies, hardly any data existed on surfactant as a delivery agent, it was, therefore, chosen to study different aspects of this topic.

The purpose of the studies presented in **Chapters 3 and 4** was to reveal possible interactions between surfactant and antibiotics which could interfere with the usefulness of surfactant as a delivery agent for antibiotics. Timed killing curves of amoxicillin, ceftazidime and tobramycin against four pulmonary pathogens in medium with and without additional surfactant revealed a reduced actvity of tobramycin in the presence of surfactant (Chapter 3). In addition, surfactant activity, tested in the lung lavage model in rats, was reduced of tobramycin-surfactant mixtures in saline but unaffected when 0.2M $NaHCO_3$ was used as solvent. The same study revealed reduced surfactant activity when combined with amphotericin B and amoxicillin, but not with pentamidine or ceftazidime (Chapter 4).

Earlier studies have reported that tobramycin can bind to phospholipids and this could be an explanation for the currently found interaction between surfactant and tobramycin. The main conclusion from both studies should be that interactions between surfactant and antibiotics may exist and can interfere with the activity of each substance. Therefore, before using surfactant-antibiotic mixtures, or mixtures of surfactant with other agents in general, alterations in activity of both substances should be considered and carefully examined.

In **Chapter 5** a model for respiratory infection with *Klebsiella pneumoniae* in mice is presented and used to explore the *in vivo* efficacy of surfactant as a delivery agent for antibiotics. It was demonstrated that intratracheal instillation of a surfactant-tobramycin mixture is more effective in protecting mice from death of a respiratory *Klebsiella pneumoniae* infection than intratracheal instillation of tobramycin alone. Surfactant instillation alone did not significantly increase survival rates in this model. These results were the first to indicate that exogenous

surfactant is effective as a vehicle.

Further investigations seem desirable to firmly establish the efficacy of this delivery method and to gain more insight into the factors determining its efficacy. Repetition of these studies with other antimicrobial agents, e.g. ceftazidime and/or other lung pathogens would be interesting. Additonal interesting comparisons with surfactant-antibiotic mixtures could include aerosolized antibiotics or liposomal-encapsulated antibiotics as these delivery methods pursue a similar goal.

The aim of the studies in **Chapter 6** was to quantify the difference between lung distribution of intratracheally instilled antibiotics with or without addition of surfactant to the antibiotic solution in infected lungs. The chapter presents a method for labeling tobramycin with technetium(^{99m}Tc-tobramycin) as well as a method for quantifying lung distribution using computerized autoradiography. With these methods, it could be demonstrated that, in *Klebsiella pneumoniae* infected rat lungs, spatial lung distribution of intratracheally instilled ^{99m}Tc-tobramycin was increased with the addition of surfactant to the antibiotic solution.

Work by other investigators had already demonstrated the superiority of surfactant as vehicle over saline indistributing intratracheally instiled agents within healthy hamster lungs. The current study extended these results to infected lungs and supports the concept of improved lung distribution as a determining factor in the previously demonstrated efficacy (Chapter 5). The methods proved to be insufficient in determining the total amounts of ^{99m}Tc-tobramycin present in infected lung areas. This would be an interesting question for further studies, as would be a comparison between aerosolized tobramycin and tobramycin intilled with surfactant as vehicle. Refinement of the currently used methods should make these comparisons possible.

Chapter 7 explores the influence of surfactant as vehicle on lung clearance of intratracheally instilled tobramycin. It describes a sophisticated experimental method for studying tobramycin pharmacokinetics using ^{99m}Tc-tobramycin and a gamma camera. It was demonstrated in rats that lung clearance of intratracheally instilled ^{99m}Tc-tobramycin is increased when surfactant is added to the antibiotic solution in comparison with lung clearance of ^{99m}Tc-tobramycin solution alone.

The results were in contrast to those described in studies by other investigators who found a decreased lung clearance of agents when instilled in combination with surfactant. Also, a

binding between tobramycin and surfactant phospholipids, as speculated upon in Chapters 3 and 4, could support this thought. However, the results have shown the opposite: an increased lung clearance of ^{99m}Tc-tobramycin when instilled in combination with surfactant which was attributed to an increased spatial lung distribution, as previously reported in Chapter 6.

The results on lung clearance of ^{99m}Tc-tobramycin in healthy animals suggested high absorption rates for ^{99m}Tc-tobramycin from the lung. Under the present experimental conditions, one should consider the occurrence of high systemic antibiotic levels after intratracheal instillation of surfactant-antibiotic suspensions. It needs to be further studied if an increased lung clearance is also found in animals with a dimished surfactant system, as in pneumonia.

In conclusion, this work has highlighted several aspects concerning the use of pulmonary surfactant as a delivery agent for antibiotics. These studies subscribe to the general expectation that pulmonary surfactant as a delivery agent for antibiotics has great potential and warrants further investigations. The clinical relevance is yet to be established; however, two important issues came to light that should be taken into account in a clinical setting. These are the possible occurrence of interactions between surfactant and antibiotics affecting each other's activity, and the occurrence of high systemic antibiotic levels when using this delivery method.

Samenvatting en conclusies

Dit proefschrift omvat vijf studies naar de toepasbaarheid van exogeen pulmonaal surfactant als vehikel, ook wel drager genoemd, voor intratracheaal toegediende antimicrobiële middelen. Deze studies zijn een voortzetting van het onderzoek naar afwijkingen in het endogene surfactant systeem en de mogelijkheid van surfactant therapie in geïnfecteerde longen. Hiervan is een overzicht gegeven in **hoofdstuk 2.** Dit hoofdstuk bevat tevens de redenen voor het gecombineerd toedienen van surfactant en antimicrobiële middelen. Tijdens de opzet van de in dit proefschrift opgenomen studies waren er vrijwel geen gegevens beschikbaar over surfactant als vehikel voor intratracheaal toegediende stoffen, daarom is gekozen voor verschillende invalshoeken van dit onderwerp.

Het doel van de studies, beschreven in **hoofdstuk 3 en 4,** was interacties op te sporen tussen surfactant en antibiotica welke kunnen interfereren met de effectiviteit van surfactant als vehikel voor intratracheaal toegediende antibiotica. Time-killing curves van amoxicilline, ceftazidime en tobramycine tegen vier longpathogenen in medium met en zonder toevoeging van surfactant, toonden een verminderde activiteit van tobramycine in de aanwezigheid van surfactant (hoofdstuk 3). De *in vitro* activiteit van amoxicilline en ceftazidime in aanwezigheid van surfactant was onveranderd in deze studies (hoofdstuk 3). Uit de studies beschreven in hoofdstuk 4 bleek dat ook de activiteit van surfactant, getest in het long-lavage model in ratten, verminderd was na mengen met tobramycine. Deze laatste interactie kon voorkomen worden door het surfactant te suspenderen in 0,2M $NaHCO_3$ in plaats van het gebruikelijke fysiologisch zout. Dezelfde studie toonde ook een verminderde activiteit van surfactant na mengen met amphotericine B en amoxicilline, maar niet na mengen met pentamidine of ceftazidime (hoofdstuk 4).Eerdere studies toonden dat tobramycine een binding aan kan gaan met fosfolipiden en mogelijk is dit een verklaring voor de in hoofdstuk 3 en 4 gevonden interactie tussen tobramycine en surfactant. Echter, de belangrijkste conclusie van deze studies is dat interacties tussen surfactant en antibiotica kunnen optreden en de activiteit van elke stof apart kan beïnvloeden. Daarom dient men voorafgaand aan het gebruik van een surfactant-antibiotica combinatie bedacht te zijn op veranderingen in de activiteit van beide stoffen en deze mogelijkheid nader te onderzoeken.

In **hoofdstuk 5** wordt een model voor een respiratoire infectie met *Klebsiella pneumoniae* in muizen geintroduceerd, waarmee de *in vivo* effectiviteit van surfactant als vehikel voor intratracheaal toegediende antibiotica is onderzocht. Aangetoond kon worden dat intratracheale toediening van een surfactant-tobramycine combinatie aan *Klebsiella pneumoniae* geinfecteerde muizen een verbetering gaf in overleving in vergelijking met het intratracheaal toedienen van tobramycine of surfactant alleen. Deze resultaten tonen voor het eerst aan dat surfactant effectief is als vehikel voor antibiotica.

Verder onderzoek is wenselijk om de effectiviteit van deze toedieningsmethode te bevestigen en om meer inzicht te verschaffen in de factoren die bepalend zijn voor het gevonden effect. Herhaling van deze studie met andere antibiotica, bijvoorbeeld ceftazidime, en/of andere longpathogenen is interessant. De *in vivo* effectiviteit van surfactant-antibiotica mengsels kan ook vergeleken worden met de *in vivo* effectiviteit van vernevelde antibiotica en de *in vivo* effectiviteit van liposomaal ingekapselde antibiotica daar deze verschillende toedieningsmethoden allen een vergelijkbaar doel nastreven.

Het doel van de studies beschreven in **hoofdstuk 6** was het verschil in intrapulmonaire distributie te kwantificeren tussen intratracheaal toegediende antibiotica met en zonder de toevoeging van surfactant. Dit hoofdstuk beschrijft een methode om tobramycine te markeren met het radioactieve technetium (^{99m}Tc-tobramycine) en tevens een methode om longverdeling van de radioactiviteit te kwantificeren met behulp van gecomputeriseerde autoradiogrammen. Met deze technieken kon, in *Klebsiella pneumoniae* geïnfecteerde rattenlongen, worden aangetoond dat de intrapulmonale distributie van intratracheaal toegediend ^{99m}Tc-tobramycine toenam door de toevoeging van surfactant aan de antibiotica oplossing.

Eerder werk van andere onderzoekers had reeds aangetoond dat surfactant superieur was ten opzichte van fysiologisch zout als vehikel in gezonde longen. De huidige studie bevestigt deze resultaten in geïnfecteerde longen en ondersteunt het concept van een verbeterde intrapulmonale distributie als bepalende factor voor het eerder aangetoonde effect (hoofdstuk 5). De methodiek bleek ongeschikt om de hoeveelheid ^{99m}Tc-tobramycine te bepalen in de geïnfecteerde gebieden, wat een belangrijke vraag blijft. Tevens zou het interessant zijn om een vergelijking in intrapulmonale distributie te maken tussen intratracheaal geinstilleerd surfactant-^{99m}Tc-tobramycine en verneveld ^{99m}Tc-tobramycine. Een verfijning of aanpassing van de huidige

methodiek moet zulke vergelijkingen mogelijk maken.

In **hoofdstuk 7** is de invloed van surfactant als vehikel op de longklaring van intratracheaal geïnstilleerd ^{99m}Tc-tobramycine onderzocht in gezonde ratten. In dit hoofdstuk wordt een methode beschreven voor het bestuderen van tobramycine pharmacokinetiek met behulp van ^{99m}Tc-tobramycine en een gamma camera. De studies toonden aan dat de longklaring van intratracheaal toegediend ^{99m}Tc-tobramycine significant toenam wanneer surfactant toegevoegd werd aan de oplossing in vergelijking met de longklaring van de ^{99m}Tc-tobramycine oplossing alleen.

Deze resultaten zijn tegengesteld aan de resultaten van twee eerdere studies door andere onderzoekers die een afname van de longklaring rapporteerden wanneer stoffen in combinatie met surfactant werden toegediend. Ook een binding tussen tobramycine en surfactant phospholipiden waarop gespeculeerd wordt in hoofdstuk 3 en 4, zou een vertraagde klaring tot gevolg kunnen hebben. De resultaten in hoofdstuk 7 tonen echter het tegenovergestelde: een toename van de longklaring van ^{99m}Tc-tobramycine wanneer geinstilleerd in combinatie met surfactant wat toegeschreven werd aan een toegenome distributie binnen de long zoals dit gevonden is in hoofdstuk 6. Het blijft onduidelijk waarom de resultaten verschillen met de eerdere studies.

De resultaten van de huidige studie in gezonde dieren suggereren hoge absorptie snelheden voor ^{99m}Tc-tobramycine vanuit de long. Onder de huidige experimentele condities moet men bedacht zijn voor hoge systemische antibiotica spiegels na intratracheaal geinstilleerde surfactant-antibiotica suspensies. Het moet verder onderzocht worden of de longklaring ook toegenomen is in dieren met een verstoord endogeen surfactant systeem, zoals dit bestaat tijdens longinfecties.

Concluderend zijn in dit proefschrift enkele aspecten belicht betreffende het gebruik van exogeen longsurfactant als vehikel voor intratracheaal toegediende antibiotica. Deze studies ondersteunen de algemene verwachting dat longsurfactant als vehikel voor antibiotica veelbelovend is. De klinische relevantie moet op dit moment nog vastgesteld worden; echter twee belangrijke overwegingen voor de kliniek zijn naar voren gekomen in dit proefschrift. Deze zijn het mogelijk optreden van interacties tussen surfactant en antibiotica die elkaars activiteit kunnen beinvloeden en het risico op hoge antibiotica serumspiegels met deze toedieningsmethode.

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Curriculum vitae

De schrijfster van dit proefschrift werd geboren op 28 mei 1968 te Den Haag. In 1986 behaalde zij het VWO-diploma aan de christelijke scholengemeenschap 'Overvoorde' te Den Haag. Aansluitend begon zij met de studie geneeskunde aan de 'Erasmus Universiteit' te Rotterdam, waar zij in 1991 het docteraalexamen behaalde. In 1991 trad zij als AIO in dienst van de Erasmus Universiteit te Rotterdam alwaar tot 1996 op de afdeling Experimentele Anaesthesie dit proefschrift werd bewerkt onder begeleiding van professor B. Lachmann. Bij de opzet en uitvoering van de studies is nauw samengewerkt met de afdeling Klinische Microbiologie aan de Erasmus Universiteit Rotterdam, in het bijzonder met dr. J. W. Mouton. In 1995 heeft de schrijfster vier maanden als gastonderzoeker gewerkt op de afdeling Klinische Fysiologie van het Universiteits Ziekenhuis te Malmö in Zweden, onder supervisie van professor P. Wollmer. De daar uitgevoerde studies zijn opgenomen in dit proefschrift. September 1996 heeft zij haar studie geneeskunde aan de 'Erasmus Universiteit' hervat. Op 18 september 1998 behaalde zij het artsexamen.

List of Publications

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