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

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1 The use of pulmonary surfactant has been proposed 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.

2 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 h for 8 consecutive days.

3 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.

Keywords: Pulmonary surfactant; antibiotics; tobramycin; drug carrier; bacterial pneumonia; tracheal instillation

Introduction

The efficacy of locally administered antibiotics for prevention or treatment of lower respiratory tract infection has been studied extensively (Ilowite *et al.*, 1987; Brown *et al.*, 1990; Mukhopadhyay *et al.*, 1994; Touw *et al.*, 1995). 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 (Ilowite *et al.*, 1987; Mukhopadhyay *et al.*, 1994). Moreover, with increased airway obstruction and lung damage the amount of aerosol deposited in peripheral regions of the lung decreases (Ilowite *et al.*, 1987; Mukhopadhyay *et al.*, 1994). 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 (Brain *et al.*, 1976; Kharasch *et al.*, 1991).

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 (Liu *et al.*, 1991). Pulmonary surfactant, a mixture of phospholipids and specific surfactant proteins, has the capacity to lower surface tension. Karasch and colleagues (1991) 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 (Lachmann & Gommers, 1993; Günther *et al.*, 1996). 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 (Eijking *et al.*, 1991; van Daal *et al.*, 1991; 1992; Gommers & Lachmann, 1993; Mikawa *et al.*, 1993; Harms & Herting, 1994). 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 (Brown *et al.*, 1990; Touw *et al.*, 1995). 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 of *Klebsiella pneumoniae*.

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 (±s.d.) 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.

An inoculum of 1×10^7 colony forming units (CFU) ml^{-1} of *Klebsiella pneumoniae* (ATCC 43816) was prepared from an overnight culture in Mueller Hinton Broth (MHB; Difco Laboratories, Detroit, Michigan, U.S.A.) as follows: 100 μl of the

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overnight culture was added to 10 ml MHB and incubated at 37°C for 1.5 h, then the culture was washed twice with saline. The inoculum was stored on ice until use. 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 anaesthetized by placing them in a container through which a mixture of $O_2:N_2O$ (1:2) and 3% ethrane was passed. Anaesthetized mice, held in the vertical position, were injected with 50 μl of the inoculum intranasally using a catheter (o.d. 0.62 mm) connected to a syringe (0.25 ml, Hamilton, Bonaduz, Switzerland). All animals recovered within 2 min 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 (Gommers *et al.*, 1993). 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^{-1}) was diluted with 0.2 M $NaHCO_3$ to a concentration of 12.5 mg ml^{-1}. For a surfactant-tobramycin preparation, the surfactant was suspended in a 12.5 mg ml^{-1} tobramycin solution to a concentration of 25 mg total lipids ml^{-1} and hand shaken. Surfactant only, was suspended in 0.2 M $NaHCO_3$ in a concentration of 25 mg ml^{-1}.

Animals, anaesthetized by inhalation (see above), 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, U.S.A.) was inserted 0.5 cm into 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 min after the procedure. Death from infection was registered every 12 h 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., U.S.A.). Significance was accepted at $P \leqslant 0.05$, two tailed.

Results

Figure 1 shows the survival curves of the intratracheally treated groups. At day 8 after infection, none 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.

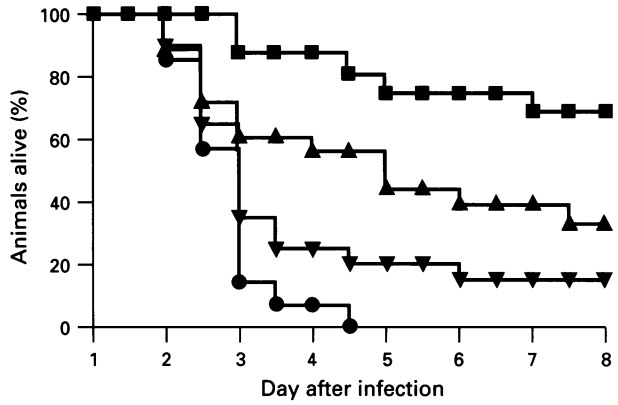

Figure 1 Survival rates of the treatment groups: surfactant-tobramycin mixture (■, $n=16$), tobramycin alone (▲, $n=18$), surfactant alone (▼, $n=20$), sham-treatment (●, $n=14$). Survival in the surfactant-tobramycin group was significantly increased compared to the tobramycin group, $P<0.05$ (Log-rank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

All animals in the non-treated group died within 6 days, mean ± s.d. 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 from 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 (van't Veen *et al.*, 1995; 1996). *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 (van't Veen *et al.*, 1995). 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 (van't Veen *et al.*, 1996). 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 proved to be more effective than tobramycin alone, it seems that the previously reported interactions between surfactant and tobramycin play little part 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^{-1} body weight (b.w.t.) in mice weighing 25 g. The reported surfactant doses for treatment of respiratory failure in subjects with pneumonia are generally several times higher, ranging from 50–300 mg kg^{-1} or more (Gortner *et al.*, 1990; Auten *et al.*, 1991; Eijking *et al.*, 1991; van Daal *et al.*, 1991; 1992; Gommers & Lachmann, 1993; Harms & Herting, 1994). However, these subjects are mechanically ventilated and receive the surfactant dose in a larger instillation volume, 2–4 ml kg^{-1}. 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^{-1}). Accordingly, the surfactant concentration was limited to 25 mg ml^{-1} as higher surfactant concentrations would become more viscous.

The tobramycin dose instilled in the present study corresponds to 10 mg kg^{-1} b.wt. in mice weighing 25 g. This high dose was chosen on the basis of the clinical daily intravenous dose used for treatment of serious infection. Few experimental results are available on intratracheally instilled aminoglycosides and most is focused on pharmacokinetics (Valcke & Pauwels, 1991; Demaeyer *et al.*, 1993; Omri *et al.*, 1994). After intratracheal instillation of 1.5 mg kg^{-1} tobramycin, in healthy rats, high levels of tobramycin were found in the bronchoalveolar lavage fluid up to 6 h after instillation (Valcke & Pauwels, 1991). Other studies showed that encapsulation of gentamicin (Demaeyer *et al.*, 1993) or tobramycin (Omri *et al.*, 1994) in liposomes can result in a significant increase of the antibiotic residence time in the lungs. However, in the study by Omri *et al.* (1994) 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 (Brown *et al.*, 1990). 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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