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Comparison of rSP-C surfactant with natural and synthetic surfactants after late treatment in a rat model of the acute respiratory distress syndrome

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1 In a previous paper we showed that an SP-C containing surfactant preparation has similar activity as bovine-derived surfactants in a rat lung lavage model of the adult respiratory distress syndrome. In this study surfactant was given ten minutes after the last lavage (early treatment). In the present investigation we were interested how different surfactant preparations behave when they are administered 1 h after the last lavage (late treatment).

2 Four protein containing surfactants (rSP-C surfactant, bLES, Infasurf and Survanta) were compared with three protein-free surfactants (ALEC, Exosurf and the phospholipid (PL) mixture of the rSP-C surfactant termed PL surfactant) with respect to their ability to improve gas exchange in this more stringent model when surfactant is given one hour after the last lavage. For better comparison of the surfactants the doses were related to phospholipids. The surfactants were given at doses of 25, 50 and 100 mg kg^{-1} body weight. The surfactants were compared to an untreated control group that was only ventilated for the whole experimental period.

3 Tracheotomized rats (8–12 per dose and surfactant) were pressure-controlled ventilated (Siemens Servo Ventilator 900C) with 100% oxygen at a respiratory rate of 30 breaths min^{-1}, inspiration expiration ratio of 1 : 2, peak inspiratory pressure of 28 cmH_2O at positive endexpiratory pressure (PEEP) of 8 cmH_2O. Animals were ventilated for one hour after the last lavage and thereafter the surfactants were intratracheally instilled. During the whole experimental period the ventilation was not changed.

4 Partial arterial oxygen pressures (PaO_2, mmHg) at 30 min and 120 min after treatment were used for statistical comparison. All protein containing surfactants caused a dose-dependent increase of the reduced PaO_2 values at 30 min after treatment. The protein-free surfactants showed only weak dose-dependent increase in PaO_2 values at this time. This difference between the protein-containing and the protein-free surfactants was even more pronounced when comparing the PaO_2 values at 120 min after treatment. Only rSP-C surfactant, bLES and Infasurf showed a dose-dependent increase in PaO_2 at this time.

5 With this animal model of late treatment it is possible even to differentiate between bovine derived surfactants. The differences between protein-containing and protein-free surfactants become even more pronounced. From the comparison of rSP-C surfactant with bovine-derived surfactants and the PL surfactant without rSP-C, it can be concluded that addition of rSP-C is sufficient to achieve the same activity as that of natural surfactants.

Keywords: ARDS-model; dose-response comparisons; rSP-C; synthetic surfactant; bovine-derived surfactant; gas exchange

Introduction

The acute respiratory distress syndrome (ARDS) is characterized by a severe deterioration in gas exchange (Bernard *et al*., 1994). This deterioration is due to atelectasis, lung oedema and protein leakage leading to formation of hyaline membranes (Seeger *et al*., 1993b). These pathophysiological and histopathological changes are also present in the rat lung lavage model (Berggren *et al*., 1986; Kawano *et al*., 1987; Häfner *et al*., 1994). In a recent investigation (Häfner *et al*., 1998) we have demonstrated that these histopathological changes show a time-dependent increase in severity in this animal model. At 10 min after lung lavage there was less formation of hyaline membranes detectable than at 60 min after the last lung lavage. This was also present when looking at margination of polymorphonuclear neutrophil leukocytes (PMNL) and intra-alveolar and interstitial oedema but the differences between both times were not as prominent.

In a previous study (Häfner *et al*., 1995) we have shown in this rat lung lavage model that a surfactant protein C (SP-C) containing surfactant had equal activity to bovine-derived surfactants. In this study the surfactants were administered ten minutes after the last lavage. This situation can be compared to early treatment of ARDS-patients. In the present investigation we analysed how surfactants behave if administered one hour after the last lavage. This can be described as late treatment and reflects the clinical situation of ARDS even better because of the increased formation of hyaline membranes and the more intense infiltration of PMNL (Häfner *et al*., 1998). ARDS patients have an accumulation of PMNL in the lungs (Repine, 1992). In addition, an increased formation of hyaline membranes is present which is accompanied by a massive influx of factors like albumin or fibrinogen (Seeger *et al*., 1993a) which can inhibit the function of endogenous surfactant. As Ito *et al*. (1996) have shown, timing of treatment

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leads to an altered activity of the surfactants. The surfactant they used exhibited a decrease in oxygenation during the experimental time when the treatment was delayed. We were interested whether this effect could also be seen in the rat lung lavage model. In this study we therefore, tested some of the surfactants that we had used previously (Survanta and Exosurf) and we compared them with another synthetic surfactant (ALEC) and two other protein containing surfactants (bLES and Infasurf). These last two surfactants were described to be superior to Survanta. Lewis *et al*. (1996) have shown in a sheep lung lavage model that bLES gave superior oxygenation to Survanta. Bloom *et al*. (1997) have shown in preterm babies with respiratory distress syndrome of the neonate that Infasurf has superior outcome to Survanta and Hudak *et al*. (1997) have shown superiority of Infasurf above Exosurf in preterm babies who suffered from respiratory distress syndrome.

Up to now four surfactant proteins (Possmayer, 1988) have been identified. Two high molecular weight proteins, SP-A and SP-D, that are both water soluble and two low molecular weight proteins, SP-B and SP-C, that are both highly hydrophobic. The bovine derived surfactants contain both surfactant proteins B and C. However, the content and the ratio of these proteins in each of these surfactants varies and is not specified (Bloom *et al*., 1997; Hudak *et al*., 1997). There are some publications in which the amount and ratio of these surfactant proteins were determined (Seeger *et al*., 1993a; Mizuno *et al*., 1995). Since we were able to show that a surfactant which contains only SP-C (Häfner *et al*., 1994; 1995) was equal in respect to efficacy as bovine-derived surfactant preparations that contain both hydrophobic surfactant proteins (SP-B and SP-C), we were further interested to investigate the role of SP-C in a standardized way in this more severe rat lung lavage model (after late treatment). Therefore, we have now tested two surfactant preparations which are based on the same phospholipid (PL) mixture with recombinant surfactant protein C (rSP-C surfactant) and without rSP-C (PL surfactant) in the rat lung lavage model.

For a better comparison of the data obtained with the protein-free to those of the protein containing surfactants, the doses used are given in relation to the amount of PL. All surfactant preparations were tested at doses of 25, 50 and 100 mg PL kg^{-1} body weight (b.w.). All surfactants were compared to an untreated control group with respect to improving gas exchange. The surfactant preparations were tested under conditions standardized with respect to the ventilator settings as well as the mode and volume of surfactant administration.

The specific aims were: (1) to estimate how the different surfactants behave in this new model of 'late treatment'; (2) to test if the addition of rSP-C is sufficient to achieve activity comparable to bovine-derived surfactants; and (3) to compare the activity of surfactant protein containing surfactants with surfactant protein-free preparations (synthetic surfactants) with respect to the maintenance of an improved gas exchange in this animal model of ARDS.

Methods

Preparation of the animals

This study protocol was reviewed and approved by the Laboratory Animal Care Committee at the district presidency of Freiburg, Germany. The study was performed with a total of 304 male Sprague Dawley rats (Harlan CBP, Zeist, the Netherlands) at a body weight (b.w.) of 230–270 g. The anaesthetic and surgical procedures were the same as previously described (Häfner *et al*., 1995). Briefly, after induction of anaesthesia with a halothane, nitrous oxide (N_2O), oxygen (O_2) mixture (1–2% halothane, 70% N_2O and 28–29% O_2) a catheter was placed into one carotid artery. These catheters contained heparin-treated, isotonic saline solution (from the stock solution (5000 iu heparin-Na ml^{-1}) 0.5 ml were diluted in 250 ml 0.9% NaCl solution). Before tracheotomy the animals received an intraperitoneal (i.p.) injection of pentobarbitone (stock solution: 60 mg ml^{-1}; 1 ml kg^{-1} b.w.). A tube was secured in the trachea of each animal. Before artificial ventilation was started, the animals were given an i.m. injection of pancuronium bromide as muscle-relaxant (1 ml kg^{-1} b.w., concentration of the solution 2 mg ml^{-1}). During the experiment additional pentobarbitone (0.25 ml kg^{-1} of the stock solution) was given i.p. every 90 min. Pancuronium bromide was additionally injected i.m. (1 ml kg^{-1} b.w.) if spontaneous breathing was observed. The tracheal tubes of six animals were connected to a distributor unit that in turn was connected to a Servo Ventilator. The animals were ventilated simultaneously at a respiratory rate of 30 breaths min^{-1}, a fraction of inspired oxygen (FiO_2) of 1.0, an inspiration expiration ratio of 1 : 2 and a peak inspiratory pressure (PIP) of 15 cmH_2O which included a positive end-expiratory pressure (PEEP) of 2 cmH_2O.

Protocols for the animal experiments

The variables reported are partial arterial oxygen pressure (PaO_2) and partial arterial carbon dioxide pressure ($PaCO_2$). At the start of the experiment blood was taken from the arterial catheter to determine pretreatment values of the animals under the described ventilatory settings. Only animals with PaO_2 values of more than 480 mmHg were included in the experiments. Before lavage the peak inspiration pressure (PIP) was raised to 28 cmH_2O and PEEP to 8 cmH_2O. The animals were then subjected to multiple lung lavage (6–8 times) with 1 ml 30 g^{-1} b.w. of isotonic saline solution warmed to body temperature. Only those animals which had PaO_2 values between 50 and 110 mmHg after lavage were included into the study. Blood gases were determined at 5, 30 and 60 min after the last lavage. One hour after the last lavage the surfactants were instilled intratracheally as described below. Untreated controls did receive sham treatment with air and were then ventilated only. Subsequently, 30, 60, 90, 120 and 150 min after surfactant instillation (equivalent to 90, 120, 150, 180 and 210 min after the last lavage) blood gases were determined. During the whole experimental period the PIP and PEEP was kept constant at 28 cmH_2O and 8 cmH_2O, respectively. For the experimental scheme see Figure 1.

Instruments

Blood gas analysis was performed with a blood gas analyser (Radiometer Copenhagen ABL 500, Radiometer Deutschland GmbH, Willich, Germany). Ventilation of the animals was performed with a Servo Ventilator (900C, SIEMENS-ELEMA, Solna, Sweden). For introduction of anaesthesia a halothane vaporiser (Draegerwerk GmbH, Lübeck, Germany) was used.

Surfactants

The following surfactants were used:

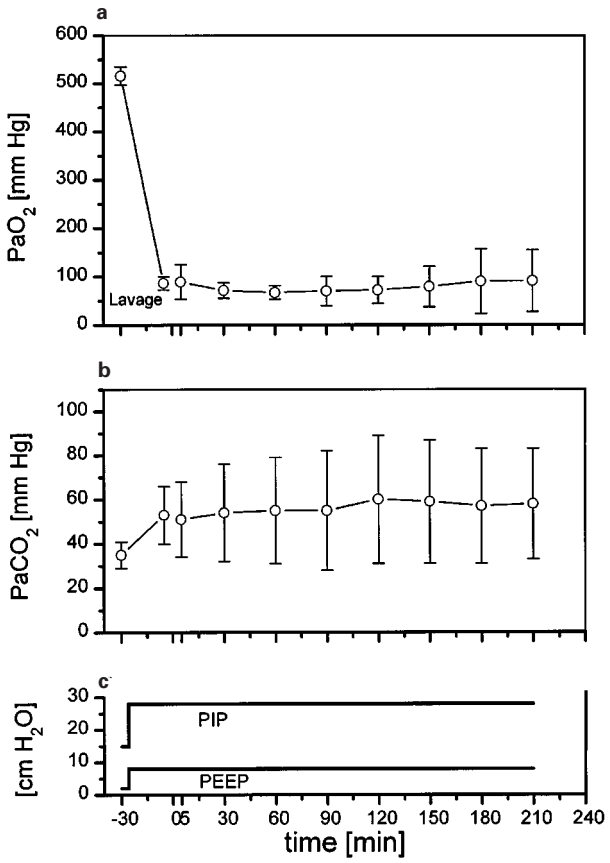

Figure 1 Mean values and s.d. (vertical lines) of PaO_2 (mmHg; a) and $PaCO_2$ (mmHg; b) after ventilation only of lung lavaged rats (control group, $n=36$). Lavage marks the time period where the repetitive lavage is performed. (c) The corresponding time course of the peak inspiratory pressure (PIP (cmH_2O)) and the positive end-expiratory pressure (PEEP (cmH_2O)) during the experiment.

Protein containing surfactants rSP-C surfactant (Byk Gulden, Konstanz, Germany) contains 2% recombinant surfactant protein C (rSP-C) associated with phospholipids (PL). rSP-C is an analogue of human SP-C where the two cysteines in positions 4 and 5 of the human SP-C sequence were exchanged by two phenylalanines and methionine in position 32 was exchanged by isoleucine. The PL consist of dipalmitoylphosphatidylcholine and palmitoyloleoylphosphatidylglycerol at a ratio of 70 : 30 plus 5% (w/w) palmitic acid related to PL. The rSP-C surfactant was supplied as dry powder and was resuspended with 0.9% NaCl solution to achieve a concentration of 25 mg PL ml^{-1}.

bLES (bovine lipid extract surfactant, BLES Biochemicals Inc., London ON, Canada; batch no. 960219) is a phospholipid-fraction of cow lungs obtained by lavage. Each vial contains a suspension that is ready to use. According to the product information of the manufacturer there are no specifications available regarding the different amounts of phospholipids and surfactant proteins B and C. Only the total phospholipid concentration is known (27 mg PL ml^{-1}).

Infasurf (a kind gift of G. Enhorning, Buffalo NY, USA batch C016 Lot B024) is a calf lung surfactant extract. Only the total phospholipid concentration is given (35 mg PL ml^{-1}). Regarding the amounts of different phospholipids and surfactant protein B and C, there are no further specifications available according to the product information of the manufacturer. Each vial contains a suspension that is ready for use.

Survanta (bovine derived surfactant, Abbott GmbH, Wiesbaden, Germany; batch no. 14-755-Z7) is a phospholipid-fraction obtained by mincing cow lungs consisting of phospholipids (72.8 – 211.2 mg). The specifications are as follows: 18.4 – 157.6 mg 1,2-dipalmitoyl-sn-glycero(3)phosphocholine (DPPC), 1.4 – 11.1 mg palmitic acid and 2.2 – 10.5 mg glyceroltripalmitate equivalent to 200 mg total PL 8 ml^{-1}. It also contains unspecified amounts of surfactant protein B and C. Each vial contains a suspension that is ready for use. The total phospholipid concentration is 25 mg PL ml^{-1}.

Synthetic surfactants ALEC (pumactant, Britannia Pharmaceuticals Ltd., Redhill, Surrey, United Kingdom; batch No. lot38) is a pure phospholipid containing surfactant that contains two phospholipids (dipalmitoylphosphatitylcholine (DPPC) and palmitoyloleoylphosphatidylglcerol (POPG)) in a ratio of 70 : 30 without any surfactant protein. Each vial contains a total of 100 mg of the above mentioned phospholipids. The content of each vial was resuspended with 0.9% NaCl solution to achieve a concentration of 25 mg PL ml^{-1}.

Exosurf (pure phospholipid containing surfactant, Wellcome GmbH, Burgwedel, Germany; batch no. R4797A) consisting of 108 mg Colfoscerilpalmitate (dipalmitoyl-sn-glycero(3)phosphocholine (DPPC)), 8 mg Tyloxapol and 12 mg Cetylacohol per vial plus 46.76 mg NaCl (total amount of lyophilized dry substance: 174.76 mg) without any surfactant protein. Resuspension was performed with the supplied water for injection. For the doses of 25, 50 and 100 mg PL kg^{-1} b.w. each vial was resuspended with 5.18 ml to achieve a concentration of 25 mg DPPC 1.2 ml^{-1}. For the dose of 67.5 mg PL kg^{-1} b.w. each vial was resuspended with 8 ml of the supplied water for injection to simulate the dosing regimen that is used to treat IRDS patients. The total phospholipid concentration in this case was 13.5 mg PL ml^{-1}.

Protein-free plain phospholipid (PL) surfactant (Batch No. EB 122, Byk Gulden, Konstanz, Germany) contained the same PL as the rSP-C surfactant but without rSP-C. It consists of dipalmitoylphosphatidylcholine and palmitoyloleoylphosphatidylglycerol at a ratio of 70 : 30 plus 5% (w/w) palmitic acid as related to phospholipids. The PL surfactant was processed similarly to the rSP-C surfactant. The content of each vial of EB 122 was resuspended with 0.9% NaCl solution to achieve a concentration of 25 mg PL ml^{-1}.

Dosage The different surfactant preparations were instilled intratracheally (i.t.) at doses of 25, 50 and 100 mg total PL kg^{-1} body weight (b.w.) in a volume of 1.2 ml per animal. To achieve the required concentrations of 6.25, 12.5 and 25 mg total PL 1.2 ml^{-1} the surfactant preparations were diluted with 0.9% saline solution (except for the dose of 67.5 mg of Exosurf the above mentioned concentration was used).

Mode of surfactant administration The surfactants were administered as one bolus as previously described (Häfner *et al*., 1995). In the control group the animals underwent multiple lavage and received sham treatment with air.

Statistics

The experiment was started with 8 – 12 rats for each dose level of all surfactant preparations and 12 animals in the untreated control group. Due to the time interval from the first to the last experiment the controls were repeated three times. The influence of the surfactant instillation on the parameter PaO_2

is shown by time-effect curves using means ± s.d. Dose-response curves were plotted using means ± s.d. of the PaO_2 values at 30 min ($PaO_2(30')$) as well as the values of PaO_2 at 120 min ($PaO_2(120')$) after treatment (equivalent to 90 and 180 min of experimental time, respectively). The results for $PaCO_2$ values were presented in tabular form. The primary parameters $PaO_2(30')$, and $PaO_2(120')$ were analysed for monotone dose-dependency by the non-parametric Jonckheere-Terpstra test (Hollander & Wolfe, 1973). Based on each dose level the effects of rSP-C surfactant on these variables were compared to the effects of the other surfactants by one-sided Wilcoxon tests. An adjustment for the multiple type I error was done according to Bonferroni-Holm (Holm, 1979).

Results

Feasibility of the comparison

The experimental procedures were well standardized in pilot experiments before the study. The whole study was performed within fourteen months without any dropouts. Due to the long interval from the first to the last experiment the control groups, with $n = 12$ animals, were repeated three times. After lavage only four of the 36 animals in the control group showed a spontaneous improvement of the PaO_2 values and the $PaCO_2$ values. This effect lead to a greater standard deviation toward the end of the experimental period (Figure 1).

Time course of the variables PaO_2 *and* $PaCO_2$

The time course of the PaO_2 values after administration of the different protein containing surfactants is given in Figure 2 and the respective data for the protein-free surfactants are shown in Figure 3. At the lowest dose there are great differences detectable between the protein containing surfactants. The rSP-C surfactant showed stable activity at all doses for the whole observation period. The time response curve was similar to bLES. Infasurf showed the weakest activity and the activity of Survanta was in-between bLES and Infasurf. With the high dose of 100 mg kg^{-1} Survanta developed an initial good response but the activity decreased towards the end of the observation period. With increasing doses the protein contain-

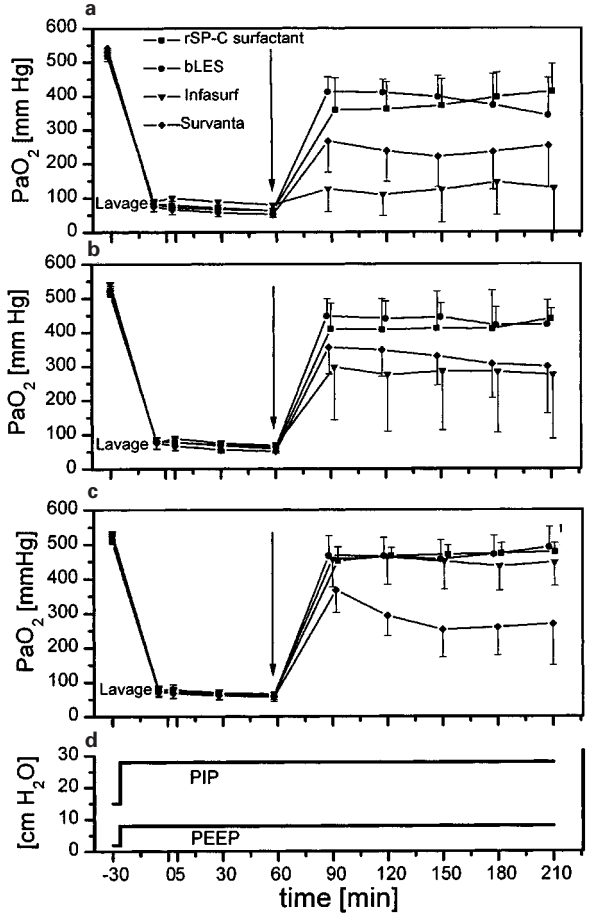

Figure 2 Time response curves of PaO_2 (mmHg) after administration of the protein containing surfactants: rSP-C surfactant, bLES, Infasurf and Survanta. All values are given as means and vertical lines show s.d. (a) The values after administration of 25 mg, (b) after administration of 50 mg and (c) after administration of 100 mg surfactant kg^{-1} body weight; $n = 11-12$ for each dose. Lavage marks the time period where the repetitive lavage was performed, while the arrow marks the time when surfactant was administered. (d) The corresponding time course of the peak inspiratory pressure (PIP (cmH_2O)) and the positive end-expiratory pressure (PEEP (cmH_2O)) during the experiment.

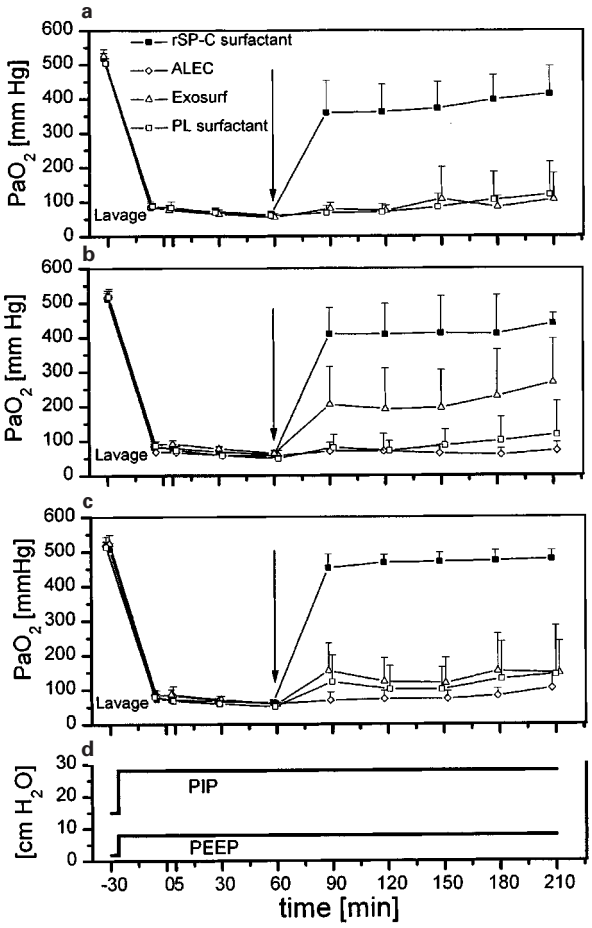

Figure 3 Time response curves of Pao2 (mmHg) after administration of the protein-free surfactants in comparison to rSP-C surfactant: ALEC, Exosurf and PL surfactant without rSP-C. All values are given as means and vertical lines show s.d. (a) The values after administration of 25 mg, (b) after administration of 50 mg and (c) after administration of 100 mg surfactant kg^{-1} body weight; $n = 8-12$ for each dose. Lavage marks the time period where the repetitive lavage was performed, while the arrow marks the time when surfactant was administered. (d) The corresponding time course of the peak inspiratory pressure (PIP (cmH_2O)) and the positive end-expiratory pressure (PEEP (cmH_2O)) during the experiment.

ing surfactants showed similar activity and, except Survanta, all protein-containing surfactants showed stable PaO_2 values during the entire observation period.

The protein-free surfactants showed no activity at the lowest dose (Figure 3). At the dose of 50 mg kg^{-1} the PaO_2 values after administration of Exosurf showed better activity but on a lower level than rSP-C surfactant. At the dose of 67.5 mg Exosurf (data not shown) showed nearly the same activity as at the dose level of 50 mg kg^{-1}. At the highest dose the activity of Exosurf decreased. ALEC did not show any improvement at the doses of 50 and 100 mg kg^{-1} and, therefore, the lowest dose was not tested. The PL surfactant showed a little initial response at the highest dose. The values remained far below the values of rSP-C surfactant and were in the range of untreated controls (see Figure 1).

$PaCO_2$ values showed an almost inverse pattern of response to the PaO_2 values. After lavage the values increased as compared to the values before lavage (Tables 1 and 2). Even 60 min after the last lavage the $PaCO_2$ values remained increased compared to the values before. In some cases there was even a further increase detectable between the value after lavage and 60 min later. After administration of all surfactants the values decreased when compared to the 60 min value after the last lavage (before treatment). The $PaCO_2$ values of the untreated controls remained unchanged during the rest of the observation period. However, there were no dose-dependent effects detectable. Due to the inhomogeneity of the values before lavage and the fact that none of the surfactants tested showed a dose-dependent effect, no statistical comparisons were performed.

Statistical evaluation of the dose-response effects and statistical comparisons of rSP-C surfactant with the other surfactant preparations

Based on the PaO_2 values at 30 as well as 120 min after treatment dose response curves were plotted (Figures 4 and 5), and dose-response calculations were performed. At 30 min after administration only the protein containing surfactants showed increasing dose-dependent improvements of the PaO_2 values (Figure 4). In contrast, among the synthetic surfactants only Exosurf and the PL surfactant showed some dose-dependent improvement (Figure 4), while ALEC lead to no significant dose-dependent effect. At a dose of 25 mg PL kg^{-1} the PaO_2 values of rSP-C surfactant were significantly higher than those of Infasurf ($P<0.01$). There was no statistically significant difference between rSP-C surfactant and the other protein containing surfactants. Between rSP-C surfactant and the PL surfactant as well as Exosurf there was a statistically significant differences ($P<0.001$) with respect to the PaO_2 values at 30 min after treatment. At a dose of 50 mg kg^{-1} there were no statistically significant differences between rSP-C

Table 1 Comparison of $PaCO_2$ (mmHg) values after administration of the different doses (related to phospholipids per kg b.w.) of the protein containing surfactants in comparison to untreated controls

		Before lavage	*After lavage*	*60 min*	*90 min*	*180 min*	*210 min*
					after lavage		
Controls	–	35±6	53±13	55±24	55±27	57±26	58±25
	Dose (mg kg^{-1})				*30 min*	*120 min after treatment*	*150 min*
rSP-C surfactant	25	38±4	59±6	60±14	53±9	56±12	54±13
rSP-C surfactant	50	44±6	64±8	73±22	60±11	62±13	58±16
rSP-C surfactant	100	37±5	61±7	62±16	52±8	57±9	56±9
bLES	25	37±5	64±9	70±18	61±9	63±14	65±13
bLES	50	39±5	58±10	58±18	55±10	55±9	53±9
bLES	100	39±6	57±10	58±20	58±10	50±21	54±21
Infasurf	25	38±7	59±6	50±8	44±8	44±15	45±19
Infasurf	50	43±8	66±11	67±16	60±15	47±16	44±15
Infasurf	100	43±8	68±14	71±25	63±13	54±17	60±14
Survanta	25	41±8	67±10	83±26	63±13	64±21	64±27
Survanta	50	43±4	69±7	83±18	61±11	65±24	68±26
Survanta	100	40±4	66±12	80±28	62±14	68±20	69±24

The values are given as mean±s.d.

Table 2 Comparison of $PaCO_2$ (mmHg) values after administration of the different doses (related to phospholipids per kg b.w.) of the protein-free surfactants in comparison to untreated controls

		Before lavage	*After lavage*	*60 min*	*90 min*	*180 min*	*210 min*
					after lavage		
Controls	–	35±6	53±13	55±24	55±27	57±26	58±25
	Dose (mg kg^{-1})				*30 min*	*120 min after treatment*	*150 min*
ALEC	50	33±6	57±13	61±26	60±25	54±18	48±21
ALEC	100	34±6	54±11	59±24	54±18	50±21	42±28
Exosurf	25	37±6	56±11	67±21	60±16	60±21	59±25
Exosurf	50	35±8	54±11	55±19	49±14	45±19	43±20
Exosurf	100	36±6	56±8	55±12	55±11	52±11	52±9
PL surfactant	25	38±6	61±10	59±15	57±14	46±21	45±23
PL surfactant	50	37±6	62±13	72±22	66±20	58±27	50±24
PL surfactant	100	37±5	66±11	75±24	63±12	61±27	58±22

The values are given as mean±s.d.

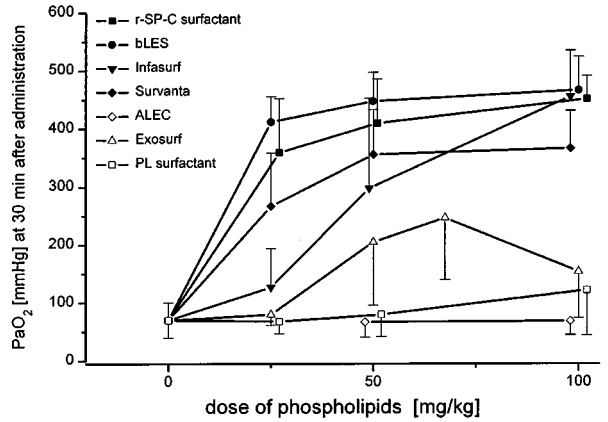

Figure 4 Dose-response curves based on the values for the PaO_2 at 30 min after administration of the different doses of the surfactant preparations: rSP-C surfactant, bLES, Infasurf, Survanta, ALEC, Exosurf and PL surfactant. The values represent means and vertical lines show s.d. Dose response calculations based on all three doses resulted in a monotone increasing dose-dependence for rSP-C surfactant: $P<0.01$; bLES: $P<0.05$; Infasurf: $P<0.01$; Survanta: $P<0.01$; Exosurf (using all four doses): $P<0.01$ and the PL surfactant ($P<0.05$) but not for ALEC. Comparisons of all three doses of rSP-C surfactant with the synthetic surfactant Exosurf and the PL surfactant resulted in significance with $P\leqslant 0.001$ for each dose.

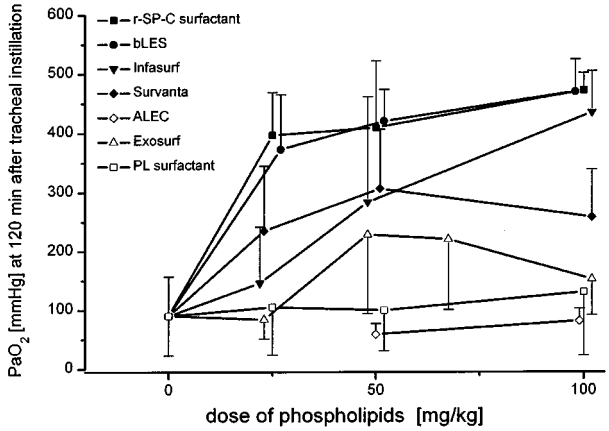

Figure 5 Dose-response curves based on the values for the PaO_2 at 120 min after administration of the different doses of the surfactant preparations: rSP-C surfactant, bLES, Infasurf, Survanta, ALEC, Exosurf and PL surfactant. The values represent means and vertical lines show s.d. Dose-response calculations based on all three doses resulted in a monotone increasing dose-dependence only for rSP-C surfactant: $P<0.01$; bLES: $P<0.05$ and Infasurf: $P<0.01$ but not for Survanta and the three synthetic surfactants ALEC, Exosurf and the PL surfactant. Comparisons of all three doses of rSP-C surfactant with all three synthetic surfactants resulted in significance with $P\leqslant 0.01$ for each dose.

surfactant and the other protein containing surfactants. However, again there were great differences between rSP-C surfactant and the synthetic surfactants. The rSP-C surfactant showed superior activity to ALEC, the PL surfactant and Exosurf with $P<0.001$. At a dose of 100 mg kg^{-1} the rSP-C surfactant was statistically significantly better than Survanta with $P<0.01$. There were no statistically significant differences between rSP-C surfactant and bLES as well as Infasurf. Whereas there were great differences between rSP-C surfactant and the synthetic surfactants. This difference was statistically significant with $P<0.001$ compared to ALEC, the PL surfactant and Exosurf.

At 120 min after administration only rSP-C surfactant, bLES and Infasurf showed a monotone increasing dose-dependent improvement of the PaO_2 values (Figure 5). Due to the decreasing activity of Survanta after the highest dose, the test for monotone increasing dose-dependence resulted in no statistical significance at this time (Figure 5). Furthermore, calculations for monotone increasing dose-dependence for the three synthetic surfactants resulted in no statistical significance at this time. Exosurf and Survanta showed a bell-shaped dose-response curve (Figure 5). At a dose of 25 mg kg^{-1} the rSP-C surfactant had statistically significant higher PaO_2 values than Infasurf ($P<0.001$) and Survanta ($P<0.01$) and there was no difference from bLES at 120 min after administration. Again the rSP-C surfactant was statistically significantly ($P<0.001$) better than Exosurf and the PL surfactant. At a dose of 50 mg kg^{-1} the rSP-C surfactant showed statistically significantly higher PaO_2 values than Survanta ($P<0.05$), whereas there were no differences from the other protein containing surfactants. At this dose level rSP-C surfactant was statistically significantly ($P<0.01$) better than ALEC, Exosurf and the PL surfactant. At a dose of 100 mg kg^{-1} there were no differences detectable between rSP-C surfactant and bLES as well as Infasurf. At this dose Survanta had statistically significantly lower PaO_2 values than rSP-C surfactant ($P<0.001$). The rSP-C surfactant showed superior activity to the synthetic surfactants. At 120 min after treatment with 100 mg kg^{-1} the rSP-C surfactant developed statistically significantly ($P<0.001$) higher PaO_2 values than ALEC, Exosurf and the PL surfactant.

This dose-response comparison shows that rSP-C surfactant and bLES displayed essentially identical activity after administration of the different doses, whereas there were great differences from the other protein containing surfactants. The protein-free surfactants showed no improvements of the PaO_2 values comparable to the protein containing surfactants. Exosurf showed a bell-shaped dose-response curve with a maximum response between the dose of 50 and 67.5 mg kg^{-1}. But even after this dose the activity was significantly lower than that after treatment with any of the protein containing surfactants.

Discussion

With this late treatment it is possible to characterize the activity of different surfactant preparations under even more severe conditions. In a separate investigation (Häfner *et al*., 1998) we showed that a time-dependent increase in histopathological changes is present in this model. This was shown with respect to formation of hyaline membranes, intra-alveolar and interstitial oedema and margination of PMNL when comparing histopathology at 10 and at 60 min after the last lung lavage. Further evidence that the late treatment strategy described here reflects an even more severe situation can be derived from the comparison with the results obtained with the early treatment (Häfner *et al*., 1995). Survanta, SP-C surfactant, Exosurf and PL surfactant were tested in the early treatment model (Häfner *et al*., 1995). When comparing at the time of 120 min after treatment all surfactants showed nearly comparable oxygenation after early treatment. Only the PL surfactant lead to less oxygenation compared to Survanta, SP-C surfactant and Exosurf. In the new 'late treatment' strategy the differences between the SP-C surfactant and the PL surfactant were even more pronounced (Figure 5). Even Survanta and Exosurf showed less oxygenation after late treatment than after early treatment. However, only the rSP-C

surfactant developed nearly similar oxygenation when comparing both treatment strategies. Therefore, we conclude that the decreased oxygenation of some surfactants after late treatment compared to early treatment gives further evidence that late treatment is more stringent than early treatment.

Dose-response calculations were used to study the effectiveness of the different surfactants. They were based on two different time points: shortly after treatment (at 30 min) and two hours after treatment to investigate the duration of activity of the surfactants. This could also be shown by the time-effect curves. The duration of activity was different for Survanta and the PL surfactant, at the highest dose used both show an initial response with a decrease in activity toward the end of the experiment. Therefore, we believe that the activity of a surfactant at two hours after treatment gives even more information on a surfactant than the activity shortly after treatment. The dose-response calculations also show whether a surfactant leads to a bell-shaped dose-response curve, an effect which leads to the assumption that this preparation may have negative effects if this preparation is used above a certain dose. The surfactants which showed this behaviour are Survanta and Exosurf. Both surfactants showed, in clinical trials, no further improvement above a certain dose (Weg *et al*., 1994; Gregory *et al*., 1997). Therefore, we believe that these doses response calculations may give additional information for planning of clinical studies.

With the comparisons based on each dose level between the different surfactants it should be demonstrated that some surfactants may have no difference in activity when the dose is high enough, but the differences may be pronounced at lower dose levels. This also gives information on the safety margins of surfactants. These comparisons show the critical role of surfactant proteins and especially of SP-C. The comparisons on the different doses used show that rSP-C surfactant is as effective as bLES, although bLES contains SP-B and SP-C. Both surfactants were found to be superior to the other bovine-derived surfactants that also contain SP-B and SP-C. All protein-free surfactants were inferior to the protein containing surfactants at all dose levels in this model of late treatment. The activity of Exosurf is clearly below that of the protein containing surfactants, and particularly the activity of the PL surfactant is clearly below the activity of the rSP-C surfactant. ALEC did not show any activity in this more stringent model. This observation is in good accord with a case report described by Haslam *et al*. (1994), where treatment with ALEC did not lead to clinical improvements. The general failure of the synthetic, protein-free surfactants to restore PaO_2 values in this animal model may be explained by the observations of other authors who have found that surfactant can be inhibited by certain proteins (Hallman *et al*., 1991; Kobayashi *et al*., 1991). The inhibitory effects of plasma proteins can only be neutralized by protein containing surfactants, while high doses of the synthetic surfactants had no additional beneficial effects. It is also likely that only protein containing surfactants are able to influence transcapillary-interstitial-epithelial lung leakage (Lewis & Jobe, 1993), thereby inhibiting the influx of plasma proteins (Lachmann *et al*., 1994) which is essential to prevent the formation of hyaline membranes (Enhörning, 1989; Nosaka *et al*., 1990).

With respect to the synthetic surfactants, it was clearly demonstrated that without any surfactant protein no full restorage of the decreased PaO_2 values can be achieved in this more severe animal model. Furthermore, a direct comparison of the rSP-C surfactant and bLES with the PL surfactant, that contains no rSP-C, led to the conclusion that rSP-C is essential to achieve an active surfactant. The crucial role of SP-C also derives from the efficacy of Survanta that contains only small amounts of SP-C (Mizuno *et al*., 1995) and the comparison with Infasurf, which is reported to contain more surfactant proteins. From the data presented we also conclude that for treating ARDS patients with surfactants the addition of at least one surfactant protein leads to superior activity as compared to protein-free surfactants.

The present results for Exosurf are in good accordance with results published by Weg *et al*. (1994). After administration of Exosurf to ARDS patients these authors were also unable to detect dose-dependent activity. However, this surfactant was used in a large clinical trial with more than 700 patients (Anzueto *et al*., 1996). In this trial Exosurf failed to show efficacy, and it was questioned whether treatment with surfactant could have any beneficial effects in patients who suffer from ARDS (Matthay, 1996). Nevertheless, it should be noted that the negative outcome of this trial can be in part due to the nebulization technique which was used for administration of the substances to the patients (MacIntyre *et al*., 1994). This has been shown to result in less than 5% deposition of Exosurf in the lungs and to result in deposition predominantly in the ventilator circuitry. Despite the negative trial with Exosurf (Anzueto *et al*., 1996), we believe that surfactant treatment may be an effective treatment for patients with ARDS. Supporting evidence for this hypothesis derives from the trials with the bovine-derived surfactants Alveofact (Walmrath *et al*., 1996), bLES (Lewis *et al*., 1997) and Survanta (Gregory *et al*., 1997). All of them contain surfactant protein B and C and were shown to be efficacious in ARDS patients. This is further evidence that an active surfactant preparation should contain at least one surfactant protein. From the present investigation comparing the effects of rSP-C surfactant with protein containing surfactants that were shown to be effective in clinical trials of ARDS, it can be assumed that the rSP-C surfactant should lead to a better outcome in patients with ARDS than that seen after Exosurf.

In conclusion, our results suggest that protein-free surfactants based only on phospholipids (such as ALEC, PL surfactant or even Exosurf) do not reach the biological activity of surfactant preparations that contain surfactant proteins. However, as can be concluded from the present investigation, the activity of such phospholipid-based surfactant preparations can be enhanced by addition of recombinant surfactant protein C (rSP-C).

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