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In vitro modulation of induced neutrophil activation by different surfactant preparations

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In vitro *modulation of induced neutrophil activation by different surfactant preparations. F.K. Tegtmeyer, L. Gortner, A. Ludwig, E. Brandt. ©ERS Journals Ltd 1996.*
ABSTRACT: Endotracheal surfactant administration has gained an important role in the treatment of respiratory failure. Polymorphonuclear neutrophil granulocyte (PMN) activation mediated by chemoattractants, such as interleukin-8 (IL-8), neutrophil-activating peptide-2 (NAP-2) and formylated bacterial oligopeptides, has been found to be involved in the pathophysiology of acute respiratory failure.

We investigated potential modulating effects of commercial surfactant preparations (Exosurf®, Alveofact®, Curosurf® and Survanta®) on spontaneous and chemoattractant-induced PMN function. Isolated cytochalasin B (CytB)-treated PMNs from healthy adults were incubated with increasing concentrations of surfactant. The response of the cells was measured in terms of elastase release from the lysosomes within 30 min.

The PMNs showed no direct activation by any of the surfactants tested. However, when cells were stimulated with suboptimal dosages of chemokines, such as IL-8 (2 nM) or NAP-2 (100 nM), or formyl-methionyl-leucyl-phenylalanine (fMLP) (50 nM), and co-incubated with increasing concentrations of surfactant (0.05–8 mg·mL^{-1}) the release of elastase was markedly modulated depending on the surfactant preparation used. Whilst Exosurf® and Alveofact® showed only modest effects on the elastase release induced by all three mediators, Curosurf® and Survanta® markedly inhibited the cellular response in a dose-dependent manner. At concentrations above 1 mg·mL^{-1}, Curosurf® and Survanta® decreased the IL-8-, NAP-2- and fMLP-induced elastase release by 83, 67 and 90%, and by 82, 75 and 80%, respectively.

In conclusion, exogenous surfactant may modulate the inflammatory response of the airways by affecting the chemoattractant-induced polymorphonuclear neutrophil activation. Surfactant preparations with inhibiting properties on neutrophil activation may participate in the prevention of neutrophil-induced lung damage.
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Lung surfactant, a complex mixture of various phospholipids, neutral lipids and apoproteins is essential for lung function [1, 2]. Surfactant replacement therapy has proved to be beneficial for the treatment of the neonatal respiratory distress syndrome [2], and is also considered as a therapeutic option for term infants and adults with acute respiratory failure [3]. Previous studies have shown that the different surfactant preparations in clinical use vary greatly with regard to their *in vitro* biophysical properties and *in vivo* physiological effects [4, 5]. Under pathological conditions leading to respiratory failure, such as immaturity, haemodynamic impairment and inflammation, the close proximity of the vascular bed and alveolar space may, however, favour an undesirable bidirectional protein leak and cell exchange. Plasma proteins and blood cells inactivate surfactant [6–8], whilst surfactant preparations and their isolated constituents exert various effects on cell systems involved in the local and systemic immune response [9–12].

Polymorphonuclear neutrophils (PMNs) are known to be the primary effector cells of the inflammatory process and the most potent inducers of lung damage and surfactant inactivation by generation of oxygen free radicals and release of proteases, such as elastase [13, 14]. However, data on surfactant-related effects on neutrophil activation are still limited [15, 16]. Chemokines, such as interleukin-8 (IL-8) and neutrophil-activating peptide-2 (NAP-2) potently stimulate various biological functions of neutrophils. Both chemokines have been shown to induce degranulation of lysosomal enzymes [17, 18], directed chemotactic migration [17, 19, 20], and oxygen radical formation [17, 21]. High levels of IL-8 and NAP-2 are found in the bronchoalveolar lavage (BAL) fluid of patients with adult respiratory distress syndrome (ARDS) [22, 23], where they may be responsible for activating destructive neutrophil effector functions [24, 25]. Moreover, surfactant preparations themselves may act either as a source for potent phospholipid-derived inflammatory mediators, such as platelet-activating factor (PAF) [26], phospholipid metabolites [15], and proteins that may affect immune cell function [27]. Thus, differences in the composition of surfactant, *i.e.* synthetic or natural preparations, could be crucial for regulation of the acute inflammatory response of the lung

with respect to the mediator-induced neutrophil activation.

The aim of this study was to investigate the potential effects of various surfactant preparations on neutrophil activation in response to IL-8, NAP-2 and formyl-methionyl-leucyl-phenylalanine (fMLP) by the assessment of extracellular elastase release from activated neutrophils.

Materials and methods

Surfactant preparations and chemokines

Four surfactant preparations of different origin designed for clinical use were investigated. Exosurf® (Wellcome, USA) was the only artificial preparation tested, whilst the other surfactants were natural products. According to the manufacturers, Alveofact® (Thomae, Germany) was prepared from bovine BAL fluid; Curosurf® (Chiesi, Italy) and Survanta® (Abbott, USA) were obtained from minced porcine or bovine lung tissue.

Human recombinant IL-8 (72 residue isoform) was purchased from Pepro Tech Inc. (Rocky Hill, NJ, USA). Human natural NAP-2 was prepared at the Department of Immunology and Cell Biology, Forschungsinstitut Borstel (Borstel, Germany) as described previously [28].

PMN purification

PMNs from citrated blood of healthy single donors were isolated by gradient centrifugation on Ficoll-Hypaque (Pharmacia LKB, Freiburg, Germany) and purified by 1% (w/v) polyvinyl alcohol (Merck, Darmstadt, Germany) sedimentation followed by hypotonic lysis of red blood cells as described previously [29]. The PMNs were finally washed with PBS, pH 7.4 and resuspended in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumen (BSA) (PBS/BSA) without Ca^{2+}/Mg^{2+}. The remaining cells were 95–98% PMNs as determined by fluorescence-activated cell sorter (FACS) using forward-side scatter analysis, and were more than 99% viable as determined by trypan blue exclusion. For all these studies, cells were prepared under sterile conditions using sterile pharmaceutical reagents.

Assay for measurement of PMN activation

Activation of PMNs was measured in terms of lysosomal elastase released from cytochalasin B (CytB)-treated cells according to established methods [29]. Purified PMNs suspended at 1×10^7 cells·mL^{-1} in Ca^{2+}/Mg^{2+}-free PBS/BSA were warmed for 30 min at 37°C under agitation and then incubated with CytB (5 μg·mL^{-1}) for 10 min. Thereafter, cells were supplemented with $CaCl_2$ and $MgCl_2$ to final concentrations of 0.9 and 0.5 mM, respectively, and 100 μL aliquots of the suspension were added to 100 μl of appropriately diluted test samples (stimuli, surfactants or both in combination) and controls in PBS/BSA, containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. After incubation for 30 min under agitation at 37°C, the cellular response was complete and the supernatants were recovered by centrifugation. Elastase enzymatic activity in serially diluted aliquots (50 μl volumes) of supernatant was assayed in duplicate after the addition of 50 μl of substrate solution containing 0.44 mM N-t-BOC-Ala-Pro-NVA p-chlorothiobenzyl ester, 0.75 mM Ellman's reagent (both from Sigma, Deisenhofen, Germany), 29.4% dimethylsulphoxide, 0.7 M NaCl in 0.14 M N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer, pH 7.5. Hydrolysis of the substrate was monitored photometrically at 405 nm, and results were expressed as the percentage of total elastase activity in detergent-treated PMN lysates, prepared with 0.1% hexadecyltrimethylammonium bromide.

Firstly, we evaluated the direct effect of surfactant on cell activation. For this, different surfactant preparations were assayed at final phospholipid concentrations of 0.05–8 mg·mL^{-1}. Surfactant preparations added to the buffer solution were prepared according to the instructions for clinical use. We then examined the potential influence of surfactants on the IL-8-, NAP-2- and fMLP-induced elastase release from PMNs. In these experiments, PMNs were stimulated with different concentrations of IL-8, NAP-2 and in presence and absence of surfactants. The viability of the cells was not altered during this treatment, as determined by trypan blue exclusion.

Appropiate controls were performed to exclude interference of surfactants with the assay system. The problem with turbid solutions of surfactant at high concentrations (above 3.3 mg·mL^{-1}), leading to an unspecific increase in absorbance at 405 nm, could be circumvented by diluting the recovered supernatants down to a maximum concentration of 2.7 mg·mL^{-1} of surfactant. None of the surfactant preparations was found to interfere with the enzyme activity of the marker elastase. This was seen upon co-incubation of surfactants with aliquots of detergent-lysed PMNs, where elastase activity (as estimated by the increase of absorbance at 405 nm) remained unchanged with surfactant concentrations up to 10 mg·mL^{-1}. In these assays, increases in absorbance due to turbidity of high surfactant concentrations was subtracted. Moreover, surfactants (up to 10 mg·mL^{-1}) did not mimic elastase enzymatic activity, as examined by incubation of surfactants with elastase substrate alone.

Statistics

Data are presented as mean±SD from three independent experiments, each carried out in duplicate. Statistical significance was analysed by the two-tailed t-test.

Results

Direct effects of surfactant on PMN activation

None of the four surfactant preparations under investigation induced a significant release of elastase in the PMN degranulation assay. As shown in figure 1, the cellular responses to increasing concentrations of surfactant did not exceed those of corresponding controls without surfactants. Slight stimulation of the cells was observed

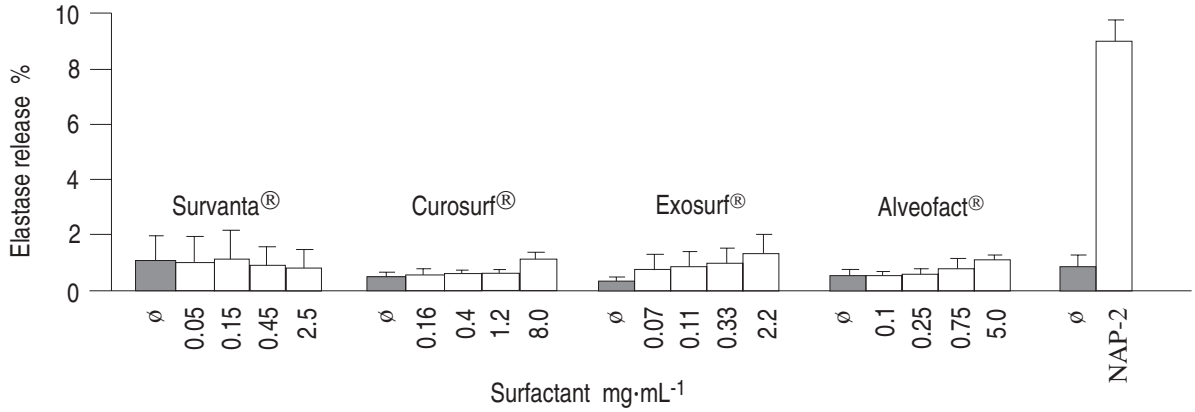

Fig. 1. – Elastase release from isolated CytB-treated PMNs exposed to various surfactant preparations at different concentrations. Elastase release was calculated as the percentage of total release from detergent-lysed cells. As a control, cells were challenged with 100 nM NAP-2. For comparison, the release of unstimulated cells (Ø) is indicated by shaded bars. Data are presented as mean±SD from three independent experiments. CytB: cytochlasin B; PMNs: polymorphonuclear neutrophils; NAP-2: neutrophil-activating peptide-2.

with the highest concentrations of Exosurf® (2.2 mg·mL^{-1}). Curosurf® (8 mg·mL^{-1}), and Alveofact® (5 mg·mL^{-1}), yielding up to 1.5% of the total elastase content in detergent-lysed control PMN. However, this was negligible in comparison to a positive control using 100 nM NAP-2, where about 8.5±0.7% of elastase was liberated. IL-8 and fMLP induced an even stronger release (fig. 2).

Effects of surfactant on mediator-induced PMN activation

Quite different results were obtained when surfactants were incubated with PMNs that were simultaneously stimulated with mediators NAP-2, IL-8 or fMLP. For these experiments, suboptimal (nonsaturating) dosages of these stimuli (100 nM NAP-2, 2 nM IL-8, 50 nM fMLP) were chosen (fig. 2) making it possible to analyse positive and negative modulatory effects of surfactants on induced neutrophil activation. Upon stimulation with NAP-2, IL-8 and fMLP elastase release was modified in the presence of surfactant, the effect being dependent on the preparation tested. By contrast, the type of stimulus did not influence the general characteristics of modulation induced by a given preparation of surfactant. Thus, as shown in figure 3, co-incubation with Exosurf®, the only artificial preparation, slightly enhanced NAP-2-, and IL-8- and induced elastase release in a concentration-dependent manner, amounting to about 120% at the highest dosage tested (2.2 mg·mL^{-1}). Two of the natural surfactants, Curosurf® and Survanta®, exhibited the opposite effect, consisting in a drastic concentration-dependent inhibition of the cellular response. Curosurf® was optimally effective at concentrations about 1 mg·mL^{-1}, reaching approximately 60, 80 and 90% of inhibition with NAP-2, IL-8 and fMLP, respectively. Similar rates of inhibition were obtained with Survanta® at 2.5 mg·mL^{-1}. Because this was the highest concentration tested, it is not known whether even stronger effects would occur at more elevated dosages.

Results with Alveofact® were less consistent. Whilst low concentrations (up to 0.33 mg·mL^{-1}) co-incubated with NAP-2-stimulated cells led to an increase in elastase release, higher concentrations of Alveofact® reversed this effect. This was observed with 11 different batches of the surfactant. With IL-8 and fMLP stimulation, no modulating effect of Alveofact® was seen at lower concentrations, but slight dose-dependent inhibition occurred with 0.75 mg·mL^{-1} and higher amounts of the surfactant. However, the reductions in elastase release rates were considerably weaker than those observed with Curosurf® and Survanta®.

To examine the possibilitv that reduction of elastase release was due to absorption or destruction of stimuli by surfactants the amount of NAP-2 was measured prior to and following incubation (30 min) with Survanta®. Exosurf®, and Alveofact® in an enzyme-linked immunosorbent assay (ELISA)-system specific for NAP-2. At none of the surfactant concentrations tested (up to 2.5, 2.2 and 5.0 mg·mL^{-1}, respectively), was there any measurable reduction in NAP-2 concentration.

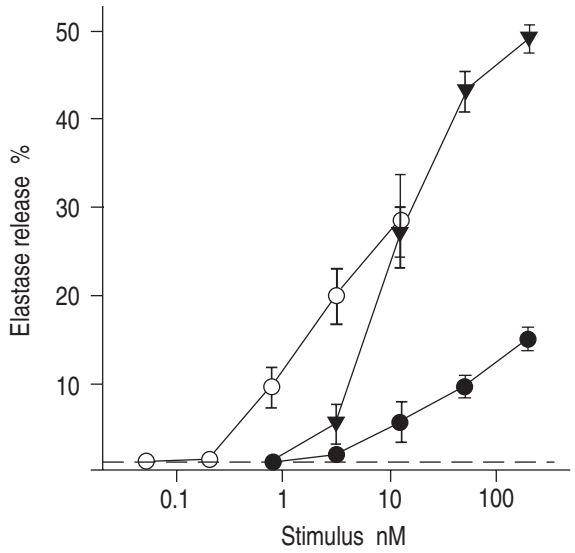

Fig. 2. – Elastase release from isolated CytB-treated PMNs induced bv increasing concentrations of NAP-2, IL-8, and fMLP. Elastase release was calculated as the percentage of total release from detergent-treated cells. The release from unstimulated cells is given as a broken line. Data are presented as mean±SD from three independent experiments. —●—: NAP-2; —○—: IL-8; —▼—: fMLP. IL-8: interleukin-8; fMLP: formyl-methionyl-leucyl-phenylalanine. For further abbreviations see legend to figure 1.

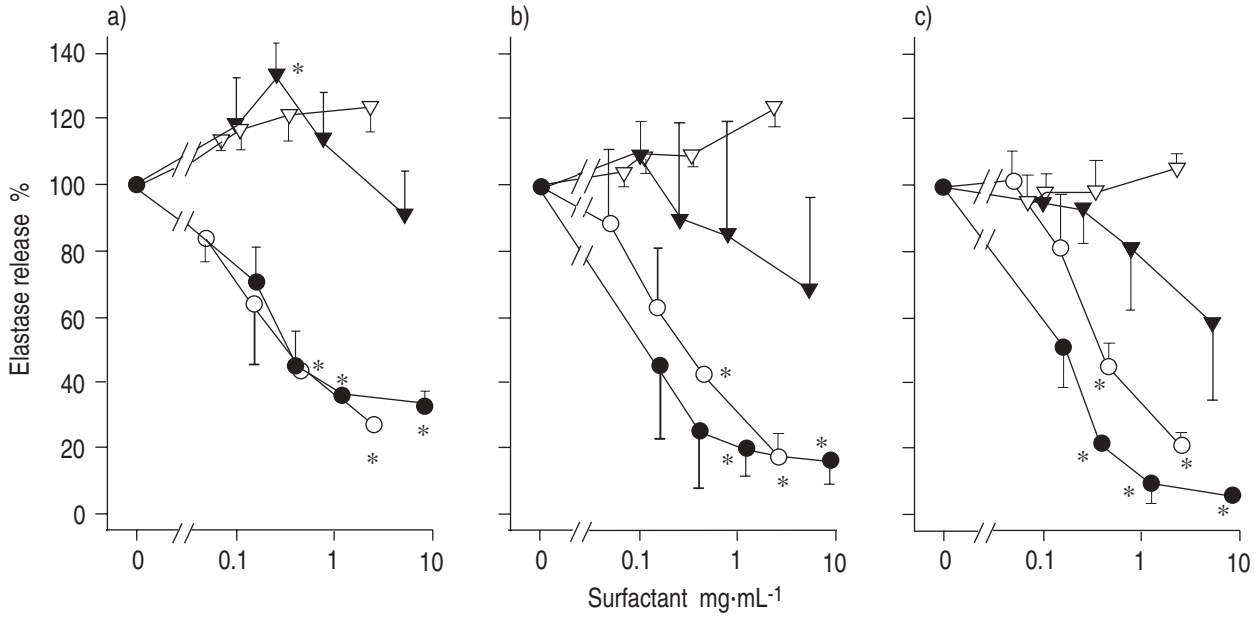

Fig. 3. – Effect of different surfactant preparations on elastase release from isolated CytB-treated PMNs. PMNs were suboptimally stimulated with: a) NAP-2 (100 nM); b) IL-8 (2 nM); or c) fMLP (50 nM) and co-incubated with increasing concentrations of surfactant. Surfactant preparations were: Exosurf® (—∇—), Survanta® (—○—), Curosurf® (—●—), and Alveofact® (—▼—). The amount of elastase released in response to NAP-2, IL-8 or fMLP alone (8.5±0.7, 17.5±1.2 and 50±6%, of total elastase content in detergent-treated controls) was set at 100%. Values were corrected for background release (1.1±0.4% of total release) measured in unstimulated control cells. Data are presented as mean±SD of three independent experiments. Statistical significant differences between the release rates of PMNs co-incubated with surfactant and those in the absence of surfactant are indicated (*: $p<0.05$). For further abbreviations see legends to figures 1 and 2.

Discussion

During respiratory failure in neonates and adults, chemotactic forces and disintegration of the alveolar-capillary membrane may cause PMNs to accumulate in the lung capillaries, to leave the circulation and enter the airspace [30–32]. Treatment of these patients may include endotracheal administration of exogenous surfactant at high doses, even exceeding 100–200 mg·kg^{-1} body weight (BW) [3].

In the present study, it was demonstrated that separated PMNs from healthy adults were not significantly activated by synthetic or natural surfactant. However, when these cells were stimulated by peptide chemoattractants, such as IL-8, NAP-2 and fMLP, the release of lysosomal elastase from PMNs was markedly modulated, the effect being dependent on the surfactant preparation used. The measurement of elastase release from CytB-treated cells was chosen because it represents a well-established method for the determination of PMN activation in response to chemotactic stimuli. Whether high elastase levels detectable in biological fluids of patients with ARDS are a result of stimulation by chemokines, is still under discussion [24]. Surfactants modulated neutrophil responses in a manner typical for each preparation, irrespective of the chemotaxin used for stimulation. Whether these effects are due to biologically active inhibitors, such as constituents of natural surfactants prepared from minced lung tissue, in contrast to synthetic preparations or surfactant extracted from lung lavage fluid, such as Alveofact® or other differences in the relative composition of the various surfactants remains unsolved.

IL-8 that can be isolated from BAL fluid of ARDS patients [22] was found to be a major inducer of transendothelial migration of neutrophils *in vitro* [32], and could act as a potent activator of neutrophils that appear in the alveolar airspaces. NAP-2 is of special interest in this concern [23], since this chemokine is thought to be locally generated from connective tissueactivating peptide III (CTAP-III) [29], a precursor peptide released in high amounts from activated platelets during the inflammatory response [33]. PMNs appear to be the only blood cells that can efficiently cleave CTAP-III into NAP-2, thereby generating their own activator [34]. Our investigations, thus, link the haemostatic and the inflammatory process and resemble the situation that may be found during lung failure [35–37], and during surfactant therapy associated complications such as pulmonary haemorrhage. A study suggesting an increased incidence of this complication in preterm infants following endotracheal administration of a synthetic preparation (Exosurf®) [38] would support this assumption. Thus, in the context of our findings, surfactant related modulation of neutrophil-response to inflammatory mediators may be operative in the pathogenesis of pulmonary haemorrhage.

In contrast to our investigations, other studies have revealed no surfactant (Curosurf®) related effects on the functions of isolated neutrophils, such as adherence, migration and chemotactic response to zymosan-activated serum and fMLP, whilst phagocytosis was significantly reduced [16]. In the latter study, PMNs were incubated with surfactant at lower concentrations than used by us and were washed after preincubation. We investigated the elastase release indicating the mediator-induced PMN response in the presence of surfactant, according more closely to the *in vivo* situation.

Surfactant is a complex of several lipids and proteins. It is not clear which components exert the effects on PMNs. Possible mechanisms which may mediate alterations of PMN function include interference by surfactant

constituents with the cell membrane and related functions, such as expression, internalization, or arrangement of receptors within the cell membrane [39], with signal transduction pathways at the ligand or receptor level [40, 41], or with processing of lipid mediators and cytokines [42, 43]. As shown by others, Fc- and complement-receptors of alveolar and peritoneal macrophages from the rat were reduced in the response to surface active material and purified phospholipids [41]. In our study using PMNs from adults, mediator-induced PMN-response was found to be maximally modulated (suppressed or enhanced) at surfactant concentrations suggested by others to be present in the bronchoalveolar lining fluid [44]. Whilst free arachidonic acid, lysophospholipids and surfactant protein (SP)-A may increase the inflammatory response [45], surfactant preparations lacking the hydrophilic apoproteins, SP-A and SP-D, as well as the phosphatidyl fraction may contribute to reduced host defence by downregulation of inflammatory processes [11, 45]. However. further studies are needed to confirm whether these results are applicable both for neonates and adults.

Based on our findings, we suggest that in addition to macrophage-derived factors that inhibit PMN function within the alveoli [46], surfactant itself may contain constituents that protect the airways from PMN-induced damage. With respect to the increasing insights into pulmonary immune response, it is easily conceivable that alterations in surfactant composition may have multiple effects on the inflammatory process in the alveolar compartment. The clinical relevance of our results is supported by the finding that, after immunisation of guinea-pigs with Freund's adjuvant and subsequent challenge with aerosolized purified protein derivatives, surfactant attenuated the acute inflammatory response [47]. However, further studies are needed to explain contradictory results and to elucidate the mechanisms and clinical implication of the interaction between surfactant constituents and cells participating in the local inflamrnatory response in different kinds of pulmonary failure.

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