

Therapeutic Surfactants Modulate the Viability of Eosinophils and Induce Inflammatory Mediator Release

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Key Words

Allergic asthma · Eosinophil cationic protein · Chemotaxis · Surfactant

Abstract

Background: There is increasing interest in testing surfactant preparations for asthma therapy. Previously, Curosurf was demonstrated to increase inflammation in allergic asthmatics. So far, little is known about the immunomodulatory effects of therapeutic surfactants, in particular concerning the interaction of surfactant components with eosinophils as key effector cells of the allergic airway inflammation. The aim of the present study was to determine the effect of different therapeutic surfactants on cellular functions of eosinophils. **Methods:** Eosinophils were isolated from peripheral blood of atopic volunteers and incubated with the natural animal-derived surfactants Curosurf or Alveofact or the synthetic recombinant human surfactant Venticute at different concentrations for up to 42 h. **Results:** Curosurf and Venticute modulated the viability of eosinophils. While incubation with Curosurf increased the number of necrotic eosinophils after 1, 20 and 42 h, Venticute increased the number of apoptotic and necrotic cells after 1 h, but there were no differences compared with control cells at later time points. All surfactant preparations increased the levels of eosinophil cationic protein after 20 h and, in addition, Curosurf en-

hanced eosinophil cationic protein release after 42 h. The supernatant of eosinophils induced chemotaxis against autologous eosinophils, and the presence of Curosurf, but not Alveofact or Venticute, augmented the chemotactic effect. Chemotaxis was partly blocked by inhibition of eotaxin but not by inhibition of leukotrienes or platelet-activating factor. **Conclusions:** Therapeutic surfactants differ in their effects on eosinophil viability and the accompanying release of inflammatory mediators and chemotactic signals. Proinflammatory effects were most pronounced for the natural surfactant Curosurf.

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Introduction

In recent years, it has been demonstrated that dysfunction of pulmonary surfactant adds to the pathophysiological changes in allergic asthma [1]. Treatment with surfactant components in asthma patients has been tried in clinical studies with varying results [2–4]. While positive effects were demonstrated on airflow and lung volumes, bronchial hyperresponsiveness remained unaf-

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fects. In a study with asthma patients that underwent segmental allergen challenge, we even demonstrated that local treatment with the natural porcine-derived surfactant preparation Curosurf caused an increase in allergic inflammation [2]. The increased eosinophilic inflammation was accompanied by high levels of IL-5 and eotaxin and a shift towards Th2 cells. However, the mechanisms that led to these effects remained unclear. Curosurf itself did not induce an eosinophilic inflammation and had no chemotactic effect on eosinophils *in vitro*. In addition, biophysical effects regarding allergen spreading by the surfactant instillation were excluded by mouse *in vivo* experiments [2]. We speculated that Curosurf had an effect on local cells or inflammatory cells that enter the lung during inflammation and might enhance mediator production (e.g., eotaxin) leading to an increased chemotaxis of eosinophils.

Several *in vitro* studies demonstrated that synthetic and natural surfactant preparations interact with immune cells that are of relevance in asthma. For example, Alveofact, Survanta and Exosurf led to a concentration-dependent suppression of lymphocyte function and proliferation [5, 6]. Curosurf decreased the production of superoxide anions and proinflammatory mediator release from monocytes [7, 8]. In addition, commercial synthetic (Exosurf) and natural surfactant preparations (Alveofact, Curosurf, Survanta) inhibited the activation of neutrophils [9]. Suwabe et al. [10] demonstrated that a natural surfactant (surfactant TA) decreased the adherence and superoxide production of neutrophils and enhanced their apoptosis.

Eosinophils play a key role during allergic inflammation in the lung since they release large amounts of harmful proteins including eosinophil cationic protein (ECP) and eosinophil peroxidase, which are capable of damaging the tissue. Besides inflammatory proteins, eosinophils secrete mediators like eotaxin [11], leukotrienes or platelet-activating factor (PAF) that might enhance chemotaxis [12, 13]. While surfactant protein (SP)-A and SP-D have been demonstrated to inhibit the release of e.g. IL-8 and ECP from eosinophils [14, 15], the effect of therapeutic surfactants on eosinophil functions have not been determined so far. Thus, the aim of the present study was to determine possible effects of surfactants on the release of proinflammatory and chemotactic mediators from eosinophils.

Eosinophils were isolated from peripheral blood and incubated with 2 natural (Curosurf and Alveofact) and 1 synthetic surfactant preparation (Venticute). Eosinophil viability and mediator release were determined. In addition, the chemotactic activity of the supernatant from

surfactant-incubated eosinophils was analyzed. We hypothesized that an interaction of surfactant with eosinophils might have caused the inflammatory changes observed in our previous study.

Material and Methods

Isolation of Eosinophils

Eosinophils were isolated from heparin-anticoagulated venous blood from atopic donors with allergy against either house dust mite allergens or grass pollen allergens using a negative selection of CD16+ granulocytes, as described previously [2]. Control experiments were performed with human alveolar macrophages derived by bronchoalveolar lavage from healthy non-smoking subjects. All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the studies which were approved by the Ethical Committee of Hannover Medical School.

Cell Count

For the determination of cell viability, a Trypan blue stain was performed on a cell aliquot immediately after incubation. Total cells and nonviable cells were counted in an improved Neubauer hemocytometer. The purity of eosinophils used in these experiments was $98 \pm 0.3\%$.

To differentiate between necrosis and apoptosis, additional staining was performed with Annexin V FITC (BD Biosciences, Heidelberg, Germany), 7-amino-actinomycin D (7-AAD; Beckman Coulter, Krefeld, Germany) and CD11b-PE-Cy7 (BD Biosciences) or isotype control (Invitrogen, Karlsruhe, Germany). Cells were then measured by flow cytometry using the Cytomics FC 500 (Beckman Coulter).

Surfactant Preparations

Three different surfactant preparations were used for the experiments. Curosurf (Nycomed Deutschland GmbH, Konstanz, Germany) is a lipid extract surfactant from minced porcine lungs which contains 99% phospholipids and about 1% SP-B and SP-C [16]. Alveofact (Boehringer Ingelheim, Ingelheim am Rhein, Germany) is an organic solvent extract of a fraction isolated from cow lung lavage. It contains 88% phospholipids, 4% cholesterol, 8% other lipids and 1% hydrophobic proteins (SP-B, SP-C) [17]. In addition, a synthetic surfactant preparation (Venticute; Nycomed Deutschland GmbH, formerly Altana Pharma AG) was used which is well characterized and consists of 63.4% 2-dipalmitoyl-sn-glycero-3-phosphocholine, 27.8% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol, 4.5% palmitic acid, 2.5% calcium chloride and 1.8% recombinant SP-C (lusupultide) [18]. The surfactant preparations were tested at concentrations of 50, 500 and 5,000 μg phospholipids per milliliter which is within the range of concentrations that are achieved during therapeutic administration in the lung [2, 19].

Cell Culture Medium

The culture of human eosinophils and alveolar macrophages was performed in RPMI-1640 medium supplemented with fetal calf serum (10% v/v; Biochrom, Berlin, Germany), glutamine

(2 mM), nonessential aminoacids (1 fold), sodium pyruvate (1.0 mM), penicillin (15 U/ml) and streptomycin (15 µg/ml; all from Invitrogen, Karlsruhe, Germany).

In vitro Culture of Eosinophils and Alveolar Macrophages

Blood eosinophils or alveolar macrophages (1×10^5 cells/100 µl/well) were cultured with or without Curosurf, Alveofact or Venticute at a concentration of 50, 500 or 5,000 µg/ml in a 96-well plate at 37°C and 5% CO₂ for 1, 20 or 42 h. After cell culture, the supernatant was harvested, contaminating cells were removed by centrifugation and the supernatant was stored at -80°C until it was used for the chemotaxis assays or analyses of mediators.

Degranulation of Cultured Eosinophils with Serum-Opsonized Sephadex Beads

To determine the ECP release in comparison with the total ECP content of the cells, maximum degranulation was induced by serum-opsonized Sephadex beads. For these experiments, the Sephadex G15 beads (150 mg/ml; Sigma-Aldrich, Taufkirchen, Germany) were incubated for 30 min at 37°C with human serum, washed with isotonic sodium chloride solution and resuspended in 1 ml of Hanks buffered salt solution. Opsonized beads (100 µl) were added to 1×10^5 eosinophils and incubated at 37°C for 30 min. The supernatant and bead-cell pellet were separated, and each fraction was solubilized with a final concentration of 0.3% cetyltrimethylammonium bromide (Sigma-Aldrich). The mixtures were immediately frozen at -80°C until testing for ECP content.

Electron Microscopy

Eosinophils were pelleted and fixed by immersion in 4% paraformaldehyde and 0.5% glutaraldehyde buffered in 0.15 M HEPES for at least 3 h. After repeated rinsing, stabilization of predominantly unsaturated lipids was achieved by postfixation with 1% osmium tetroxide in 0.1 M cacodylate buffer followed by a rinsing in 0.1 M cacodylate buffer (2×10 min). After dehydration through ascending concentrations of acetone, the pellets were embedded in Epon. The suspensions were centrifuged at 5,600 g for 10 min after each processing step. Finally, ultrathin sections (70 nm) were stained with lead citrate and uranyl acetate and examined in an EM 10 (Zeiss, Oberkochen, Germany).

Determination of Mediators (ECP and Eotaxin)

In the supernatants of cultured eosinophils, ECP was measured by the Uni-CAP-ECP system (detection limits 2–200 µg/l; Pharmacia Diagnostics AB, Uppsala, Sweden). The eotaxin duo-set ELISA was used to determine eotaxin levels (detection limits 15.6–1,000 pg/ml; R&D Systems, Wiesbaden, Germany). All measurements were performed in duplicate. By using this ELISA, eotaxin was not detected in any of the samples (data not shown).

Chemotaxis Assay

Eosinophils release mediators that can induce eosinophil chemotaxis. To determine whether eosinophils that are incubated with surfactant preparations release increased amounts of chemotaxis-inducing mediators, supernatant was obtained from eosinophils that were cultured for 42 h in the presence or in the absence of Venticute, Alveofact or Curosurf.

The chemotactic activity of autologous eosinophils against this supernatant was assessed with the modified Boyden cham-

ber technique [20]. Briefly, Boyden chambers (Corning Costar, Bodenheim, Germany) were filled with 100 µl of a 1:4 dilution of supernatant from eosinophils that had been incubated with the surfactant preparations in chemotaxis buffer (PBS supplemented with 10% BSA, 0.1 mM CaCl₂ and 0.1 mM MgCl₂). Chemotaxis buffer without supernatant served as negative control. The addition of eotaxin (0.1 µg/ml; Peprotech Inc., London, UK) to chemotaxis buffer was used as positive control. Chemotactic activity against the surfactant preparations themselves was excluded in separate experiments (data not shown). All experiments were performed in duplicates.

The chamber was covered with polycarbonate filters (pore size 5 µm; Millipore, Schwalbach, Germany), and 100 µl of a human eosinophil suspension at a concentration of 1×10^6 cells/ml was added to each chamber. In some experiments, eosinophils were mixed with the diluted supernatants immediately before being applied to the chemotaxis chamber to determine chemokinesis. After incubation for 1.5 h at 37°C, migrated cells in the lower part of the Boyden chambers were counted in an improved Neubauer hemocytometer.

Blocking Experiments with Chemotaxis Inhibitors

To determine relevant chemotactic substances in the cell supernatant, additional experiments were performed with chemotaxis inhibitors.

Eosinophils were pretreated with either herbimycin A, a protein tyrosine kinase inhibitor that inhibits eotaxin-induced chemotaxis by blocking the activation of the receptor for eotaxin, CCR3 [21], montelukast, a specific cysteinyl leukotriene receptor antagonist [22], or BN 52021, a specific PAF antagonist derived from Ginkgo biloba [23], each at a concentration of 10 µM for 1 h. The cells were then washed, resuspended in chemotaxis buffer and placed in the upper part of the Boyden chamber. The lower part was filled with a 1:4 dilution of supernatant of eosinophils that were incubated with the surfactant preparations in chemotaxis buffer. Migrated cells were counted as described above. All experiments were performed in duplicate.

Statistical Analysis

For statistical analysis the Kruskal-Wallis test in combination with Dunn's multiple comparison test was used (GraphPad Prism 4, version 4.03 for Windows). Values of $p < 0.05$ were considered to be significant. Unless otherwise stated, the data in the text and figures were expressed as the mean $\pm$ standard error of the mean.

Results

Effects of Surfactant Preparations on Eosinophil Viability

In the absence of surfactant, the viability of eosinophils, as determined by Trypan blue staining, was $98.1 \pm 0.9\%$ after 1 h and decreased to $87.0 \pm 4.4\%$ after 20 h and to $41.7 \pm 8.3\%$ after 42 h (fig. 1). When eosinophils were incubated with Curosurf or Venticute at a concentration of 5,000 µg/ml, the viability was strongly de-

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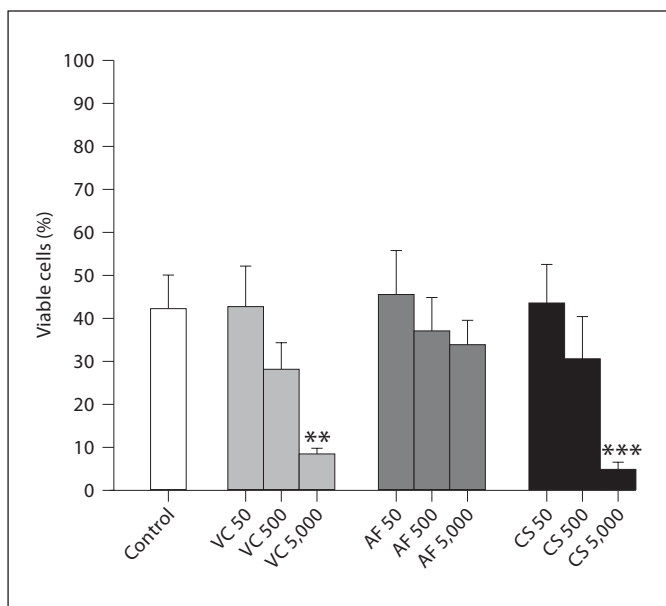
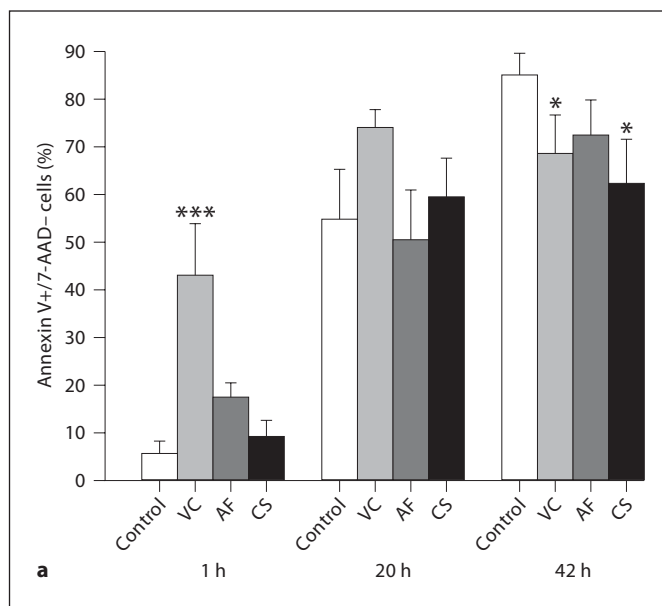
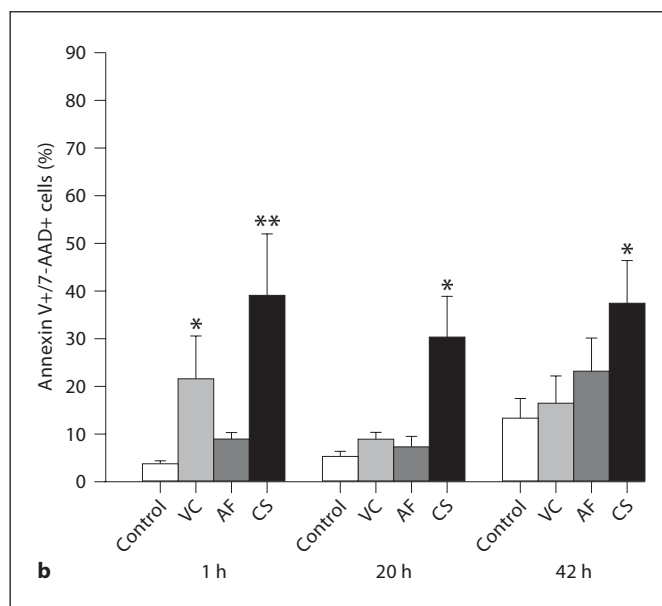


Fig. 1. Viability of eosinophils determined by Trypan blue staining. Eosinophils were incubated without (control) or with surfactants for 42 h. Venticute (VC), Alveofact (AF) and Curosurf (CS) were used at concentrations of 50–5,000 µg/ml. ** $p < 0.01$ and *** $p < 0.001$, respectively, compared with the control ($n = 4-8$).

Fig. 2. Determination of apoptosis and necrosis by annexin V and 7-AAD staining. Eosinophils were incubated without (control) or with surfactants – Venticute (VC), Alveofact (AF) and Curosurf (CS) – for 1, 20 or 42 h at a concentration of 5,000 µg/ml. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, respectively, compared with the control ($n = 6-10$). Annexin V-positive/7-AAD-negative cells were regarded as apoptotic cells (a), while double-positive cells (annexin V+/7-AAD+) were regarded as necrotic cells (b).



a



b

2

creased after 42 h compared with eosinophils incubated without surfactant (fig. 1). The viability was not affected at lower concentrations (50 and 500 µg/ml) or at earlier time points (1 or 20 h; data not shown). Alveofact, even at the highest concentration, did not affect the viability of eosinophils (fig. 1).

Staining of eosinophils with annexin V and 7-AAD allowed to determine the fraction of early apoptotic or late apoptotic/necrotic cells. In these experiments, no effects were observed at concentrations of 50 or 500 µg/ml

for Alveofact or Curosurf. However, there was an increased percentage of Venticute-treated cells that stained positive for annexin V and negative for 7-AAD after 1 h at concentrations of 500 ($p < 0.05$) and 5,000 µg/ml ($p < 0.001$; fig. 2a). This fraction of cells was considered as early apoptotic. After 1 h, the high concentration of Venticute also increased the percentage of annexin V and 7-AAD double-positive cells that were considered as late apoptotic/necrotic (fig. 2b). The strongest effects were observed for Curosurf at 5,000 µg/ml. Curosurf increased

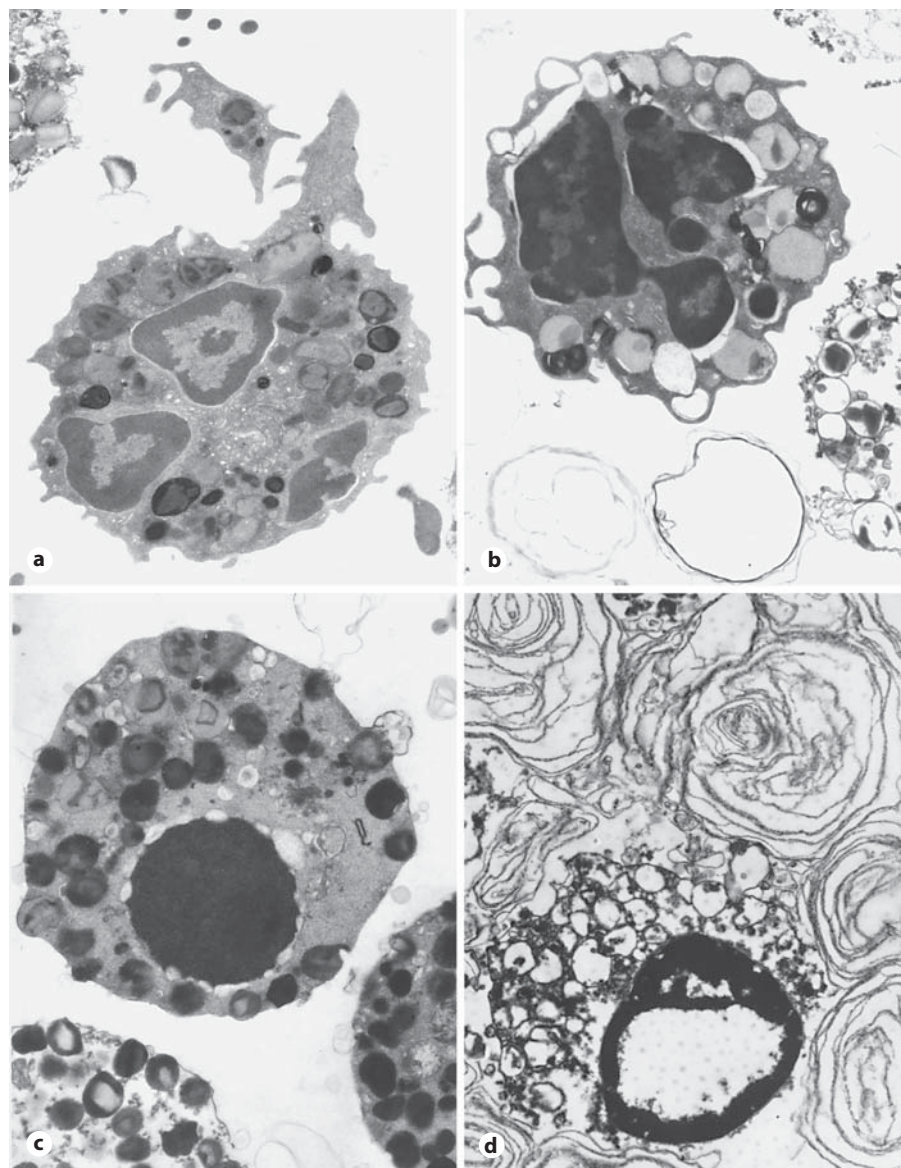


Fig. 3. Transmission electron micrographs of purified blood eosinophils that were incubated for 42 h demonstrating a normal eosinophil (**a**), an eosinophil at an early stage of apoptosis with chromatin condensation after incubation with Venticute (**b**), an apoptotic eosinophil with a pyknotic nucleus and extensive blebbing at the nuclear envelope after incubation with Alveofact (**c**), and a necrotic, lysed eosinophil after incubation with Curosurf (**d**). Surfactant concentration 5,000 $\mu\text{g}/\text{ml}$. Magnification $\times 11,000$.

the percentage of annexin V and 7-AAD double-positive cells after 1 h to $38.7 \pm 13.1\%$ compared with $3.6 \pm 0.8\%$ within the control cells ($p < 0.01$; fig. 2b). This increase was also observed after 20 and 42 h (fig. 2b). These effects of Curosurf fit well to the decreased amounts of viable cells that were determined by Trypan blue staining. However, for Venticute, the effects observed by Trypan blue staining were not reflected by the staining with annexin V and 7-AAD.

The viability of alveolar macrophages that were incubated under the same culture conditions as the purified blood eosinophils was $85.3 \pm 8.5\%$ after 42 h. No significant difference occurred when the macrophages were

incubated with Venticute ($97.3 \pm 2.7\%$), Alveofact ($95.3 \pm 4.7\%$) or Curosurf ($100 \pm 0.0\%$) at a concentration of up to 5,000 $\mu\text{g}/\text{ml}$ ($n = 3$). Similar observations were made for lower surfactant concentrations (50 and 500 $\mu\text{g}/\text{ml}$) at earlier time points (1 and 20 h; data not shown).

Eosinophils Incubated with Surfactant Show Ultrastructural Signs of Apoptosis and Cytolysis

Transmission electron micrographs of eosinophils that were incubated for 42 h show lucent cores indicating degranulation, and some cells already showed signs of apoptosis (fig. 3a). In the presence of Venticute and Alveofact at high concentrations (5,000 $\mu\text{g}/\text{ml}$), cells dis-

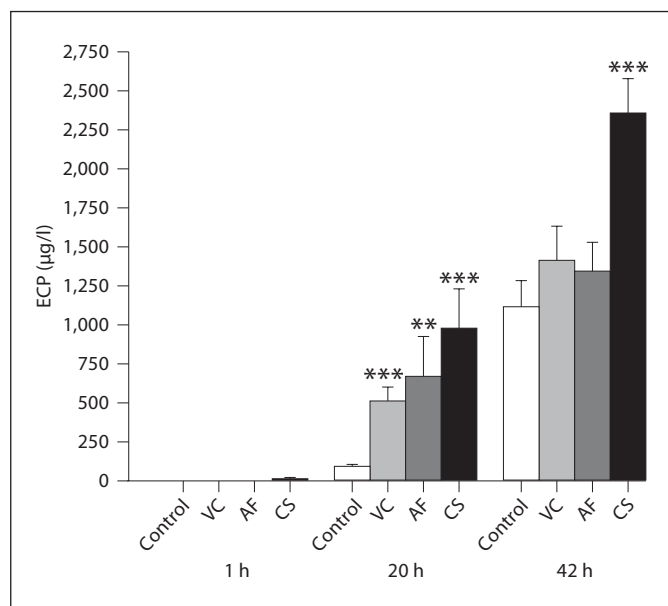


Fig. 4. ECP levels in the supernatants of eosinophils that were incubated without (control) or with Venticute (VC), Alveofact (AF) or Curosurf (CS) for 1, 20 or 42 h at a concentration of 5,000 µg/ml. ** $p < 0.01$ and *** $p < 0.001$, respectively, compared with the control ($n \geq 3$).

played similar signs of degranulation, although signs of apoptosis (including chromatin condensation and nuclear bleb formation) were more frequent (fig. 3b, c), and more necrotic cells were observed in these samples as compared with samples without surfactant. The most pronounced effects were seen in the presence of Curosurf. In that case, almost all cells were necrotic and showed chromatolytic nuclei (fig. 3d). This qualitative analysis indicates that eosinophils partially undergo apoptosis and necrosis, in particular after Curosurf treatment which might lead to increased release of granule proteins.

ECP Release from Eosinophils in the Presence of Surfactant

The release of intracellular contents by eosinophils was reflected by an increase in ECP in the supernatant during incubation. After 1 h, the ECP concentration was 4 ± 2 µg/l and increased to 85 ± 16 µg/l after 20 h and to $1,103 \pm 179$ µg/l after 42 h. Curosurf induced a significant increase in ECP release already at a concentration of 500 µg/ml (after 1 h, 15 ± 4 µg/l, $p < 0.01$; after 20 h, 709 ± 112 µg/l, $p < 0.001$; after 42 h, $2,135 \pm 192$ µg/l, $p < 0.001$). At concentrations of 5,000 µg/ml, all

surfactants augmented ECP release compared with the control after 20 h (fig. 4). After 42 h, ECP release from control cells was increased compared with the earlier time points, and only Curosurf but not Venticute or Alveofact augmented the release of ECP (fig. 4).

Further experiments were performed with serum-opsonized Sephadex beads to estimate the ECP release in relation to the total ECP content of the cells. The incubation with beads released 21% of total ECP after 1 h, 16% after 20 h, and 29% after 42 h. Without surfactants, eosinophils released approximately 0.1% of the total ECP content after 1 h, 2% after 20 h, and 25% after 42 h of incubation. Comparable percentages were obtained after treatment with Alveofact at 5,000 µg/ml (0, 15 and 30% for 1, 20 and 42 h, respectively) or Venticute at 5,000 µg/ml (0, 12 and 32% for 1, 20 and 42 h, respectively). However, after treatment with Curosurf at 5,000 µg/ml, eosinophils released higher relative amounts of ECP (after 1 h, 0.2%; after 20 h, 22%; after 42 h, 54%).

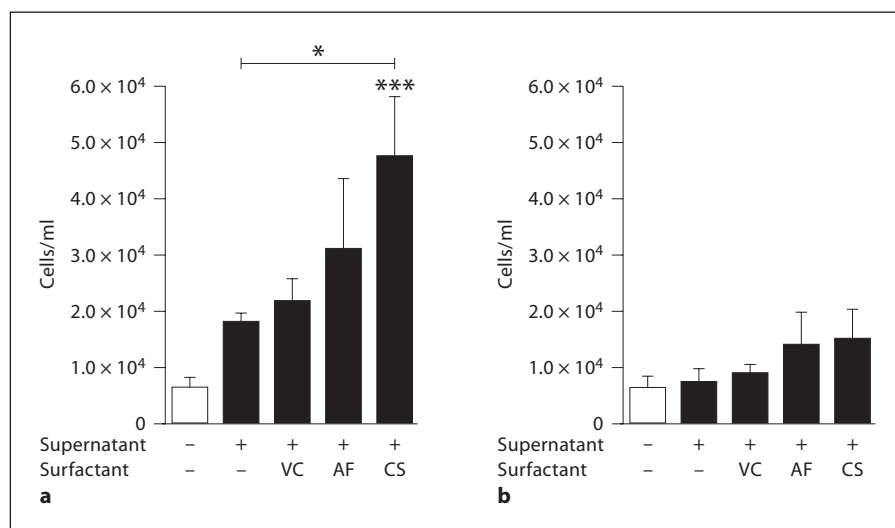
Chemotactic Activity of Supernatant from Eosinophils Incubated with Surfactant

The supernatant from eosinophils that were incubated for 42 h induced chemotaxis (fig. 5a). Compared with controls without supernatant (white bar), supernatants from surfactant untreated and treated cells (black bars) led to an increased chemotaxis of eosinophils. But only Curosurf-treated eosinophils released more chemotactic mediators leading to a significantly increased chemotaxis compared with control condition (white bar; $p < 0.001$) or supernatants from eosinophils that were not treated with surfactant ($p < 0.05$; fig. 5a). In contrast, supernatants from Alveofact- or Venticute-treated cells did not enhance chemotaxis (fig. 5a). Significant chemokinesis was not induced by any of the supernatants (fig. 5b). Eotaxin (0.1 µg/ml) was used as a positive control that strongly induced chemotaxis ($32.1 \times 10^4 \pm 4.7 \times 10^4$ cells/ml; $p < 0.01$) and chemokinesis ($7.4 \times 10^4 \pm 1.4 \times 10^4$ cells/ml; $p < 0.05$). The chemotactic activity of the surfactants alone was excluded with separate experiments (data not shown).

Inhibition of Chemotactic Activity by Herbimycin A

Pretreatment with herbimycin A reduced the eosinophil chemotaxis against supernatant to $53 \pm 13\%$ ($p < 0.05$) of the chemotaxis without inhibitor (control, 100%). In contrast, the PAF receptor antagonist BN 52021 and the leukotriene receptor antagonist montelukast did not inhibit chemotaxis (126 ± 44 and $123 \pm 16\%$ of control levels, respectively). While montelukast or BN 52021

Fig. 5. Chemotactic (a) and chemokinetic activity (b) of supernatants from eosinophils that were incubated without surfactant (control), or with Venticute (VC), Alveofact (AF) or Curosurf (CS) at 500 $\mu\text{g}/\text{ml}$ for 42 h. * $p < 0.05$ and *** $p < 0.001$, respectively, compared with the control without supernatant (white bars; $n = 7$).



failed to block chemotaxis when the supernatants from Curosurf-treated cells were used (108 ± 46 and $96 \pm 30\%$, respectively), herbimycin A decreased chemotaxis ($53 \pm 22\%$; $p = 0.07$; $n = 6$).

Discussion

In a previous study with asthma patients, we demonstrated that the natural animal-derived surfactant preparation Curosurf induced an increase in the eosinophilic allergic inflammation when it was administered immediately prior to segmental allergen challenge [2]. We hypothesized that the surfactant had proinflammatory effects on eosinophils which might have been responsible for the observed changes. Our present data support this hypothesis since Curosurf decreased eosinophil survival and increased the release of eosinophil mediators in vitro which also caused eosinophil chemotaxis. These effects might explain the augmentation of the inflammatory response when Curosurf was tested in asthma patients.

During the 2 days of culture, the number of viable eosinophils decreased continuously and, at the end, only 40% of the control cells were viable as determined by Trypan blue staining. Venticute and Curosurf further reduced the number of viable cells at this time point. Interestingly, Venticute already enhanced the fraction of apoptotic and necrotic cells compared with control cells after 1 h, but there were no differences at later time points, and a link to increased ECP release after 1 h was missing. In contrast, Curosurf was a much stronger modulator and

increased the fraction of necrotic cells as determined by annexin V/7-AAD staining and also increased the ECP release at all time points. Thus, Curosurf was the only surfactant preparation that demonstrated a link between increased number of necrotic cells and increased ECP release. At the highest concentration of 5,000 $\mu\text{g}/\text{ml}$, all surfactants increased ECP release after 20 h but neither Venticute nor Alveofact induced raised numbers of necrotic or apoptotic cells at this time point. This might indicate that modulation of ECP release may be independent of cell viability.

The observed effects of therapeutic surfactants on eosinophil survival are in line with previous observations made by Suwabe et al. [10] who demonstrated that a natural bovine surfactant (surfactant TA) increased the apoptosis of human neutrophils. The surfactant-treated neutrophils showed picnotic nuclei and expressed Fas antigens. In addition, the adherence and superoxide production of neutrophils was reduced, which was also described in an earlier study [24]. The authors concluded that the observed effects might protect against neutrophil activation during inflammation. Our data fit these observations regarding the surfactant-induced reduction in the cell viability. However, eosinophils seem to differ from neutrophils regarding the release of proinflammatory mediators while undergoing apoptosis or necrosis. Our data demonstrate that the decreased eosinophil survival in the presence of Curosurf was accompanied by an increased release of ECP. ECP like eosinophil peroxidase and eosinophil-derived neurotoxin is an eosinophil granular protein which is stored in the matrix of the eosino-

phil granules. High concentrations of Curosurf induced an increased release of these granular proteins. These effects might rather be proinflammatory in an eosinophil-driven disease. In a recent review, Uller et al. [25] suggested that airway tissue eosinophils either undergo apoptosis followed by secondary necrosis or primary necrosis/cytolysis. Both mechanisms lead to the release of eosinophil mediators and thereby aggravate the allergic inflammation. Previously, it was assumed that apoptosis-inducing drugs might be useful for triggering the resolution of unwanted eosinophilic inflammatory responses [26]. This assumption was based on findings in peripheral blood eosinophils from asthma patients that survived longer during *in vitro* culture than eosinophils from healthy controls [27]. Similar observations were made for airway eosinophils from allergen-challenged asthma patients that were compared with cells from unchallenged patients [28]. However, a recent study by Uller et al. [29] demonstrated that inducing eosinophil apoptosis and cytolysis might lead to amplification of the allergic inflammation. The authors used an ovalbumin asthma mouse model to investigate the effects of a monoclonal anti-Fas antibody. This antibody treatment reduced eosinophil viability as expected, but it enhanced the allergic inflammation instead of reducing it. The authors concluded that Fas receptor-mediated eosinophil apoptosis in airway tissue may cause severe disease exacerbation. Our data fit these observations since Curosurf induced late apoptotic/necrotic eosinophils which was accompanied by a release of eosinophil granule proteins. We assume that this effect is accountable for the proinflammatory effects observed after Curosurf administration in asthma patients in our previous study.

While undergoing necrosis, eosinophils might release other mediators besides ECP that are able to enhance the allergic inflammation. Uller et al. [29] suspected that the observed Fas-induced inflammation was probably associated with the *de novo* recruitment of eosinophils because the expression of several eosinophil-recruiting chemokines was upregulated. Some of these chemokines might be secreted in an autocrine fashion from eosinophils that undergo apoptosis. Therefore, we tried to identify different mediators in the supernatant of cultured eosinophils that have the potential to act as chemotactic substances. In the current study, we investigated the chemotaxis of autologous eosinophils. The supernatants obtained from eosinophils cultured for 42 h with or without surfactant induced chemotaxis. Supernatants from Curosurf-treated cells increased the chemotaxis significantly compared with supernatant from untreated cells.

Major chemoattractants for eosinophils in the asthmatic airways are eotaxin, PAF and leukotriene C₄ [11]. In our previous study, increased levels of eotaxin were detected in BAL fluid after surfactant treatment prior to allergen challenge [2]. Eosinophils are known as a potential source of eotaxin [30], and the increased levels at inflammatory sites may partly be due to a release from eosinophils themselves. We hypothesized that eotaxin might have contributed to the chemotactic effects of the supernatant from *in vitro* cultured eosinophils. Although, we did not detect eotaxin in the supernatant by ELISA, herbimycin A partly reduced the eosinophil chemotaxis towards the supernatant of untreated cells and it inhibited eosinophil chemotaxis against the supernatant of Curosurf-treated cells. Blocking of PAF or leukotriene C₄ alone did not reduce chemotaxis in our experiments. Therefore, we assume that the autologous chemotaxis was induced by a combination of chemotactic substances released from eosinophils which may include eotaxin and, less likely, leukotriene C₄ [31] or PAF. In addition, chemokines like RANTES [32] might have been involved. The presence of Curosurf enhanced the chemotactic effects because it might have increased the release of chemotactic substances. Referring to our previous study, we assume that this mechanism is accountable for the increased eosinophil influx after Curosurf administration.

The surfactant concentrations of 50–5,000 µg phospholipids/ml that we used in our experiments were within the range of *in vivo* concentrations that would be achieved during therapeutic surfactant administration. A recent report by Clayton et al. [33] demonstrated that the deposition of 8.9 mg of radio-labeled synthetic surfactant (lucinactant) to asthma patients led to a phospholipid concentration in the epithelial lining fluid (ELF) of about 300 µg/ml. This study was performed with a low amount of surfactant because it was a feasibility and tolerability study that was also used to determine the deposition of the administered surfactant. Babu et al. [19] deposited a total dose of 200 mg of the synthetic surfactant Pumactant in patients with asthma by inhalation. Assuming a total volume of ELF in the lung of about 30 ml [34], the local surfactant concentration in these patients would have reached more than 6,000 µg phospholipids/ml. In our previous study, 120 mg phospholipids were administered to one segment of the lung [2]. Assuming that this is about 1/20 of the total lung with a total ELF volume of about 1.5 ml, we have even reached much higher local concentrations.

Importantly, none of the surfactant preparations reduced the viability of alveolar macrophages when they were incubated under the same culture conditions as the eosinophils. Alveolar macrophages, which are regularly exposed to pulmonary surfactant in the alveoli, seem to be 'resistant' against the effects of surfactant. These findings might indicate that eosinophils and neutrophils receive specific signals when they reach the natural surfactant layer in the alveolar space. Locally residing cells like alveolar macrophages are not affected by these signals. It can be speculated that in this way, pulmonary surfactant accelerates the clearance of invading inflammatory cells in the alveolar lumen [25].

The responsible component(s) of the surfactants and the molecular events that lead to increased apoptosis or necrosis of eosinophils were not identified. One possible explanation could be that surface-active phospholipids contained in the surfactants have a lytic effect. These phospholipids might adsorb to the cell surface resulting in incorporation and alteration of the membrane fluidity

and membrane integrity. In addition, an alteration in the transmembrane signaling might occur [8, 35].

In conclusion, the present findings demonstrate that therapeutic surfactants might affect the allergic inflammation in the lung by acting directly on eosinophils. The observed effects on viability and mediator release of the cells were most pronounced for the natural surfactant preparation Curosurf and have to be considered when surfactant preparations are used for therapeutic approaches in allergic asthma.

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