

Natural Porcine Surfactant Augments Airway Inflammation after Allergen Challenge in Patients with Asthma

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There is increasing evidence for a role of pulmonary surfactant in asthma and allergic inflammation. In murine asthma models, recent studies have demonstrated that surfactant components downregulate the allergic inflammation. Therefore, we tested the hypothesis that in individuals with mild asthma, a natural porcine surfactant preparation (Curosurf) given before segmental allergen challenge can reduce the allergic airway inflammation. Ten patients with asthma and five healthy control subjects were treated in two segments with either Curosurf or vehicle followed by local allergen challenge. Six additional patients with asthma received Curosurf before allergen challenge in one segment as above, but the second segment was instilled with Curosurf without allergen challenge. Unexpectedly, surfactant treatment augmented the eosinophilic inflammation 24 hours after allergen challenge. A direct chemotactic effect of Curosurf was excluded. However, levels of eotaxin and interleukin-5 were increased in bronchoalveolar lavage after Curosurf treatment, whereas IFN- γ -levels and numbers of IFN- γ ⁺ T cells were decreased. Curosurf had no influence on spreading and retention of allergen determined by allergen uptake in mice. These findings demonstrate that treatment with a natural porcine surfactant results in an augmentation of the eosinophilic inflammation after allergen challenge that is more likely due to immunomodulatory effects than to biophysical properties of the surfactant.

Keywords: asthma; allergy; immunomodulation; pulmonary surfactant; segmental allergen provocation

In recent years evidence has accumulated that pulmonary surfactant plays a role in asthma. There are different mechanisms by which surfactant is important for the pathophysiologic sequel in asthma. One mechanism relates to the biophysical function of surfactant. Airway obstruction, which is commonly thought to be caused by smooth muscle constriction, mucosal edema, and secretion of fluid into the airway lumen, may, in addition, be due to a poor function of pulmonary surfactant. A dysfunction of surfactant has been demonstrated both in asthma models (1) and in patients with asthma (2–4). Poor-functioning surfactant loses the ability to stabilize airways and to prevent airway collapse (5). Therefore, surfactant dysfunction in asthma might contribute to airway obstruction, and treatment strategies that act on improving surfactant function might be of benefit in asthma. Accordingly, animal studies have shown that surfactant treatment in ovalbumin-challenged guinea pigs prevented allergic bronchial constriction (6) and that lung function and blood

gases in guinea pigs were less affected after treatment with calf lung surfactant extract before antigen challenge (7). In patients with an asthmatic attack, Kurashima and coworkers found improved lung function variables after surfactant inhalation (8). In contrast, a clinical study in children with asthma showed that surfactant nebulization had no effect on airflow obstruction and bronchial hyperresponsiveness to histamine (9). Recently, inhalation of a dry powder surfactant (Pumactant) abolished the early asthmatic response after allergen provocation in patients with allergic asthma, whereas the late-phase reaction was unaffected (10).

A further important mechanism in the pathophysiologic sequel of asthma relates to immunomodulatory properties of surfactant. Airway obstruction in asthma occurs on inhalation of a variety of stimuli due to bronchial hyperresponsiveness. This bronchial hyperresponsiveness is caused and sustained by a chronic inflammation of the airways (11). Surfactant components like the lung collectins surfactant protein (SP)-A and SP-D have been found capable of downregulating this allergic inflammation in a murine asthma model leading to decreased bronchial hyperresponsiveness (12, 13). Furthermore, several *in vitro* studies demonstrated that synthetic and natural surfactant preparations interact with immune cells that are of relevance in asthma (reviewed in Reference 14). For example, Alveofact, Survanta, and Exosurf led to a concentration-dependent suppression of lymphocyte function and proliferation (15, 16). In addition, commercial synthetic (Exosurf) and natural (Alveofact, Curosurf, Survanta) surfactant preparations also inhibited activation of neutrophils (17). Production of superoxide anions and proinflammatory mediator release from monocytes was decreased by Curosurf, a natural porcine surfactant (18, 19). These effects were dependent on intracellular signaling events because activation of nuclear factor- κ B and L-selectin-induced signal transduction was inhibited by various surfactant preparations (20, 21). So far, data on modulating the allergic inflammation by surfactant treatment in patients with asthma are missing.

On the basis of these findings we tested the hypothesis that in patients with mild asthma a natural porcine surfactant preparation (Curosurf) given before a segmental allergen challenge can reduce the allergic airway inflammation. Curosurf is a lipid extract surfactant from minced porcine lungs with good surface activity that contains surfactant phospholipids and the hydrophobic SP-B and SP-C but no SP-A or SP-D (22). It was used because it is commercially available and approved for the use in humans. At present, no surfactant preparation for human use contains SP-A or SP-D, which might be an interesting treatment option in asthma. Because of the public discussion on bovine spongiform encephalopathy, we decided to use a porcine instead of a bovine surfactant preparation.

We describe here an unexpected augmentation of the eosinophilic allergic response after treatment with Curosurf. To identify possible mechanisms for the eosinophil influx, we assessed the chemotactic activity of Curosurf on eosinophils *in vivo* and

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in vitro. Furthermore, we analyzed the chemokine receptors CCR3 and CCR5 on eosinophils that migrated into the lung and measured bronchoalveolar lavage (BAL) levels of the CC chemokine eotaxin. To test for effects of Curosurf on the T cell balance between a Th1-type and a Th2-type response we examined BAL levels of interleukin-5 (IL-5) and IFN- γ and determined intracellular expression of IL-5 and IFN- γ in BAL T cells. To control for biophysical effects of surfactant treatment, we examined spreading and retention of allergen in mice lungs. Therefore, we quantified the uptake of intratracheally administered Alexa488-labeled allergen (dextran) by alveolar macrophages after Curosurf and control treatment.

METHODS

Study Design

Sixteen patients with mild asthma and five healthy volunteers were enrolled in this study and underwent two bronchoscopies 24 hours apart. On Day 1, all participants received a BAL as a baseline procedure (B) and were segmentally given 10 ml of sterile saline as a control challenge (C). Ten patients with asthma and all control subjects were treated in two segments with either Curosurf (SA) or vehicle (CA) followed by local allergen challenge. Six patients with asthma received Curosurf before allergen challenge as above in one segment (SA), but the additional segment was instilled with Curosurf without allergen challenge (S). On Day 2, all subjects were rebronchoscoped and underwent three BAL procedures (C, CA, SA or C, S, SA, respectively). For details see later.

Study Subjects

Patients with asthma had mild intermittent allergic asthma as defined in the GINA Workshop Report 2002 (www.ginasthma.com). They had a history of episodic wheeze with reversible airflow obstruction, and asthma had previously been diagnosed by an independent physician. Each patient had a positive skin-prick test, defined as more than 4 mm diameter skin wheal response to 1 or more out of 12 common allergens (mixed grass pollen, mixed tree pollen, rye pollen, mugwort pollen, ribwort pollen, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat fur, dog hair, mixed feather, *Alternaria*, *Cladosporia* from ALK-Scherax, Hamburg, Germany). Bronchial hyperresponsiveness and concentration of methacholine that reduced FEV₁ by 20% (PC₂₀FEV₁) (methacholine) were determined as described (23). Patients were defined eligible for study inclusion when they were skin prick positive for either grass pollen or house dust mite allergen. For safety reasons, segmental allergen provocation was performed out of season. Therefore, airway hyperreactivity was not necessarily present as was elevated total IgE. The allergen extract used for segmental allergen challenge (mixed grass pollen or *D. pteronyssinus*; ALK-Scherax) was that which produced the largest wheal response on skin-prick testing, and the chosen concentration was one-tenth the dilution in saline that elicited a 3-mm-diameter skin wheal response. The instilled amount of antigen was 10 to 1,000 SQ-U diluted in 10 ml of saline solution. Patients were only using β_2 -agonists when required for relief of symptoms. None were treated with corticosteroids, sodium cromoglycate, theophylline, or leukotriene modifiers within 4 weeks before bronchoscopy.

The normal control subjects had no history of allergic or other diseases, negative skin-prick tests, normal serum IgE (< 100 IU/ml), normal lung function tests, and no bronchial hyperresponsiveness (PC₂₀ > 8 mg/ml). Clinical characteristics of the study subjects are summarized in Table 1.

All study subjects were nonsmokers, and no subject suffered from acute bronchitis 4 weeks before the investigations. 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.

Bronchoscopy Procedure with Segmental Allergen Challenge and Surfactant Treatment

Before bronchoscopy, all subjects received nebulized salbutamol (1.25 mg), atropine (0.5 mg, subcutaneously), and midazolam (0.05–0.1 mg/kg).

Lidocaine (maximum: 6 mg/kg) was used to achieve local anesthesia of upper and lower airways. The bronchoscope (BF 160 P; Olympus Optical, Hamburg, Germany) was wedged into the anterior bronchus of the left lower lobe and baseline BAL (B) was performed with 6 $\times$ 20 ml sterile saline solution. Lavage fluid from the first 20 ml was discarded. The instrument was passed into the anterior lingular bronchus, and 10 ml saline solution was instilled as a control challenge (C). The bronchoscope was then passed into the medial segment of the middle lobe, and 120 mg Curosurf diluted in 10 ml of saline solution was instilled into the segment through a Teflon catheter followed by allergen challenge with either grass pollen mix or *D. pteronyssinus* (SA). The concentration of the administered allergen was determined by skin-prick test before bronchoscopy. The bronchoscope was then passed into the anterior segment of the right upper lobe, and sham treatment with 10 ml saline was performed before allergen challenge (CA). In a subgroup of six patients with asthma, Curosurf alone was instilled into the anterior segment of the right upper lobe as a control without allergen challenge (S) instead of sham treatment to control for direct effects on individuals with asthma allergic to Curosurf.

After 24 hours at the time of maximum eosinophilic inflammation, all subjects were rebronchoscoped with the same premedication and the anterior lingular bronchus (C), the right anterior upper lobe bronchus (CA or S, respectively), and the medial middle lobe bronchus (SA) were lavaged.

A commercially available endotoxin detection assay (E-toxate, Limulus amoebocyte lysate; Sigma, St. Louis, MO) was used to demonstrate that the instilled saline and allergen solutions as well as the Curosurf solution were free of endotoxin (endotoxin content < 0.125 EU/ml).

Processing of BAL Cells and ELISA for IL-5, IFN- γ , and Eotaxin

BAL fluid samples were processed as described (23). Briefly, cells were filtered through a 100- μ m filter, centrifuged, and the supernatant was aliquoted and stored at -80°C until examination by ELISA. ELISA-kits for IL-5, IFN- γ , and eotaxin were obtained from R&D Systems (Wiesbaden, Germany).

The total count of nucleated cells was performed by using a Neubauer hemocytometer. Differential cell counts were performed from cytopsin slides with 300 cells per slide being counted. An aliquot of cells was separated for flow cytometric analysis.

Flow Cytometric Detection of Chemokine Receptors CCR3 and CCR5 on Eosinophils

Chemokine receptor staining of eosinophils has been performed as described previously (24). Aliquots (10 μ l) containing 1 $\times$ 10⁵ BAL cells were incubated at 4°C for 30 minutes with the primary antibodies against the human CCR3 (clone 61828.111; rat IgG2a; R&D Systems) and human CCR5 (clone 45502.111; mouse IgG2b; R&D Systems). The mouse IgG2b and the rat IgG2a isotype control antibody were obtained from Immunotech (Hamburg, Germany). In a second step, cells were stained by a fluorescein isothiocyanate-conjugated goat anti-mouse (for CCR5) or goat anti-rat antibody (CCR3) (Immunotech) and analyzed by flow cytometry using a live gate on the granulocyte population. Data are expressed as a ratio of fluorescence intensity of specific monoclonal antibody (median) and isotype control (median).

Intracellular Expression of IL-5 and IFN- γ in BAL-derived T Cells

Intracellular cytokine detection was performed after stimulation with anti-CD3/CD28 (25). Briefly, 24-well flat-bottom plates (Nunc, Wiesbaden, Germany) were coated with 1 ml anti-CD3 (OKT3, Orthoclone; Janssen Cilag, Berlin, Germany) at a concentration of 150 ng/ml supplemented with coating buffer containing sodium carbonate and sodium hydrogen carbonate for 2 hours at 37°C and 5% carbon dioxide. After washing with Roswell Park Memorial Institute medium 1640, 1 $\times$ 10⁶ BAL cells/ml resuspended in Roswell Park Memorial Institute medium 1640 supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) were added. Cells were stimulated with anti-CD28 (0.5 mg/ml; Becton Dickinson, Heidelberg, Germany) in the presence of Brefeldin A (1 μ M). For intracellular staining, a Cytofix/Cytoperm Kit (BD Pharmingen, San Diego, CA) was used. After incubation for 6 hours at 37°C in a humidified atmosphere of 5% carbon dioxide in air, cells were

TABLE 1. CLINICAL CHARACTERISTICS OF THE STUDY SUBJECTS

Subjects	Age (yr)	Sex	FEV ₁ (% Predicted)	PC ₂₀ (mg/ml)	IgE (kU/L)	Allergen Sensitivity†	Allergen Provocation SQ-U‡	Allergen Challenge (Segments)
Control subjects	20	M	115	> 8	8	–	Dp 1000	2
	18	M	114	> 8	9	–	Dp 1000	2
	26	F	123	> 8	14	–	gp 1000	2
	23	M	115	> 8	26	–	Dp 1000	2
	22	F	113	> 8	15	–	gp 1000	2
Median (range)	22 (18–26)	2 F/3 M	115 (113–123)	> 8	14 (8–26)			
Patients with asthma	22	M	118	> 8	28	gp, Ry, tp, Mu, Ri, Dp, Df, d	Dp 1000	2
	23	F	105	> 8	27	gp, Ry, Dp, Df, d, c	Dp 1000	2
	25	F	105	> 8	305	gp, Ry, tp, Mu, Ri, Dp	gp 10	2
	18	M	88	0.54	249	gp, Ry, tp, Mu, Dp, Df, d, c, f	gp 1000	2
	33	M	114	> 8	502	gp, Ry, Df	gp 1000	2
	29	F	106	0.15	214	gp, Ry, tp, Mu, Ri, Df, d, c	gp 1000	2
	25	F	100	2.90	112	Dp, Df, d, c	Dp 1000	2
	25	M	90	4.18	77	gp, Ry, Mu, d, c	gp 1000	2
	23	M	79	0.18	210	gp, Ry, Dp, Df, d, c	gp 1000	2
	33	M	102	0.95	180	gp, Ry, tp, Dp, Df, d, c, f	Dp 1000	2
	27	M	109	1.83	833	gp, Ry, Dp, Df, d, f	Dp 1000	1
	24	M	117	2.18	368	gp, Ry, tp, Mu, Ri, Df, d, c	DP 1000	1
	24	M	103	> 8	228	gp, Ry, tp, Mu, Ri, Dp, d, c	gp 100	1
	19	M	109	> 8	920	gp, Ry, tp, Mu	gp 1000	1
	29	F	97	< 0.10	49	tp, Dp, Df, d, c	gp 1000	1
	19	M	111	2.28	382	Ri, Dp, Df, d, c	Dp 1000	1
Median (range)	24.5 (18–33)	5F/11M	105 (79–118)	2.59* (0.10–> 8)	221* (27–920)			

Definition of abbreviations: Al = *Alternaria*; c = cat fur; Cl = *Cladosporia*; d = dog hair; Df = *Dermatophagoides farinae*; Dp = *Dermatophagoides pteronyssinus*; f = mixed feather; gp = mixed grass pollen; Mu = mugwort pollen; PC₂₀ = provocative concentration of methacholine causing a 20% drop in FEV₁; Ri = ribwort pollen; Ry = rye pollen; tp = mixed tree pollen.

* $p < 0.01$ compared with control subjects.

† Allergen sensitivity was determined by skin-prick test.

‡ Allergen provocation was done with either grass pollen mix (gp) (ALK-Scherax) or *Dermatophagoides pteronyssinus* mix (Dp) (ALK-Scherax) of the indicated amount (SQ-U) diluted in 10 ml of saline solution.

washed in staining buffer (phosphate-buffered saline containing 1% heat-inactivated fetal calf serum and 0.09% sodium azide, pH 7.4–7.6) as recommended by the manufacturer. Anti-CD4-fluorescein isothiocyanate (25 µg/ml; Coulter, Krefeld, Germany) and anti-CD8 peridinin chlorophyll protein (6.25 µg/ml; Becton Dickinson) were then added, and cells were incubated for 30 minutes at 4°C in the dark. After a further wash, cells were fixed and permeabilized with Cytotfix/Cytoperm buffer for 20 minutes at 4°C followed by a wash with Perm/Wash buffer. For intracellular staining, mouse anti-human IFN-γ or rat anti-human IL-5, both phycoerythrin conjugated (Becton Dickinson), were added and cells were incubated for 30 minutes at 4°C. As control antibodies, nonspecific fluorescein isothiocyanate-, phycoerythrin-, and peridinin chlorophyll protein-labeled antibodies were used (Becton Dickinson). Flow cytometric analysis was performed with scatter gates on the lymphocyte fraction using a flow cytometer (Calibur; Becton Dickinson) as described previously (23). Positive staining for cytokines was considered when cells were detected above the background level of cells stained with isotype-matched, nonspecific control antibodies in identical concentrations and labeled with the same fluorochrome (phycoerythrin). Cytokine-positive cells were expressed as percentage of CD4⁺ or CD8⁺ T cells.

Chemotaxis Assay for Eosinophils after Incubation with Curosurf

Eosinophils were isolated from heparin-anticoagulated venous blood from healthy donors ($n = 9$) using negative selection of CD16⁺ granulocytes as described previously (24). Chemotactic activity assay was performed using the modified Boyden chamber technique (26). Briefly, Boyden chambers (Nuclepore GmbH, Tübingen, Germany) were filled with either 100 µl medium alone or 100 µl medium containing eotaxin (100 ng/ml; Peprotech Inc., London, UK) or Curosurf (500 µg/ml) or both substances in combination and covered with polycarbonate filters (pore size, 3 µm; Nuclepore). Hundred microliters of a human eosinophil suspension at a concentration of 5×10^5 cells/ml was added to each chamber. After incubation for 1 hour at 37°C, migrated cells in the lower part of the Boyden chambers were lysed by adding 0.1%

Triton X-100, and β-glucuronidase activity in the lysates was determined photometrically using *p*-nitrophenyl β-D-glucuronide as a substrate (all substances from Sigma). For calculation of the number of migrated cells, equivalent to β-glucuronidase activity determined in the lower part of the Boyden chamber, values were calculated by a computer-assisted technique from a standard curve using known numbers of unchallenged eosinophils. Chemotactic activity was expressed as chemotactic index: ratio of the number of migrating cells in presence of stimulus to the number of migrating cells in presence of medium.

Quantification of Allergen Spreading and Retention after Curosurf Treatment in Mice

The effect of Curosurf on allergen spreading and retention was investigated in a mouse model. Mice (BALB/c) were anesthetized with halothane (4.5%) and intraperitoneal injection of propofol (1 mg) and intubated with an intravenous catheter. One group of animals received Curosurf at a body weight-adapted dose (0.7 mg diluted in saline solution to a volume of 30 µl), whereas the other group received saline solution as a control. One minute after the treatment, 5 µg of Alexa488-dextran (molecular weight: 10 kD, Molecular Probes; Göttingen, Germany) diluted in saline solution at a volume of 30 µl were instilled in animals from both groups. Animals of both treatment groups were killed after 1 or 24 hours ($n = 10$ of each group), and a BAL was performed with 2×0.8 ml phosphate-buffered saline. The BAL was centrifuged at $1,200 \times g$ for 10 minutes, and the cell pellet was fixed with paraformaldehyde (2%) for 20 minutes. After fixation, the cells were washed in phosphate-buffered saline twice and resuspended in 300 µl phosphate-buffered saline. A nuclear stain was performed with TO-PRO-3 (1:500, Molecular Probes), and the cells were measured with a FC 500 flow cytometer (Beckmann Coulter) equipped with a 488-nm argon ion laser and a 633-nm helium–neon laser. A gate was set on the macrophage population in the forward and sideward scatter dot blot and on the TO-PRO-3 positive cells measured in Channel 4. Cells out of these two gates were identified positive for ingested Alexa488-dextran, measured in Channel 1, when they were found above the background level of cells from untreated control mice that did not receive Alexa488-dextran.

The mean fluorescence intensity of Alexa488-dextran-positive cells was compared (27).

Statistical Analysis

The Mann-Whitney U test was used for intergroup comparison between patients with asthma and control subjects. For comparison of paired data within the study groups, the Wilcoxon signed rank test was used for the individual comparisons. The Bonferroni correction for multiple comparisons was used throughout. A *p* value less than 0.05 was considered as statistically significant. Unless otherwise stated, the data in the text and figures were expressed as median (with range or individual results).

RESULTS

BAL Fluid Recovery and Differential Cell Counts

In healthy control subjects, BAL fluid recovery did not differ between segments at baseline, after saline, and after the allergen challenges. In patients with asthma, there was a decreased recovery after control allergen challenge and an increased recovery after surfactant alone compared with baseline (Table 2). In control subjects, total BAL cell numbers increased after instillation of either saline or allergen independent of treatment compared with baseline, but, probably due to the low number of subjects, the differences did not reach statistical significance. In patients with asthma, there was a significant increase of total cells after saline and after the allergen challenges, whereas the increase after surfactant alone was not significant. Unexpectedly, surfactant treatment compared with vehicle further increased total cell numbers after allergen challenge (Figure 1A). The increased total cell count after allergen challenge in the patients with asthma was mainly caused by eosinophils. Interestingly, surfactant treatment further increased eosinophil numbers after aller-

gen challenge (Figure 1B). In control subjects, provocation with either saline or allergen did not change the number of eosinophils or lymphocytes but resulted in increased neutrophil numbers. However, these data did not reach statistical significance. In the patients with asthma, neutrophil numbers were also increased after saline, after the allergen challenges, and after surfactant alone. In contrast, lymphocyte numbers were only increased after the allergen challenges (Table 2). Surfactant treatment resulted in a further elevation of neutrophils and lymphocytes after allergen challenge compared with sham treatment (Table 2).

Chemotactic Activity of Curosurf on Eosinophils

Segmental instillation of Curosurf alone did not increase eosinophil numbers in patients with asthma (Table 2). To further exclude the possibility that Curosurf directly enhances eosinophil chemotaxis, the modified Boyden chamber was used to study the *in vitro* chemotaxis. Highly enriched eosinophils from healthy donors were incubated in the presence and absence of Curosurf and subsequently stimulated with eotaxin. Alternatively, eosinophils were stimulated directly with Curosurf. As seen in Figure 2, Curosurf neither altered the migration of eosinophils in response to eotaxin nor directly stimulated eosinophil migration. Therefore, the increased numbers of BAL eosinophils after surfactant treatment cannot be explained by a mechanism that solely enhances the migration of eosinophils.

Eotaxin in BAL Fluid and Chemokine Receptors on BAL Eosinophils

The potent inducer of eosinophil chemotaxis, eotaxin, was undetectable in most BAL samples from the control subjects and was low at baseline, after saline, and after Curosurf alone in the

TABLE 2. BASIC BRONCHOALVEOLAR LAVAGE FLUID DATA

Subjects	BAL	n	Recovery	Total Cells	Eosinophils		Neutrophils		Lymphocytes		Macrophages	
			(ml)	($\times 10^6$)	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)
Control subjects without atopy	B	5	78 (73–93)	6.0 (3.8–13.0)	0.0 (0.0–0.1)	0.0 (0.0–1.0)	0.2 (0.1–0.3)	3.0 (2.0–3.0)	0.4 (0.3–1.0)	10.0 (5.0–10.0)	5.5 (3.3–11.6)	87.0 (86.0–91.0)
	C	5	82 (80–94)	5.5 (3.8–22.0)	0.1 (0.0–0.2)	1.0 (0.0–1.0)	1.0 (0.2–7.0)	16.0 (5.0–32.0)	0.5 (0.3–1.8)	9.0 (7.0–11.0)	4.0 (3.2–13.0)	76.0 (59.0–85.0)
	CA	5	86 (84–88)	8.5 (5.5–38.0)	0.1 (0.1–0.4)	1.0 (0.0–1.0)	2.6 (0.6–25.1)	25.0 (10.0–66.0)	0.9 (0.5–1.6)	8.0 (4.0–11.0)	4.9 (4.3–13.2)	66.0 (30.0–81.0)
	SA	5	86 (81–94)	6.6 (4.5–137.0)	0.1 (0.0–1.4)	1.0 (0.0–2.0)	2.2 (0.4–98.6)	33.0 (8.0–72.0)	0.6 (0.2–5.5)	7.0 (4.0–10.0)	4.4 (3.5–30.1)	56.0 (22.0–81.0)
Patients with atopic asthma	B	16	73 (58–85)	4.5 (3.0–15.0)	0.0 (0.0–0.8)	1.0 (0.0–13.0)	0.1 (0.0–0.5)	2.5 (1.0–7.0)	0.6 (0.2–3.2)	11.0 (6.0–21.0)	3.7 (2.5–11.4)	83.0 (67.0–91.0)
	C	16	76 (57–90)	7.1* (1.5–150.0)	0.1 (0.0–4.5)	1.0 (0.0–10.0)	1.0† (0.1–94.5)	12.5† (2.0–70.0)	0.7 (0.1–7.5)	9.5* (5.0–14.0)	4.7 (1.3–36.0)	76.0† (16.0–88.0)
	S	6	91* (76–96)	9.7 (3.8–120.0)	0.4 (0.0–8.4)	2.5 (0.0–7.0)	2.0 (0.2–81.6)	20.0* (4.0–68.0)	0.6 (0.4–10.8)	8.0 (5.0–13.0)	6.9 (3.0–34.5)	69.5 (16.0–88.0)
	CA	10	68* (34–82)	28.8§§ (6.5–143.0)	19.5§§ (2.1–91.5)	64.0§§ (20.0–82.0)	4.6§§ (0.5–20.0)	12.5† (2.0–40.0)	2.0*§ (0.8–18.6)	7.5* (4.0–13.0)	6.1 (1.7–12.9)	17.5§§ (4.0–44.0)
	SA	16	77 (34–91)	68.0§§ (23.0–260.0)	39.8§§ (2.7–215.0)	61.0†† (5.0–85.0)	14.0††§ (2.3–104.0)	15.0† (2.0–66.0)	7.5§§ (1.2–28.7)	9.0* (4.0–13.0)	8.2† (3.2–39.5)	11.0†† (3.0–28.0)

Definition of abbreviations: B = baseline procedure; BAL = bronchoalveolar lavage; C = 10 ml of sterile saline as a control challenge; CA = allergen challenge with vehicle treatment; S = Curosurf without allergen challenge; SA = allergen challenge with Curosurf.

Fluid recovery, cell counts, and percentage of cells in BAL fluid from healthy control subjects and patients with asthma. BAL fluid was obtained at baseline (B) or 24 hours after saline challenge (C) and after the allergen challenges with either Curosurf treatment (SA) or vehicle treatment (CA). In a subgroup, Curosurf alone (S) was given instead of CA.

Values are given as median (range).

* *p* < 0.05, compared with baseline values.

† *p* < 0.01, compared with baseline values.

‡ *p* < 0.05, compared with saline challenge.

§ *p* < 0.01, compared with saline challenge.

|| *p* < 0.05, compared with vehicle treatment, for allergen-challenged segments treated with Curosurf.

§ *p* < 0.01, compared with vehicle treatment, for allergen-challenged segments treated with Curosurf.

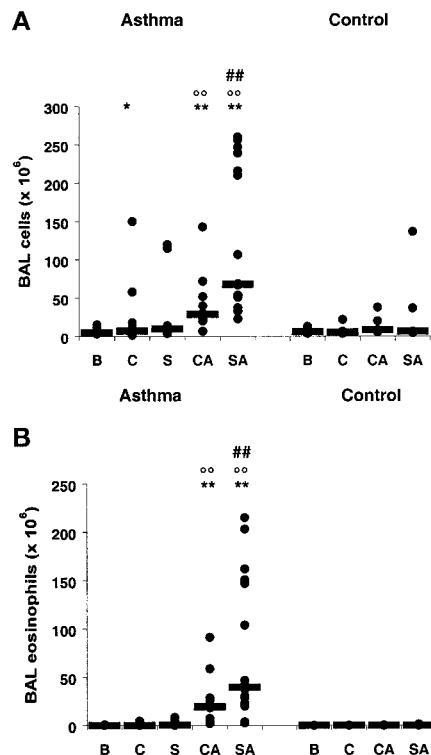


Figure 1. Total bronchoalveolar lavage (BAL) cell count (A) and eosinophil numbers (B) at baseline (B), after saline challenge (C), after Curosurf alone (S), after vehicle treatment followed by allergen challenge (CA) and after Curosurf treatment followed by allergen challenge (SA) from patients with asthma and healthy control subjects. * and ** indicate p values less than 0.05 and p values less than 0.01, respectively, compared with (B), and ∞ indicates p values less than 0.01, compared with (C). For allergen-challenged segments treated with Curosurf (SA), # indicates p values less than 0.01, compared with allergen-challenged segments treated with vehicle (CA).

patients with asthma. BAL levels of eotaxin increased after the allergen challenges, but the increase was only significant after surfactant treatment. Furthermore, these levels were significantly higher after surfactant treatment compared with vehicle treatment (Figure 3). Eotaxin is a ligand for the chemokine receptor CCR3, and recent data demonstrate that it is also an agonist for CCR5, a chemokine receptor that is involved in mouse eosinophil migration. Therefore, we measured the expres-

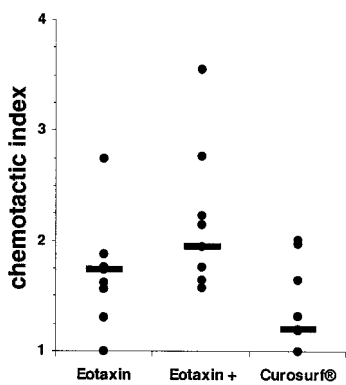


Figure 2. Eosinophils (n = 9) were pretreated with Curosurf (500 µg/ml) and medium, respectively. Cells were subsequently stimulated with eotaxin (100 ng/ml). In another set of experiments, eosinophils were directly stimulated with Curosurf (500 µg/ml). The chemotactic activity was measured using the modified Boyden chamber technique and expressed as chemotactic index: quotient of the number of migrating cells in presence of stimulus/migrating cells in presence of medium.

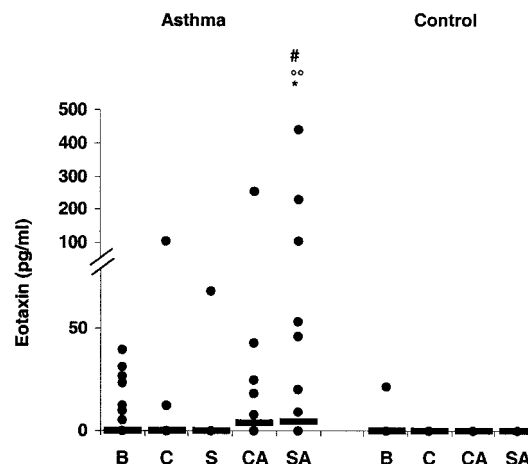


Figure 3. Concentration of eotaxin in BAL fluid measured by ELISA before (B), and 24 hours after either saline challenge (C), Curosurf alone (S), vehicle treatment followed by allergen challenge (CA) and Curosurf treatment followed by allergen challenge (SA) from patients with asthma and healthy control subjects. * indicates p values less than 0.05 compared with (B), and ∞ indicates p values less than 0.01 compared with (C). For allergen-challenged segments treated with Curosurf (SA), # indicates p values less than 0.05, compared with allergen-challenged segments treated with vehicle (CA).

sion of both chemokine receptors on eosinophils isolated from BAL 24 hours after surfactant or vehicle treatment and subsequent allergen challenge. CCR3 on eosinophils was strongly expressed, whereas CCR5 was not expressed 24 hours after allergen challenge. There was no difference in the expression of CCR3 after surfactant treatment compared with vehicle control treatment (Figure 4).

IL-5 and IFN-γ in BAL Fluid

To investigate the effect of surfactant treatment on the expression of Th1-type and Th2-type cytokines, we measured the levels of IL-5 and IFN-γ in BAL fluid by ELISA. In healthy control subjects IL-5 levels were low at baseline, and there was no difference after either provocation. In patients with asthma, IL-5 levels were low at baseline, after saline challenge, and after

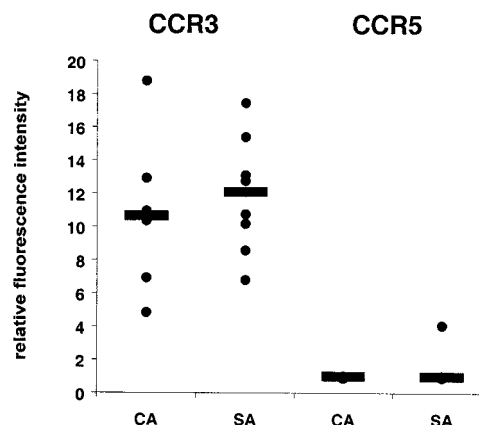


Figure 4. Expression of chemokine receptors CCR3 and CCR5 on BAL eosinophils from patients with asthma determined by flow cytometry. Cells were obtained from two segments 24 hours after treatment with either vehicle (CA; n = 6 for CCR3; n = 3 for CCR5) or Curosurf (SA; n = 9 for CCR3; n = 8 for CCR5) followed by allergen challenge.

Curosurf alone but increased significantly after the allergen challenges. Interestingly, surfactant treatment resulted in a significant increase of IL-5 in BAL fluid compared with vehicle treatment (Figure 5A).

IFN- γ levels were not significantly different between patients with asthma and control subjects at baseline. In control subjects, IFN- γ levels were unchanged after provocation with either saline or allergen, and surfactant treatment was without effect. In patients with asthma, surfactant treatment before allergen challenge reduced IFN- γ levels compared with baseline and saline controls. Compared with vehicle treatment, IFN- γ levels were lower after surfactant treatment albeit not significantly different ($p = 0.072$). A reduction of IFN- γ levels was also detectable after instillation of Curosurf alone. However, the decrease was also not statistically significant ($p = 0.125$) (Figure 5B).

IFN- γ and IL-5 in CD4 $^{+}$ and CD8 $^{+}$ BAL T Cells

Because surfactant components have been shown to influence T cell proliferation and cytokine production *in vitro*, we further analyzed the capability of T cells from BAL after surfactant treatment and vehicle treatment before allergen challenge (SA

and CA) to produce IFN- γ and IL-5. Low percentages of IL-5 $^{+}$ /CD4 $^{+}$ and IL-5 $^{+}$ /CD8 $^{+}$ T cells were found after allergen challenge, and these numbers were not influenced by treatment with surfactant (Figure 6A). In contrast, relatively high percentages of IFN- γ $^{+}$ /CD4 $^{+}$ and IFN- γ $^{+}$ /CD8 $^{+}$ T cells were present after allergen challenge. Treatment with surfactant resulted in a reduction of IFN- γ $^{+}$ T cells that was significant for the CD8 $^{+}$ subpopulation (Figure 6B).

Spreading and Retention of Alexa488-Dextran in the Presence and Absence of Curosurf

Preceding experiments revealed that Curosurf does not modulate the uptake of allergen by alveolar macrophages *in vitro* (data not shown). Our *in vivo* experiments in mice demonstrated that the intratracheal treatment with either saline or Curosurf followed by instillation of Alexa488-labeled dextran did not result in different percentages of alveolar macrophages that were found to have ingested the allergen. One hour after the instillation, 84.8% (51.2–96.3%) of the alveolar macrophages were Alexa488-dextran positive after saline treatment and 87.6% (61.8–93.9%) were Alexa488-dextran positive after Curosurf treatment. The amount of ingested Alexa488-dextran determined as mean fluorescence intensity of Alexa488-dextran-positive macrophages 1 hour after Curosurf treatment was not different from the amount of ingested Alexa488-dextran 1 hour after saline treatment (Figure 7A). These data indicate that Curosurf does not affect the distribution and accessibility of allergen by alveolar cells. Twenty-four hours after the instillations, the percentage of macrophages that were found to be Alexa488-dextran positive was 90.9% (79.9–97.2%) after saline treatment and 90.4% (82.5–96.8%) after Curosurf treatment. Furthermore, there was no effect of Curosurf treatment on the Alexa488-dextran content of macrophages (Figure 7B), suggesting that Curosurf has no effect on allergen retention and long-term accessibility of allergen by alveolar cells. The amount of intracellular Alexa488-dextran was increased at 24 hours compared with 1 hour after the instillation in both groups ($p < 0.01$), indicating an accumulation of allergen inside the macrophages during this period, which is independent of Curosurf treatment.

DISCUSSION

Studies in murine asthma models have suggested that surfactant components are effective at downregulating the allergic inflammatory response. In particular, the lung collectins SP-A and

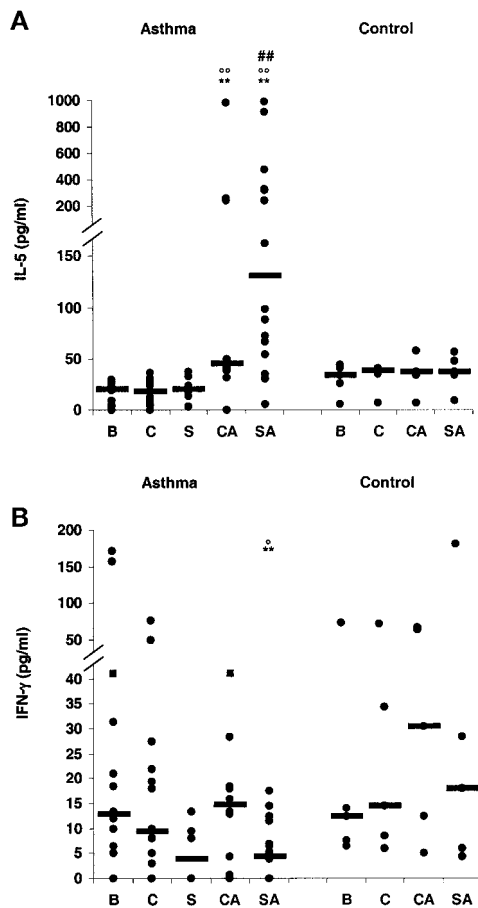


Figure 5. Concentration of interleukin (IL)-5 (A) and IFN- γ (B) in BAL fluid measured by ELISA before (B), and 24 hours after either saline challenge (C), Curosurf alone (S), vehicle treatment followed by allergen challenge (CA) and Curosurf treatment followed by allergen challenge (SA) from patients with asthma and healthy control subjects. ** Indicates p values less than 0.01 compared with (B), and ° and ∞ indicate p values less than 0.05 and p values less than 0.01, respectively, compared with (C). For allergen-challenged segments treated with Curosurf (SA), ## indicates p values less than 0.01, compared with allergen-challenged segments treated with vehicle (CA).

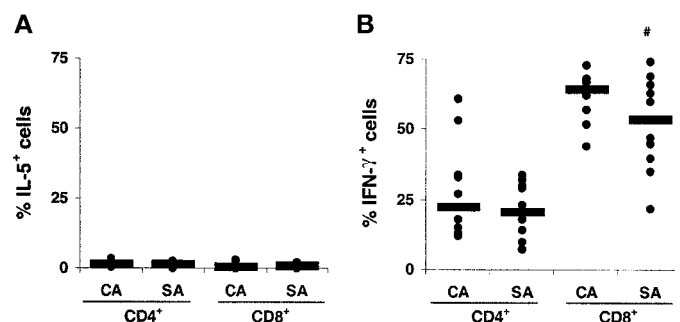


Figure 6. Intracellular expression of IL-5 (A) and IFN- γ (B) by CD4 $^{+}$ and CD8 $^{+}$ T cells from patients with asthma determined by flow cytometry after stimulation with anti-CD3/anti-CD28. Cells were obtained from two segments 24 hours after treatment with either vehicle (CA) or Curosurf (SA) followed by allergen challenge. # Indicates p values less than 0.05, compared with allergen-challenged segments treated with vehicle.

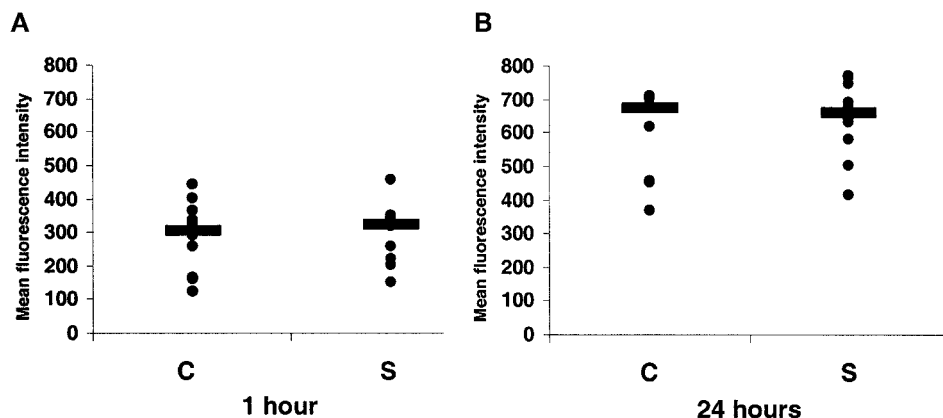


Figure 7. Flow cytometric determination of Alexa488-dextran uptake by alveolar macrophages. Mean fluorescence intensity measured at 525 nm was evaluated of Alexa488-dextran-positive macrophages obtained by BAL 1 hour (A) and 24 hours (B) after instillation of Curosurf (S) or vehicle (C) before instillation of Alexa488-labeled dextran.

SP-D have been demonstrated to modify innate and adaptive immune cell functions *in vitro* (14, 28) and to decrease the allergen-induced inflammation in animals *in vivo* (12, 13). Because of lack of data in humans and the unavailability of lung collectins for human use, the aim of the present study was to investigate the effect of the natural porcine surfactant Curosurf on the allergic inflammatory response induced by segmental allergen challenge in patients with asthma. This is the first study in humans that investigated the effect of natural surfactant on allergen-induced inflammatory changes. Previous studies have focused on lung function measurements that revealed an improvement of the early-phase bronchoconstriction after surfactant treatment (8, 10). Unexpectedly, here we demonstrate that natural porcine surfactant augmented the inflammatory response with increased numbers of total cells in BAL, the majority being eosinophils but also lymphocytes, and neutrophils were elevated compared with sham treatment.

The mechanisms by which intrabronchial surfactant augmented the eosinophilic inflammation are unclear. In an attempt to explain this increased eosinophilic inflammation, we investigated possible direct chemotactic effects of Curosurf on eosinophils *in vivo* and *in vitro*. Intrabronchial instillation of Curosurf alone did not attract eosinophils into the bronchoalveolar compartment. Moreover, there was no chemotactic effect of Curosurf on peripheral eosinophils isolated from healthy donors. Furthermore, the chemotactic effect of eotaxin in this system was unaffected in combination with Curosurf (Figure 2). Therefore, it is unlikely that a direct chemotactic effect of Curosurf accounts for increased numbers of eosinophils in BAL.

Increased numbers of eosinophils in BAL after allergen challenge are mediated by chemoattractants. Eotaxin represents the most potent chemoattractant for eosinophils, and its effect in producing tissue eosinophilia is enhanced in combination with IL-5 (29). Both mediators were elevated in patients with asthma after allergen challenge. Treatment with surfactant further increased BAL levels of eotaxin and IL-5 (Figures 3A and 4A), suggesting that these mediators are at least partly causing the eosinophilic influx into the bronchoalveolar compartment. However, it remains unclear how surfactant increases these mediators and which cellular sources are involved. In the asthmatic airways, eotaxin is secreted mainly by macrophages with a lesser contribution from T cells and eosinophils (30). The possible effect of surfactant components on chemokine release by these cells needs further research.

One further possible explanation for increased eosinophil numbers can relate to changes of their chemokine receptors under the influence of surfactant. Importantly, the chemotactic effect of eotaxin on eosinophils is mediated via chemokine recep-

tors. For example, eotaxin is the ligand for CCR3, a chemokine receptor that is strongly expressed on human eosinophils (31). Treatment of eosinophils with eotaxin results in a downregulation of CCR3 that is due to receptor internalization (24, 32, 33). A recent study has shown that eotaxin also interacts with human CCR5 (34), and a functional expression on mouse eosinophils has been described (35). Therefore, we examined the expression of both CCR3 and CCR5 on BAL eosinophils of patients with asthma after the allergen challenges. Because there were not sufficient numbers of eosinophils to isolate at baseline and after saline challenge, a comparison was only possible between eosinophils after the allergen challenges with Curosurf and vehicle treatment. CCR5 could not be detected on BAL eosinophils, which is in accordance with previous data that CCR5 is not expressed on human eosinophils (33). Interestingly, we found no difference in the expression of CCR3 between eosinophils from the Curosurf-treated and from the vehicle-treated segment. Because BAL eosinophils are already activated and had moved into the bronchoalveolar compartment, we also assessed the effect of Curosurf on chemokine receptor CCR3 and CCR5 expression on freshly isolated peripheral eosinophils. Consistently, we did not observe changes of chemokine receptor expression induced by Curosurf (data not shown). These findings suggest that the expression of CCR3 is unaffected by Curosurf treatment and that the increased numbers of eosinophils cannot be explained by a modulation of the chemokine receptor CCR3.

Because the eosinophilic inflammation in asthma is associated with a Th2-type cytokine profile with increases of Th2 cytokines and decreases of Th1 cytokines, we analyzed levels of representative cytokines and found increased concentrations of the Th2-type cytokine IL-5 but decreased concentrations of the Th1-type cytokine IFN- γ after Curosurf treatment. These data suggest that Curosurf treatment induces a shift in the direction of a Th2-type response. The mechanisms by which Curosurf modifies lymphocyte cytokine release is unclear. However, it has been reported that surfactant lipids inhibit lymphocyte proliferation and that they influence the effects of lung collectins on immune cell functions (16). Therefore, Curosurf might have affected cytokine production of lymphocytes in our study. To further elaborate on this mechanism, we investigated the ability of BAL lymphocytes to produce these cytokines on costimulation with anti-CD3/anti-CD28. Interestingly, the percentage of CD4⁺ and CD8⁺ cells that produced IFN- γ was decreased after Curosurf treatment compared with vehicle treatment. In contrast, the percentage of IL-5⁺ T cells did not change after Curosurf treatment and was relatively low in both segments, despite changes in IL-5 BAL levels. These results are consistent with our previous data that showed a decreased potential of BAL T cells to produce

IFN- γ but an unchanged potential to produce IL-5 after allergen challenge in patients with asthma (23). Curosurf treatment further reduces the potential of CD4⁺ and CD8⁺ T cells to produce IFN- γ and, therefore, the dysbalance between T cell-derived Th1-type and Th2-type cytokines is further skewed toward the Th2 type.

One mechanism to explain increased allergic inflammation after surfactant treatment might relate to biophysical surfactant properties. Surface-active material in the airways promotes spreading and might therefore deliver the instilled allergen deep into the lung. In addition, it has been demonstrated that surfactant in the airways displaces particles toward the epithelial surface (36). Changes of airway surfactant balance might improve allergen contact to the underlying mucosa, which in turn might result in an increased allergic inflammation after local surfactant administration. It becomes obvious that Curosurf indeed altered airway mechanics and improved airway openness from the finding of increased BAL recovery 24 hours after Curosurf treatment, whereas allergen-induced airway inflammation even led to a decreased recovery. However, the treatment with Curosurf did not change the distribution and retention of allergen in mice lungs quantified as the uptake of Alexa488-labeled dextran by alveolar macrophages. We used alveolar macrophages to quantify the distribution of allergen throughout the lung because it has been shown that they take up fluorescently labeled dextran by endocytosis (27). In addition, a previous study has shown that monocyte-derived dendritic cells take up house dust mite allergen (Der p 1) and fluorescein isothiocyanate-labeled dextran, which were found to colocalize inside the cells (37). Therefore, we assumed that the properties of Alexa488-dextran regarding molecular size and distribution in the fluid phase are comparable with the allergens we used in humans. Because our data demonstrate that Curosurf treatment does not affect the accessibility of Alexa488-dextran by alveolar macrophages, we assume that this can be applied to other alveolar cells including dendritic cells, T cells, epithelial cells, and further cells that are involved in the allergic inflammation. However, our observations remain restricted to alveolar macrophages that were accessible by our BAL procedure, and the lavage material might not be wholly representative of all alveolar constituents. In addition, transfer of conclusions from the mouse model to the human system is limited at least because of the differences in airway anatomy between both species. Despite these limitations, our findings suggest that the effects of Curosurf on the allergic airway inflammation are more likely due to immunomodulatory properties than to biophysical properties of the surfactant.

Our study cannot answer the question whether the observed effects are restricted to Curosurf or are mediated by all commercially available surfactants. A previous comparison of surfactant preparations by Bernhard and coworkers (22) demonstrated similarities between Curosurf and the natural bovine surfactants Alveofact and Survanta regarding ultrastructural composition and quantity of SP-B and SP-C. However, Curosurf contained more sphingomyelin and had a lower phosphatidylcholine content than Alveofact and Survanta. The natural surfactant preparations have been intensively used in preterm infants for treatment of infant respiratory distress syndrome, and allergic reactions have not been reported so far. We excluded contaminations of LPS that of course have the potential of promoting allergic reactions. However, it has been reported that platelet-activating factor can be found in Curosurf (38). Platelet-activating factor might promote allergic reactions (39), but it is unlikely that contaminants add significantly to the finding of increased eosinophilic inflammation because Curosurf alone did not induce eosinophilia. Further studies will be necessary to compare surfactant preparations regarding their immunomodulatory properties.

This will be of major importance when surfactant preparations are tested as treatment for allergic airway diseases (10). In addition, surfactant treatment of adult lung disease may require specifically designed tailored preparations.

Curosurf consists of 99% phospholipids and 1% SP-B and SP-C but lacks SP-A and SP-D. A previous study has shown an inhibitory effect of pulmonary SP-A and SP-D on allergen-induced lymphocyte proliferation (40). In a mouse model of allergic inflammation, SP-A and SP-D protected against *Aspergillus*-induced pulmonary hypersensitivity and led to a marked shift from Th2 to Th1 response (13). These data suggest that SP-A and SP-D might be more suitable to reduce the allergic inflammation than surfactant lipid preparations that lack these proteins. Further studies are necessary to investigate the role of surfactant in allergic asthma. The potential of SP-A and SP-D to alleviate the allergic inflammation has to be tested in patients with asthma.

Taken together, the present findings demonstrate that Curosurf treatment results in an augmentation of the allergic inflammation after local allergen challenge in patients with asthma. Curosurf increases the eosinophilic inflammation driven by eosinophils and IL-5 and induces a shift toward a Th2-type response. Our results suggest that Curosurf does not affect allergen distribution and retention. We conclude that treatment with Curosurf is not suitable to decrease the allergic response in patients with asthma. This might also comprise treatment with other surfactant preparations and should be taken into consideration when studying their potential treatment effect in asthma.

Conflict of Interest Statement: V.J.E. has no declared conflict of interest; A.H. has no declared conflict of interest; Y.D. has no declared conflict of interest; J.E. has no declared conflict of interest; R.B. has no declared conflict of interest; H.K. has no declared conflict of interest; M.D. has no declared conflict of interest; A.B. has no declared conflict of interest; N.K. has no declared conflict of interest; J.M.H. has no declared conflict of interest, the pharmaceutical manufacturer of Curosurf (Nycomed) provided the drug free of charge and no other financial support was received from Nycomed.

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