

Increased surfactant protein D in rat airway goblet and Clara cells during ovalbumin-induced allergic airway inflammation

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Summary

Background Structural remodelling of airways in asthma that follows inflammation may be affected by surfactant protein D (SP-D)-mediated effects on the immune response.

Objective To determine potential sites of SP-D interaction with the pulmonary immune response, we examined the distribution of immunoreactive SP-D in an experimental model of allergen-induced airway inflammation using immunohistochemistry, biochemical methods and *in situ* hybridization.

Methods The experimental model used subcutaneous injection of ovalbumin in adult rats, which induced an airway response to inhaled nebulized ovalbumin. Three groups of rats (ovalbumin, ovalbumin + dexamethasone and saline) were challenged thrice weekly for 3 weeks. A fourth group of seven rats (naive) were taken from the same delivery of rats as the other groups. Lungs were then lavaged to determine total cell count, eosinophil count, ovalbumin-specific IgE by enzyme-linked immunosorbent assay and SP-D by immunoblot. Tissue samples were fixed and embedded, and sections were studied for the infiltration of eosinophils and for expression of SP-D protein by histochemistry and mRNA by *in situ* hybridization.

Results Ovalbumin induced perivascular and peribronchiolar eosinophilia which could be prevented by dexamethasone treatment. In addition, the ovalbumin-specific IgE levels in serum and bronchoalveolar lavage fluid of ovalbumin-challenged animals were enhanced. Increased amount of SP-D in lavage and tissue, particularly in type II pneumocytes, in Clara cells and, surprisingly, in hyperplastic goblet cells of inflamed lungs was found. SP-D mRNA was detected in goblet cells as well as in type II pneumocytes and Clara cells. Dexamethasone treatment did not affect level of SP-D immunoreactivity.

Conclusion SP-D accumulation is increased in this model of allergen-induced eosinophilia, both in upper and lower airways. The increase is unaffected by dexamethasone.

Keywords airway remodelling, asthma, BAL, dexamethasone, eosinophil recruitment, goblet cell, ovalbumin-induced eosinophilia, rat, surfactant protein D

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Introduction

Asthma is a chronic inflammatory disease accompanied by airway obstruction and hyper-responsiveness. Asthmatic inflammation is characterized by the expression of multiple genes for inflammatory mediators such as cytokines, leukotrienes, adhesion molecules, enzymes and receptors. Infiltration of eosinophils and T lymphocytes, goblet-cell hyperplasia, smooth-muscle-cell hyperplasia, subepithelial deposition of extracellular matrix proteins and neovascularization predominate in chronic asthma [1–4]. However, the pathogenesis and the functional consequences of structural alterations of bronchial tissue, so-called airway remodelling, are not well known.

Various *in vivo* rat models of pulmonary allergic response have been developed either by intratracheal installation of sephadex or by exposure to aerosolized allergens (reviewed in 5–7). Inflammatory cell infiltrates, particularly eosinophils, were observed in the peribronchiolar area in these animal models. Eosinophils are considered to be the major inflammatory leucocytes involved in asthma.

Surfactant proteins, SP-A and SP-D, members of the family of C-type lectins, are involved in the pathogenesis of airway inflammation by interaction with carbohydrate structures on the surface of pathogens, such as viruses, fungi and bacteria, and by enhancement of phagocytosis by alveolar macrophages [8, 9]. Their role in asthma is much less clear. In particular, SP-D may be important in modulating the inflammatory response in asthma. It has been shown to decrease allergen-induced lymphocyte proliferation and histamine production in peripheral blood from asthmatic and non-asthmatic children [10]. The effects of acute asthma on SP-D abundance may depend on

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species. Bronchoalveolar lavage (BAL) in asthmatic adults showed increased SP-A and SP-D accumulation [11], while both SP-A and SP-D were decreased in an experimental murine model of asthma [12]. In a study by Wright et al. [13], however, neither SP-A nor total protein concentration in the BAL of asthmatics was altered. Airway remodelling may depend on the location of the interaction of SP-D with the pulmonary immune response. Haczku et al. [14] attributed the elevated level of SP-A and SP-D in an *Aspergillus fumigatus*-induced asthma model solely to type II pneumocytes. Because airway remodelling may be affected by immune modulation, we were interested in determining whether SP-D was expressed in other cell types of the lung potentially relevant to small airway inflammation. To investigate the role of SP-D in eosinophil-rich lung inflammation we examined its immunohistochemical distribution in tissue using monoclonal SP-D antibodies and *in situ* RNA hybridization, and determined the amount of SP-D protein in the BAL fluid in a recently developed rat model of ovalbumin challenge-induced lung inflammation [7].

Methods

Immunization protocol

Male Brown Norway rats (average weight = 200 g) were actively sensitized [15] by subcutaneous injection of a suspension of 10 µg ovalbumin grade VI (Ova; Sigma-Aldrich, Steinheim, Germany) and 20 mg Al(OH)₃ gel (Merck, Darmstadt, Germany) in 0.5 mL 0.9% NaCl solution on days 1, 14 and 21. The animals simultaneously received intraperitoneal (i.p.) 0.25 mL of 400 × 10⁶/mL heat-inactivated *Bordetella pertussis* bacteria in phosphate-buffered saline (PBS).

Challenge protocol

On the 28th day the animals were exposed to a nebulized aerosol of an ovalbumin suspension (10 mg ovalbumin in 1 mL 0.9% NaCl) in a nose-only inhalation system for 60 min to provoke an influx of eosinophil granulocytes into the airways [7]. Twenty-one positive control and 21 dexamethasone-treated rats inhaled the ovalbumin suspension aerosol, and 21 control rats inhaled only 0.9% NaCl aerosol. The aerosol was generated by a nebulizer driven by compressed air at 0.2 MPa. The three groups of rats were challenged in this way on Monday, Wednesday, and Friday of each week for 3 weeks. A maximum of 3 days between challenges was allowed so that the antigen was administered before the eosinophil level dropped, an occurrence that happened on the 4th day after the last challenge [16]. On days 33, 40 and 47 (all Wednesdays), seven rats from each group were removed prior to the challenge that day: four rats for lavage and three rats for histological examination. The 21 dexamethasone-treated rats received a dosage of 1 mg/kg in 0.2 mL 0.9% NaCl solution/kg rat i.p. daily, except for weekends, 30 min before challenge. The first dose was given on day 27. The dosage was decreased on the 34th day to 0.1 mg/kg in 0.2 mL 0.9% NaCl solution/kg rat i.p. because the rats receiving dexamethasone exhibited moderate weight loss (See Results).

The fourth group of rats (naive) was taken from the same delivery of rats as the other groups. They were neither sensitized, challenged nor treated with substance or placebo. Apart

from this they were treated in the same way as the other groups of animals. They were killed on the 47th day of the experiment.

Bronchoalveolar lavage

The rats were first killed by urethane overdose (1.5 g/kg i.p.). The trachea was exposed and a catheter introduced. The BAL was performed with 3 × 4 mL Hanks' balanced salts (Sigma-Aldrich) solution. The recovered washes were pooled, yielding ~10 mL for each animal. Total cell count and count of eosinophil granulocytes per 1 µL BALF-fluid from pooled BAL of each animal were determined using an automatic cell counter (Technicon H1E, Bayer Diagnostics GmbH, München, Germany). Cells per total lavage volume were determined as follows: total cell count of eosinophil count in 1 µL × BAL-recovery in µL/animal. The supernatant was obtained from the pooled BAL of each animal by centrifugation at 1000 g for 10 min at 4 °C in K-EDTA coated tubes and stored at -18 °C.

Serum and BAL ovalbumin-specific IgE measurements

Ovalbumin-specific IgE titres were estimated by enzyme-linked immunosorbent assay (ELISA) based on a method from van Halteren [17]. The microtitre plates were first coated overnight at 4 °C with 50 µL/well of 100 µg/mL ovalbumin grade VI in 0.1 M Na₂HPO₄ buffer adjusted to pH 9. The plates were then washed in PBS with 0.05% Tween®. Afterwards a solution of PBS with 10% calf serum was added and left for 2 h at RT (20–22 °C). After a further washing, 100 µL of the samples were brought into their respective wells and incubated for 2 h at RT. The BAL and serum samples were diluted by 1 : 10 and 1 : 50 with PBS, respectively. The plates were once again washed and the captured IgE detected using 100 µL/well 1 µL/mL mouse anti-rat IgE Biotin (Biozol Diagnostica: MCA 193B; Eching, Germany) in blocking buffer at RT for 1 h. The plates were washed again and 100 µL/well, 5 µL/mL streptavidin-peroxidase conjugate added and left for 45 min at RT. The last wash was performed, 100 µL tetramethylbenzidine was placed in each well and left for approximately 20 min. The reaction was stopped by adding 50 µL/well of 2.5% H₂SO₄ and the OD recorded at 450 nm (against 620 nm).

Histology and immunohistochemistry

Whole lungs including the trachea were removed and filled with 4% formaldehyde. The proximal trachea was ligated and the lung was placed in 4% buffered formaldehyde, pH = 7, which was exchanged 24 h later with fresh fixative, then embedded in paraffin. Some sections were stained with haematoxylin and eosin, and others with Alcian blue or periodic acid-Schiff (PAS) for staining of acidic and neutral mucins, which were counterstained with haematoxylin. Antigen retrieval using microwave heating (2 × 5 min, 800 Watt) in a 0.01-M sodium citrate solution was used to optimize immunostaining.

For immunostaining we employed the streptavidin-peroxidase system (Vector Elite Kit, Vector Laboratories, distributed by ALEXIS, Grüneberg, Germany) according to the manufacturer's instructions with the following changes. Briefly, endogenous peroxidase was blocked by incubating the sections in a fresh solution of 0.3% hydrogen peroxide in absolute methanol for 30 min at RT. This step was followed by the blocking with normal horse serum (10% in PBS) for 20 min at RT. We used IIE11 and IIF6 against SP-D (dilution 1:10;

60 min incubation at 37 °C). The generation and the specificity of the monoclonal antibodies have been previously described [18]. After washing (three times for 10 min in PBS) the sections were overlaid with biotinylated horse anti-mouse IgG, which was pre-adsorbed with rat serum (Vector) diluted 1 : 100, 30 min at RT, and washed again twice for 10 min. This was followed by incubation with the peroxidase-labelled streptavidin, 30 min at RT and further washings in PBS and distilled water. The sections were developed with 0.05% 3, 3-diaminobenzidine tetrahydrochloride (Serva, Heidelberg, Germany) in PBS and 0.03% hydrogen peroxide, counterstained with Mayer's haematoxylin and coverslipped with Canada balsam.

Controls were performed by substitution with PBS or incubation with a non-relevant mouse monoclonal antibody of the same isotype and concentration.

Western blot

For western blotting of SP-D, the proteins of 3 mL of BAL fluid were precipitated by ultracentrifugation at 100 000 *g* at 4 °C for 2 h and redissolved in 150 µL of sample buffer (360 mM Tris, pH 6.8 containing 190 mM sodium dodecyl sulphate, 5% 2-mercaptoethanol, 0.03% bromophenol blue and 55% vol. glycerol). Twenty microlitres of the protein solution (containing the protein isolated from 400 µL BAL fluid) were subjected to polyacrylamide gel electrophoresis.

In addition, the supernatant of the BAL of a naive rat was examined for SP-D remaining after ultracentrifugation. The proteins of two samples (3 mL each) of supernatant were precipitated for 60 h at 4 °C: one sample by addition of ammonium sulphate up to a final concentration of 4.9 M, one sample by addition of trichloroacetic acid up to a concentration of 1.84 M. The precipitated proteins were separated by centrifugation at 20 000 *g* at 4 °C for 30 min and redissolved in 22 µL sample buffer. These samples were subjected to polyacrylamide gel electrophoresis, then transferred to a nitrocellulose membrane, and blocked with 5% non-fat milk in Tris-buffered saline, pH 7.6. Detection of SP-D was performed with monoclonal mouse anti-SP-D IIE11 (dilution 1:100) as the primary antibody and horseradish peroxidase-conjugated anti-mouse immunoglobulin (New England Biolabs, Beverly, MA, USA) as the secondary antibody (dilution 1:2000). The blots were developed with ECL reagents (New England Biolabs) and exposed to Hyperfilm (ECL, Amersham Pharmacia, Freiburg, Germany). The detected bands were analysed by densitometry.

In situ hybridizations

These were performed as previously described with the following modifications [19]. Water used for preparation of solutions was treated with diethylpyrocarbonate overnight

and then autoclaved to inhibit ribonucleases. Paraffin-embedded 5-µm sections were deparaffinized in xylene, rehydrated and treated with proteinase K, 5 µg/mL for 0.5 h at 37 °C, followed by rinsing in deionized water. Sections were dehydrated through graded ethanols, allowed to air dry, then hybridized with antisense or sense 11-digoxigenin UTP-labelled cRNA riboprobes transcribed from a cDNA encoding rat SP-D, as previously described [19]. cRNA probe was subjected to alkaline hydrolysis to reduce the transcript length to an average of 0.15 kb. Hybridization and detection employed 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5 (buffer 1), 2% blocking reagent in buffer 1 (buffer 2), 0.1 M NaCl, 0.1 M Tris-HCl, 0.05 M MgCl₂, pH 9.5 (buffer 3; Roche, Indianapolis, IN, USA).

Hybridization conditions A total of 0.5 µg/mL cRNA probe were dissolved in pre-warmed UltraHyb (Ambion, Austin, TX, USA) at 65 °C, and 100 µL/section were applied to each section, which was encircled by a hydrophobic film (PAP Pen, Research Products International, Mount Prospect, IL, USA). After overnight hybridization in moist chambers containing 5 × standard salt citrate buffer (SSC), 50% formamide at 42 °C, sections were washed in 2 × SSC for 10 min followed by a high stringency wash in 400 mL 0.1 × SSC at 60 °C for 15 min. Detection was performed by blocking the sections for at least 1 h, rinsing in buffer 1, then overlaying sections with 100 µL of antidigoxigenin antibody-alkaline phosphatase conjugate 1 : 100 in buffer 2 overnight at 4 °C. Antibody was washed off in two 10-min washes in buffer 1, followed by a 2-min incubation in buffer 3. Excess buffer was adsorbed from near the edge of the section, which was then covered with 200 µL of substrate (NBT/BCIP prepared according to the manufacturer's instructions, Roche) and incubated at RT in a moist chamber in the dark for 2 h. Controls included sections hybridized with sense 11-digoxigenin-labelled RNA probes and sections treated with RNase A as previously described before hybridization. Colour development was stopped in Tris-HCl 10 mM pH 7.5, EDTA 5 mM pH 7.5, and mounted in Probe Mount (Innovex, Richmond, CA, USA) prior to photomicroscopy.

Morphometry

H&E-stained slides were studied light-microscopically, and morphometric measurements were made with a Leica microscope (Bensheim, Germany) at 160×. The microscope was connected to a video camera (Sony DPX950P, Sony Corporation, Tokyo, Japan). Images of airway epithelium were recorded and analysis was performed on a personal computer (Soft Imaging System GmbH, Münster, Germany) with images displayed on the monitor, using the public domain image program (NIH-Image version 1.6). For each treatment group, two stained lung

Table 1. Ovalbumin-specific IgE ELISA OD values of serum collected on days 33, 40 and 47

Treatment	<i>n</i>	Day 33 OD value ± SEM	Day 40 OD value ± SEM	Day 47 OD value ± SEM
Naive group (NAI)*	3*			0.02 ± 0.02*
NaCl challenge group (NaCl)	3*	0.77 ± 0.39*	0.74 ± 0.08*	0.43 ± 0.05†
Ovalbumine challenge group (Ova)	3*	1.45 ± 0.40*	1.15 ± 0.24*	0.38 ± 0.03†
Ova challenge + dexamethasone challenge (DEXA)	3*	0.88 ± 0.12*	0.40 ± 0.03*	0.30 ± 0.02†

*Statistical significant difference versus Ova challenge control, *P* < 0.05. †Statistical significant difference versus naive group, *P* < 0.05 (one-way ANOVA followed by Tukey's post-hoc multiple comparisons).

Table 2. Ovalbumin-specific IgE ELISA OD values of BAL collected on days 33, 40 and 47

Treatment	<i>n</i>	Day 33 OD value $\pm$ SEM	Day 40 OD value $\pm$ SEM	Day 47 OD value $\pm$ SEM
Naive group (NAI)*	4*			0.00 $\pm$ 0.00*
NaCl challenge group (NaCl)	4*	0.06 $\pm$ 0.03*	0.03 $\pm$ 0.01*	0.02 $\pm$ 0.003*
Ovalbumine challenge group (Ova)	4*	0.13 $\pm$ 0.025*	0.23 $\pm$ 0.012†	0.33 $\pm$ 0.01†‡
Ova challenge + dexamethasone challenge (DEXA)	4*	0.15 $\pm$ 0.03*	0.16 $\pm$ 0.01*†	0.16 $\pm$ 0.02*†‡

*Statistical significant difference versus Ova challenge control, $P < 0.05$. †Statistical significant difference versus NaCl challenge control, $P < 0.05$.

‡Statistical significant difference versus naive group, $P < 0.05$ (one-way ANOVA followed by Tukey's posthoc multiple comparisons).

sections from three rats each were analysed. The total number of eosinophils in the bronchial wall was determined according to the method described by Palmans et al. [7]. Briefly, the total wall area (WAt) was measured as the difference between the outer adventitial area and the area defined by the basement membrane of the epithelium.

Statistics

All data are expressed as mean $\pm$ SEM. For each time-point, statistical significance of the differences between experimental groups was tested by one-way analysis of variance (ANOVA) followed by Tukey's multiple post-hoc comparisons. A level of $P = 0.05$ was considered significant.

Results

Ovalbumin-specific IgE

Serum levels of ovalbumin-specific IgE increased in sensitized rats exposed to ovalbumin for 2 and 3 weeks as compared with control animals (Table 1). Measurement of IgE values in BAL fluid revealed significant differences between ovalbumin-challenged and NaCl-challenged as well as dexamethasone-treated animals (Table 2).

Ovalbumin-induced eosinophilia in rat airways

Antigen challenge resulted in a significant increase in the total number of cells as well as in the number of eosinophils recovered from BAL as compared with controls (Fig. 1). Dexamethasone treatment before antigen challenge reduced this increase in inflammatory cells.

After primary sensitization, a 1-week challenging to aerosolized ovalbumin induced an increase of cellular cell infiltrates, particularly eosinophils, in the perivascular and peribronchiolar area of rat airway sections as compared to the NaCl-exposed animals (Figs 2a–c). More prolonged exposure to ovalbumin resulted in a further increase of eosinophilia at 2 weeks and a slight decrease to the 1-week level at about 3 weeks (Table 3). Animals that received dexamethasone treatment had significantly lower numbers of eosinophils than those treated with NaCl (Table 3). Their body weight is given in Table 4.

Ovalbumin-induced increase in SP-D expression

Histochemistry and in situ hybridization In normal rat lungs, SP-D-immunoreactivity was confined to type II pneumocytes, alveolar macrophages, Clara cells and, surprisingly, to goblet cells of the airways (Table 5; data not shown). Ovalbumin exposure induced a strong increase of SP-D

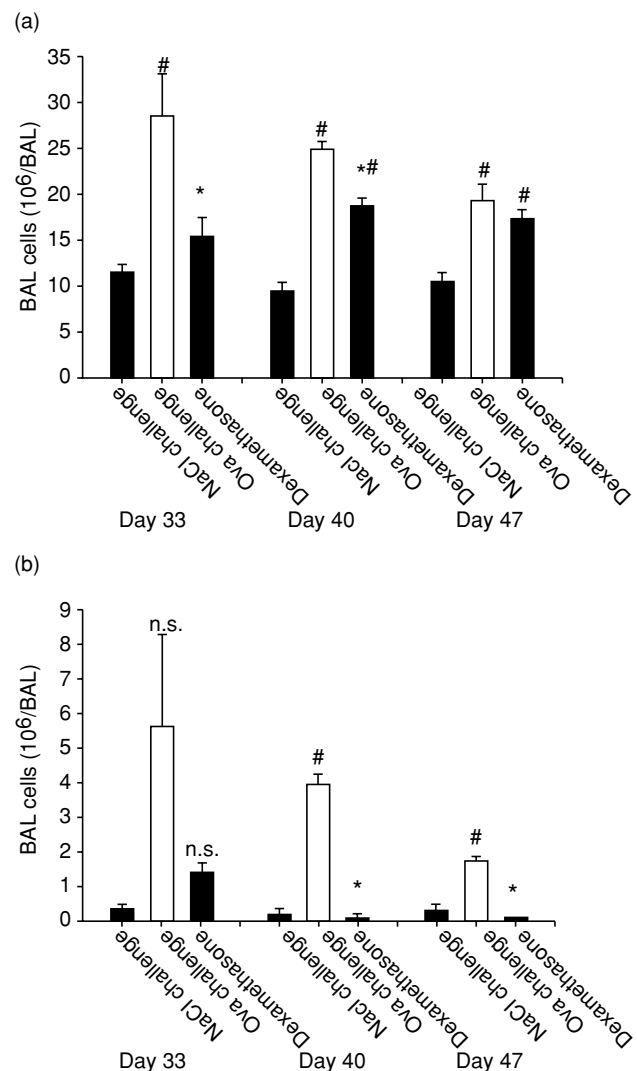


Fig. 1. Average total cell and eosinophil cell count in rat BAL fluid ($n = 4$). # = statistical significant difference vs. NaCl challenge control, $P < 0.05$. * = statistical significant difference vs. Ova challenge control, $P < 0.05$. (One-way ANOVA followed by Tukey's post-hoc multiple comparisons). (a) Naive BAL fluid total leucocyte content is 12.90 ± 2.6 SEM (10^6 /BAL). (b) Naive BAL fluid eosinophil content is 0.28 ± 0.09 SEM (10^6 /BAL).

immunoreactivity in type II pneumocytes, in Clara cells and in hyperplastic goblet cells (Figs 2e and g). Consecutive serial sections of immunostained and Alcian blue-stained lung unequivocally demonstrated the presence of SP-D in goblet cells (Figs 2f and g).

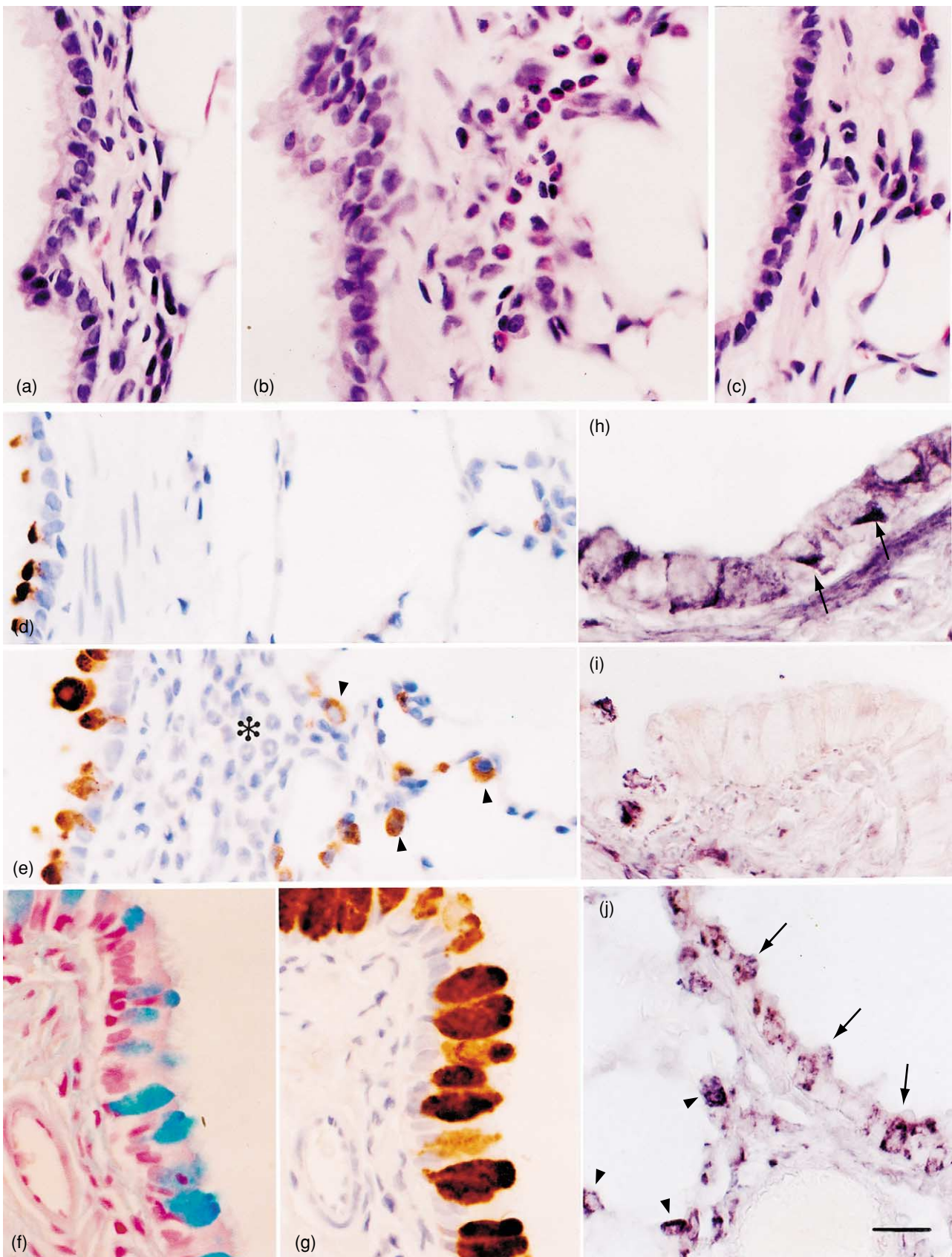


Fig. 2. Paraffin sections of rat lung (a–j), bar = 33 μ m. (a–c) H&E staining of normal (a), ovalbumin-challenged (b) and ovalbumin-challenged and dexamethasone-treated (c) lung. Note the peribronchial infiltration of eosinophils in (b). (d and e) Immunohistochemical distribution of SP-D in normal (d) and ovalbumin-challenged (e) lung. Arrowheads in (e) indicate the increased immunoreactivity in hyperplastic type II pneumocytes. Area in (e) indicated by asterisk shows the eosinophil infiltration in counterstaining. (f and g) Consecutive serial sections of hyperplastic goblet cells stained for Alcian blue (f), and SP-D (g). (h–j) *In situ* hybridization for SP-D shows positive mRNA signal in goblet cells (h, arrows), in Clara cells (j, arrows) and in type II pneumocytes (j, arrowheads). (i) No specific signal is seen in sections with sense RNA probe.

Table 3. Peribronchial eosinophils (cell profiles/mm² total airway wall)

Treatment	Day 33	Day 40	Day 47
NaCl	112 ± 55	158 ± 127	59 ± 48
Ova challenge	517 ± 251*	1088 ± 477*	599 ± 181*
Ova challenge + dexamethasone challenge	10 ± 8	50 ± 26	14 ± 12

* $P \leq 0.05$ comparing Ova group with NaCl or Ova + dexamethasone group (one-way ANOVA followed by Tukey's post-hoc multiple comparisons).

Table 4. Changes in body weight of animals harvested on day 47

	Weight day 27 (SEM)	Weight day 46 (SEM)	% difference
Naive group (NAI)	251.4 ± 3.7	287.14 ± 5.11	14.2
NaCl challenge group (NaCl)	308.1 ± 4.5	301.43 ± 6.02	-2.17
Ova challenge group (Ova)	298.1 ± 5.1	281.14 ± 6.19	-5.7
Ova challenge + dexamethasone challenge (Dexa)	302.1 ± 7.3	256.43 ± 8.00	-15.13

Table 5. Semiquantitative analysis of SP-D immunoreactivity in rat lung tissue

	NaCl challenge group	Ova challenge group	Ova challenge + dexamethasone challenge group
Type II pneumocytes	2	3	2
Alveolar macrophages	0	1	1
Goblet cells	0†	3*	3*
Clara cells	2	3	2

SP-D immunoreactivity score: 3, strongly positive (*goblet cell hyperplasia); 2, positive; 1, moderately positive; 0, negative (†only a few single goblet cells with strongly positive staining).

Sections employed for mRNA *in situ* hybridization clearly showed a positive signal in goblet cells (Fig. 2h). As internal control, type II pneumocytes and Clara cells revealed strong mRNA expression (Fig. 2j). Sections examined with the SP-D sense probe showed no specific labelling (Fig. 2i). We did not detect SP-D mRNA in other cell types in the lung.

In addition, SP-D immunostaining was present in higher amounts in alveolar macrophages, which were particularly found as cell clusters in newly formed granulomas. These granulomas were also surrounded by a higher number of SP-D-positive type II pneumocytes (not shown).

Western blot Figure 3 shows that SP-D precipitated from BAL fluid was detected by immunoblotting as one single band of protein at a molecular mass of about 47 kDa. The vast majority of SP-D was precipitated by ultracentrifugation (Fig. 3, lane 2). Only traces of SP-D were detected in ammonium sulphate precipitates of the supernatant of ultracentrifuged bronchoalveolar fluid (Fig. 3, lane 3), whereas trichloroacetic acid did not precipitate any SP-D detectable by anti-SP-D antibody binding (Fig. 3, lane 4).

SP-D amounts were determined by immunoblotting of proteins obtained from concentrated bronchoalveolar fluid

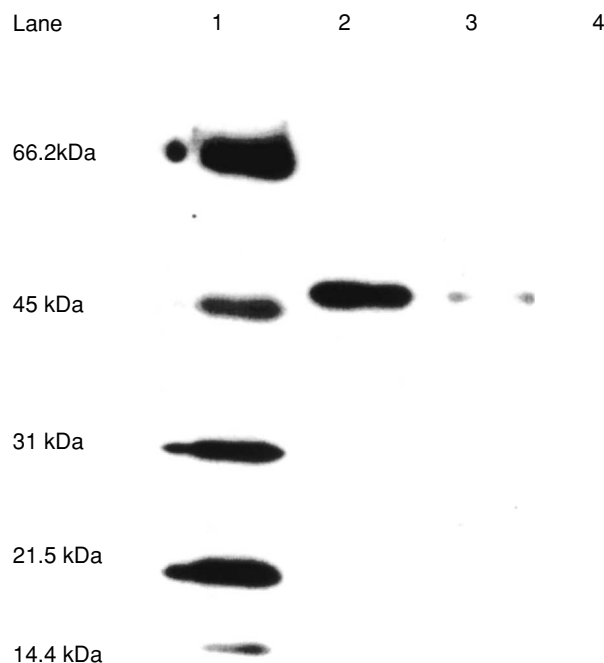


Fig. 3. Immunoblot of proteins of the bronchoalveolar fluid of a naive rat for SP-D. Lane 1: molecular mass standards. Lane 2: proteins of the bronchoalveolar fluid concentrated by ultracentrifugation. The proteins remaining in the supernatant after ultracentrifugation were precipitated by addition of ammonium sulphate (lane 3) or trichloroacetic acid (lane 4) and the precipitates were subjected to polyacrylamide gelelectrophoresis and immunoblot.

followed by densitometric evaluation of the bands. The immunoreactive SP-D changed significantly after ovalbumine challenging, as shown in Fig. 4. Dexamethasone partly prevented the increase in SP-D in the BAL fluid at 33 and 40 days, but there were no significant differences in SP-D in the fluid at 47 days.

Discussion

Our results indicate that ovalbumin treatment of rats increased SP-D accumulation in upper and lower airways, in addition to increasing the eosinophilia and enhancing ovalbumin-specific IgE levels. Surprisingly, SP-D immunopositivity and mRNA expression were not only restricted to type II pneumocytes and more distal located non-ciliated bronchiolar cells (Clara cells), but included the more proximally located goblet cells of the bronchial epithelium. In this study, the SP-D tissue immunoreactivity parallels the amount of SP-D in the BAL fluid represented by the amount of SP-D in precipitates after ultracentrifugation of the fluid.

In vivo animal models, which induce a persistent eosinophilic airway inflammation, may be a useful approach to the study of airway remodelling in asthma [5, 20, 21, for review see 6]. In the present study we employed a model of ovalbumin exposure [7]. As has been observed in other similar studies, concerning rat and mouse models of antigen-induced pulmonary inflammation [7, 22], many of the markers of inflammation normalize after chronic antigen exposure. Despite a considerable drop in eosinophil numbers in the BAL fluid of the ovalbumin challenged group by day 47, the level was still over four times that of the

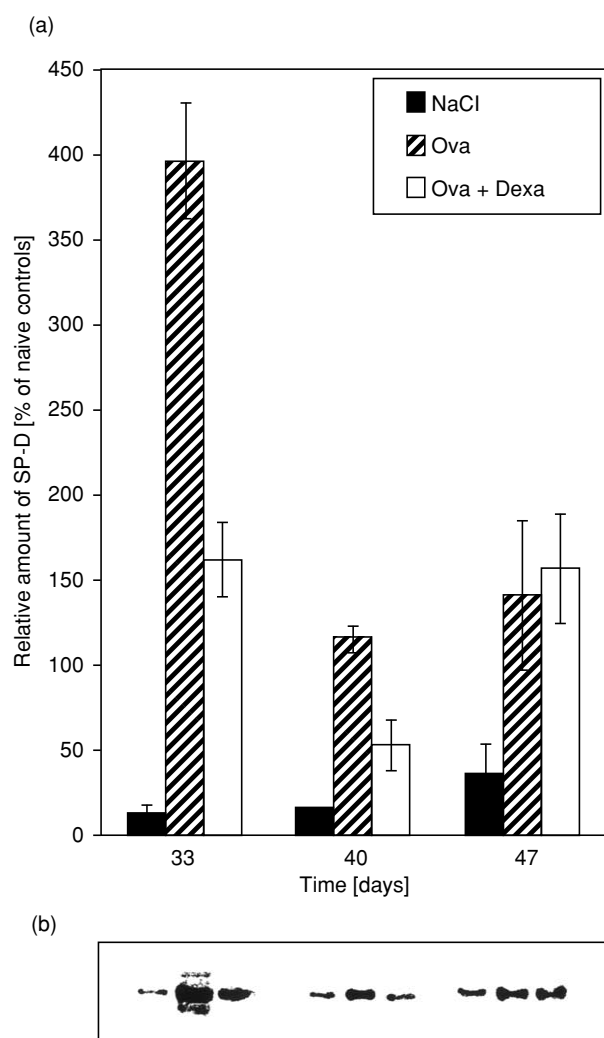


Fig. 4. Amounts of SP-D in bronchoalveolar fluid of rats after ovalbumin challenge. (a) The results of the densitometric evaluation of immunoblot data; (b) one representative blot (mean $\pm$ SD, $n = 3$).

NaCl group. The much longer, 12-week, study of Palmans et al. [7] mirrored the reduction in BAL fluid eosinophil number shown here. Additionally they showed that, despite this initial decrease, the eosinophil numbers in BAL fluid and peribronchiolar area maintained relatively high levels until the 12th week and that this was also accompanied by such characteristics of chronic lung inflammation as progressive deposition of fibronectin into the 12th week; a finding that suggests that despite such a drop in eosinophil numbers in the BAL fluid the inflammatory process is still continuing. The serum ovalbumin-specific IgE measurements supports this supposition. Although in all the sensitized groups there was a strong decrease in serum ovalbumin-specific IgE between days 33 and 47. There were still levels 10–20 times that of the NaCl group and, during this time, the levels of IgE in BAL fluid of the ovalbumin-challenged group rose.

Inflammatory reaction and its inhibition by dexamethasone was consistently reproduced in our model. Glucocorticoids have both anti-inflammatory and lung-maturational properties. Glucocorticoid treatment prevents airway hyper-reactivity in

antigen-challenged animals by inhibiting eosinophil recruitment (reviewed in [23]). Glucocorticoid effects vary depending on the model. Dexamethasone had no effect on type II cell synthesis or secretion of SP-D *in vitro* [24], and did not induce SP-D *in vivo* [25]. In the present study, SP-D was unaffected by dexamethasone in ovalbumin-challenged rats. In contrast, dexamethasone increases fetal and newborn lung SP-D mRNA and protein [26–28]. Effects of dexamethasone on SP-D expression may depend on age and the state of inflammation.

Our new finding that SP-D is expressed in goblet cells indicates that SP-D is also synthesized by epithelial cells in the upper respiratory tract. The precise role of SP-D in the upper airway has yet to be clarified. Reports describe increased SP-D and SP-A in patients with bronchial asthma [10] as well as in experimental models of airway inflammation [11, 14] and SP-D may, together with SP-A, provide protection against hypersensitivity in an *Aspergillus* inhalation model [29]. The interaction of SP-D with microbes or with mediators produced during inflammation and injury may influence the overall inflammatory response in large, goblet cell-containing airways, and itself appears to be regulated by the hypersensitivity response.

Goblet cell mucin production contributes to the pathophysiology of airway obstruction in asthma [30]. SP-D secreted by goblet cells could bind to known bacterial mucin secretogues, such as *Pseudomonas aeruginosa* [31], and may diminish mucin production and limit adverse effects on airway obstruction.

We found that ovalbumin-induced eosinophilia can be routinely reproduced in the rat. Immunoreactivity for SP-D is increased in our model of allergen-induced airway inflammation. We also demonstrate that SP-D is synthesized in goblet cells of the upper respiratory tract, particularly under hypersensitivity conditions promoting goblet cell hyperplasia. We speculate that allergen-challenged induced SP-D in the upper airway will modify the local immune response, and may enhance host defense against inhaled pathogens in the upper airway.

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References

- 1 Dunnill MS. The pathology of asthma. Ciba Found Study Group 1971; 38:35–46.
- 2 Djukanovic R, Roche WR, Wilson JW et al. Mucosal inflammation in asthma. *Am Rev Respir Dis* 1990; 142:434–57.
- 3 Beasley R, Burgess C, Crane J, Pearce N, Roche W. Pathology of asthma and its clinical implications. *J Allergy Clin Immunol* 1993; 92:148–54.
- 4 Holgate ST. Epithelial damage and response. *Clin Exp Allergy* 2000; 30 (Suppl. 1):37–41.
- 5 Guo RF, Ward PA, Jordan JA, Huber-Lang M, Warner RL, Shi MM. Eotaxin expression in Sephadex-induced lung injury in rats. *Am J Pathol* 1999; 155:2001–8.
- 6 Leckie MJ. Chronic allergen challenge as an experimental model: necessary, significant or useful? *Clin Exp Allergy* 2000; 30:1191–3.

- 7 Palmans E, Kips JC, Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. *Am J Respir Crit Care Med* 2000; 161:627–35.
- 8 Crouch EC. Collectins and pulmonary host defense. *Am J Respir Cell Mol Biol* 1998; 19:177–201.
- 9 Crouch E, Wright J. Surfactant proteins A and D and pulmonary host defense. *Annu Rev Physiol* 2001; 63:521–54.
- 10 Wang JY, Shieh CC, You PF, Lei HY, Reid KB. Inhibitory effect of pulmonary surfactant proteins A and D on allergen-induced lymphocyte proliferation and histamine release in children with asthma. *Am J Respir Crit Care Med* 1998; 158:510–8.
- 11 Cheng G, Ueda T, Numao T et al. Increased levels of surfactant protein A and D in bronchoalveolar lavage fluids in patients with bronchial asthma. *Eur Respir J* 2000; 16:831–5.
- 12 Wang JY, Shieh CC, Yu CK, Lei HY. Allergen-induced bronchial inflammation is associated with decreased levels of surfactant proteins A and D in a murine model of asthma. *Clin Exp Allergy* 2001; 31:652–62.
- 13 Wright SM, Hockey PM, Enhorning G et al. Altered airway surfactant phospholipid composition and reduced lung function in asthma. *J Appl Physiol* 2000; 89:1283–92.
- 14 Haczku A, Atochina EN, Tomer Y et al. *Aspergillus fumigatus*-induced allergic airway inflammation alters surfactant homeostasis and lung function in BALB/c mice. *Am J Respir Cell Mol Biol* 2001; 25:45–50.
- 15 Poppe H, Kuesters S, Szelenyi I. The effect of AWD 12–281, a new selective PDE4-inhibitor, luteprednol and beclomethasone in models of allergic rhinitis and airway inflammation in Brown Norway-rats. *Am J Respir Crit Care Med* 1999; 159:A95 (Abstract).
- 16 Blyth DI, Pedrick MS, Savage TJ, Hessel EM, Fattah D. Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am J Respir Cell Mol Biol* 1996; 14:425–38.
- 17 van Halteren AG, van der Cammen MJ, Biewenga J, Savelkoul HF, Kraal G. IgE and mast cell response on intestinal allergen exposure: a murine model to study the onset of food allergy. *J Allergy Clin Immunol* 1997; 99:94–9.
- 18 Kasper M, Albrecht S, Grossmann H, Grosser M, Schuh D, Muller M. Monoclonal antibodies to surfactant protein D. evaluation of immunoreactivity in normal rat lung and in a radiation-induced fibrosis model. *Exp Lung Res* 1995; 21:577–88.
- 19 Aderibigbe AO, Thomas RF, Mercer RR, Auten RL Jr. Brief exposure to 95% oxygen alters surfactant protein D and mRNA in adult rat alveolar and bronchiolar epithelium. *Am J Respir Cell Mol Biol* 1999; 20:219–27.
- 20 Carvalho C, Jancar S, Mariano M, Sirois P. A rat model presenting eosinophilia in the airways, lung eosinophil activation, and pulmonary hyperreactivity. *Exp Lung Res* 1999; 25:303–16.
- 21 Tohda Y, Kubo H, Ito M, Fukuoka M, Nakajima S. Histopathology of the airway epithelium in an experimental dual-phase model of bronchial asthma. *Clin Exp Allergy* 2001; 31:135–43.
- 22 Yiamouyiannis CA, Schramm CM, Puddington L et al. Shifts in lung lymphocyte profiles correlate with the sequential development of acute allergic and chronic tolerant stages in a murine asthma model. *Am J Pathol* 1999; 154:1911–21.
- 23 Evans CM, Jacoby DB, Fryer AD. Effects of dexamethasone on antigen-induced airway eosinophilia and m (2) receptor dysfunction. *Am J Respir Crit Care Med* 2001; 163:1484–92.
- 24 Mason RJ, Lewis MC, Edeen KE, McCormick-Shannon K, Nielsen LD, Shannon JM. Maintenance of surfactant protein A and D secretion by rat alveolar type II cells *in vitro*. *Am J Physiol Lung Cell Mol Physiol* 2002; 282:L249–58.
- 25 Ogasawara Y, Kuroki Y, Tsuzuki A, Ueda S, Misaki H, Akino T. Pre- and post-natal stimulation of pulmonary surfactant protein D by *in vivo* dexamethasone treatment of rats. *Life Sci* 1992; 50:1761–7.
- 26 Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA. Glucocorticoids and lung development in the fetus and pre-term infant. *Pediatr Pulmonol* 2001; 32:76–91.
- 27 Deterding RR, Shimizu H, Fisher JH, Shannon JM. Regulation of surfactant protein D expression by glucocorticoids *in vitro* and *in vivo*. *Am J Respir Cell Mol Biol* 1994; 10:30–7.
- 28 Dulkerian SJ, Gonzales LW, Ning Y, Ballard PL. Regulation of surfactant protein D in human fetal lung. *Am J Respir Cell Mol Biol* 1996; 15:781–6.
- 29 Madan T, Kishore U, Singh M et al. Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by *Aspergillus fumigatus* antigens and allergens. *J Clin Invest* 2001; 107:467–75.
- 30 Fahy JV. Remodeling of the airway epithelium in asthma. *Am J Respir Crit Care Med* 2001; 164:S46–51.
- 31 LeVine AM, Whitsett JA. Pulmonary collectins and innate host defense of the lung. *Microbes Infect* 2001; 3:161–6.