

Serum surfactant protein D is elevated in allergic patients

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Summary

Background There is evidence that surfactant protein (SP)-D is important in the innate, as well as in the adaptive pulmonary immune response. Serum concentrations of SP-D have been proposed as parameter of the integrity of the blood–airspace barrier in interstitial lung diseases. We hypothesized that serum SP-D concentrations are affected in allergic patients and correlate with changes in allergic airway inflammation.

Objective To determine levels of serum SP-D in allergic patients compared with non-allergic controls. Furthermore, to investigate associations between serum SP-D concentrations on the one hand and changes in commonly used markers of bronchial inflammation in allergic airways disease on the other hand.

Materials and methods Fifty allergic patients were studied and bronchial allergen challenge was used as a model to increase bronchial allergic inflammation in these patients. Serum SP-D concentrations, inflammatory parameters in induced sputum and bronchial hyper-responsiveness (BHR) were determined before and after allergen challenge. Twenty-five non-allergic volunteers served as controls.

Results Baseline serum SP-D was significantly higher in allergic patients as compared with controls (mean serum SP-D concentration (95% confidence interval): 62.7 (55.5, 70.0) in allergic patients vs. 49.5 (36.7, 62.3) ng/mL in non-allergic controls, $P = 0.006$). In addition, baseline serum SP-D appeared to be an independent predictor for the magnitude of the late asthmatic response after allergen challenge. Furthermore, serum SP-D was predictive for the sputum eosinophil cationic protein concentration after allergen challenge.

Conclusion We propose that serum SP-D concentrations are associated with allergic bronchial inflammation and may give additional information, beside BHR and sputum eosinophils, about the degree of bronchial inflammation in allergic patients.

Keywords allergy, asthma, bronchial allergen challenge, inflammation, surfactant

Submitted 5 February 2004; revised 27 May 2004; accepted 22 June 2004

Introduction

Pulmonary surfactant is a complex of several lipids and proteins covering the alveolar surface of the lung. The best-known function of this complex is to decrease surface tension at the alveolar air–liquid interface and thereby to prevent the collapse of alveoli. However, two of the four different surfactant-associated proteins that have been described, namely surfactant protein (SP)-A and SP-D, also appear to be important mediators in the innate immunity of the lung. SP-A and SP-D are members of a family of collagenous carbohydrate-binding proteins, known as collectins and are both synthesized and secreted by alveolar type II and non-ciliated bronchiolar epithelial cells [1, 2]. As for their role in the innate immunity of the lung, it has been observed that they are involved in opsonizing microorganisms for phagocytosis [3, 4], and in the clearance of viruses [5] and fungi [6,

7]. Furthermore, there is evidence that SP-A and SP-D inhibit lipopolysaccharide-induced production of pro-inflammatory cytokines [8]. *In vivo* studies indicate that SP-A and SP-D knockout mice are more susceptible to bacterial and viral infections [5, 9, 10].

In addition to the studies on SP-A and SP-D in innate immunity there is evidence to suggest that these proteins are important in the allergic pulmonary immune response. SP-A and SP-D can bind to allergens, such as house dust mite (HDM) allergens [11] and pollen [12] *in vitro*, and they can inhibit the binding of allergen-specific IgE to HDM [11], possibly by steric hindrance. Furthermore, SP-A and SP-D can inhibit *Dermatophagoides pteronyssinus* (Der p)-stimulated lymphocyte proliferation and histamine release [13], and SP-A can reduce IL-8 production by eosinophils *in vitro* [14]. Therefore, it has been suggested that these proteins inhibit the allergic inflammatory response. A protective role of both SP-A and SP-D has been confirmed in a murine model of allergic bronchopulmonary aspergillosis [15]. Mice that were pre-treated in this model with SP-A or SP-D exhibited a lower degree of bronchial inflammation after allergen challenge

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when compared with control challenge, as reflected by lower levels of IL-2, IL-4 and IL-5, reduced eosinophilia and specific antibody levels.

In humans, the serum concentration of particularly SP-D has been proposed as a non-invasive parameter of the permeability or integrity of the blood–airspace barrier in interstitial lung diseases like interstitial pulmonary fibrosis [16]. In asthma, increased permeability of the bronchoalveolar epithelial barrier, leading to mucosal oedema and the accumulation of plasma proteins in the alveolar spaces, has been recognized [17]. In addition, bi-directional increase in permeability in asthma is indicated by studies that showed increased passage of macromolecules from the lower respiratory tract into the blood, which was increased during inflammatory conditions [18, 19]. Increased synthesis of SP-A and SP-D in the lung has been shown in murine models of allergy [20–22]. Taken together the mentioned data suggest that serum concentrations of SP-D may be affected also in patients with allergic bronchial inflammation, and might correlate with changes in the allergic inflammatory process. SP-A is thought to be tightly bound to surfactant lipid aggregates [16]. Serum concentrations of SP-A are therefore less likely to be affected in allergic lung disease.

Hence, we aimed at answering the following questions in the present study:

- Are serum concentrations of SP-D elevated in allergic patients when compared with non-allergic controls?
- Are serum concentrations of SP-D associated with commonly used markers of the bronchial inflammatory process in allergic patients? Bronchial hyper-responsiveness (BHR), the number of eosinophils and the concentration of eosinophil cationic protein (ECP) in induced sputum were measured.
- Finally, are serum concentrations of SP-D affected by bronchial allergen challenge, a generally used model to increase bronchial allergic inflammation. Changes in serum SP-D after bronchial allergen challenge and associations with parameters of bronchial inflammation were compared with results with regard to serum SP-A.

Materials and methods

Fifty allergic patients were investigated (Table 1). Twenty-five of these patients had symptoms of mild-to-moderate asthma according to GINA guidelines (grades I–III) [23], and 25

had perennial rhinitis without any asthma symptoms [24]. All patients were sensitized to HDM, as judged by RAST. The following inclusion criteria were used: (a) Forced expiratory volume in 1 s (FEV_1) = 70% pred, (b) serum IgE to *D. pteronyssinus* > 0.5 IU/mL, and (c) no significant change in allergen exposure because of allergen avoidance during at least 6 months. Whenever co-sensitization to pollen was present, the study was performed outside the relevant pollen season. Exclusion criteria were: (a) immunotherapy in the history, (b) respiratory tract infection within 6 weeks prior to the study, (c) use of oral or inhaled corticosteroids during or 6 weeks prior to the study, (d) use of antihistamines during or 2 weeks prior to the study, (e) immunosuppressive medication, and (f) smoking. Twenty-five volunteers served as controls. They were non-allergic, as determined by RAST and skin prick test (SPT) with a panel of common inhaled allergens, non-smoking, had no history of rhinitic or asthmatic symptoms and no BHR to histamine. The Medical Ethic Committee of the Academic Medical Center approved the study, and all subjects gave written informed consent.

Study design

The study had a prospective experimental design. Sputum induction, FEV_1 , provocative concentration of allergen needed to decrease FEV_1 by 20% of the baseline values (PC_{20}) histamine and blood sampling were performed on the first day of the study period. In addition, diurnal change in FEV_1 was recorded after a control challenge with diluent on this day. Bronchial allergen challenge took place on the second day of the study period. Twenty-four hours after bronchial allergen challenge blood sampling, sputum induction and PC_{20} histamine were repeated. Blood sampling was repeated 1 week after allergen challenge.

Bronchial challenges

Bronchial histamine challenge was performed according to the 2 min tidal breathing method as described by Sterk et al. [25]. Bronchial allergen challenge was done with a standardized *D. pteronyssinus* extract (1 mg/mL, which contained 85.1 µg Der p 1 and 9.7 µg Der p 2 per mg protein, representing 10^6 SQU/mL or 50 000 BU/mL; ALK, Houten, the Netherlands) as described [25], with modifications [26]. Briefly, a collapsible reservoir of approximately 30 L, made of static field dissipative material (RCAS 1206, Richmond Redlands, CA, USA) and filled with dry air, was connected

Table 1. Baseline characteristics

	Allergic patients	Non-allergic controls	Rhinitics	Asthmatics	Single responders	Dual responders
<i>n</i>	50	25	25	25	21	29
Age† (years)	27 ± 6	27 ± 7	27 ± 5	27 ± 6	27 ± 6	27 ± 6
Sex (M/F)	11/39	6/19	5/20	6/19	3/18	8/21
SplgE‡ (kU/L)	18.8 (3.2)	< 0.3	13.8 (3.3)	25.5 (2.8)	12.7 (3.9)**	24.9 (2.5)
Total IgE‡ (kU/L)	170 (4.0)	22.0 (2.3)	127.3 (3.9)	235.9 (3.9)	158.5 (4.9)	178.6 (3.5)
FEV_1 † (% pred)	98.8 ± 14.4	107.6 ± 7.7	102.9 ± 12.9	94.6 ± 14.9	102.3 ± 10.8	96.3 ± 16.2
PC_{20} histamine‡ (mg/mL)	3.48 (4.6)	> 16.0	9.1 (2.6)*	1.3 (4.0)	8.1 (2.8)***	1.9 (4.7)

SplgE, specific IgE against house dust mite; FEV_1 , forced expiratory volume in 1 s.

*Non-asthmatic rhinitis vs. asthma: $P = 0.001$, **Dual vs. single responders: $P < 0.05$, ***Dual vs. single responders: $P = 0.001$.

†Age and FEV_1 percentage of predicted are expressed as mean ± SD.

‡SplgE, total IgE and PC_{20} for histamine are expressed as geometric mean (GSD).

to a nebulizer (Mallinckrodt Diagnostica, Petten, the Netherlands) producing allergen aerosols from a 0.5 mL sample. The entire volume of the reservoir was inhaled by tidal breathing through a three-way valve system with the nose clipped. Previous studies demonstrated a 70% recovery of nebulized allergen. The starting allergen dose was calculated from the SPT threshold and the PC₂₀ histamine according to the method of Cockcroft et al. [27]. FEV₁ was measured on a dry rolling-seal spirometer (SensorMedics BV, Bithoven, the Netherlands). Drop in FEV₁ (mean of two measurements) relative to baseline values (median of three measurements within 5%) was measured 0.5 and 10 min after inhalation of allergen. With intervals of 10 min doubling doses of allergen were inhaled from the reservoir, until a decline of 20% in FEV₁ was reached. Subsequently, FEV₁ was measured hourly for at least 10 h. The bronchial response to allergen was expressed as the percentage change from baseline and was corrected for diurnal variation as determined after control challenge. The late asthmatic response (LAR) was defined as the maximal decline in FEV₁ between 4 and 12 h after allergen challenge and was corrected for the change in FEV₁ during a control challenge, which was performed on the day before the bronchial allergen challenge. Patients who had an LAR of 20%, regardless whether they had isolated rhinitic symptoms or asthmatic symptoms, were considered as dual responders. Patients who had an LAR of <20% were considered as single responders.

Sputum induction

Sputum induction and processing were performed as described earlier [28]. Shortly, induction was performed by inhalation of nebulized hypertonic saline (NaCl) during quiet tidal breathing. An Aerodyne Omega Ultrasonic nebulizer (Kendall, Neustadt/Donau, Germany) was used with plastic tubes of 30 cm length. This nebulizer generates aerosols with a mass median aerodiameter of 4.5 µm. No sputum induction was performed when the FEV₁ was less than 80% of the baseline value or in case of complaints of dyspnoea. Sputum induction was started with 3% saline inhalation for 15 min, thereafter the saline concentration was raised to 4% and 5% at 15 min intervals. Subjects were encouraged to expectorate through several attempts of forced expiration following every inhalation interval. Sputum was collected in plastic tubes and kept on ice until processing.

Sputum processing

An equal volume of dithiotreitol (10 mM DTT in 135 mM Tris buffer, pH 8.0) was added to the sputum (DTT; Sigma Chemical Company, St Louis, MO, USA). The sample was incubated in a shaking bath of 37 °C for 15 min with periodical gentle manual mixing to ensure complete homogenization. Cell numbers were counted in a Bürker counting chamber. Slides were stained with Jenner–Giemsa staining and Romanovsky (Diff-Quick, Dade Behring, Düringen, Switzerland). Sputum samples, which contained less than 20% non-squamous cells or less than 50% viable cells were excluded from analysis. For differential cell counts a total number of 500 non-squamous cells were examined. If less than 2% eosinophils was found, another 500 non-squamous

cells were counted in order to decrease the confidence limits of low eosinophil percentages. All differential cell counts were performed by one investigator blinded for patient identity and sample time-point.

Protein assays

SP-A was measured by ELISA with the SP-A Test Kokusai-F (Sysmex Corporation, Kobe, Japan). The kit uses the method of Shimizu et al. [29], with minor modifications. The same monoclonal antibodies (Mabs) PC6 and PE10, raised against SP-A obtained from bronchoalveolar lavage fluid (BALF) from patients with pulmonary alveolar proteinosis (PAP) were used as in the original procedure. Plastic tubes instead of plastic beads were used as the solid phase. The SP-A standard, was obtained from PAP patients. Briefly, 50 µL of SP-A standards (ranging from 0 to 500 ng/mL) or serum samples twofold diluted with sample dilution buffer (phosphate-buffered saline (PBS), pH 7.2 containing 150 mM NaCl, 100 mM SDS and 6% Triton X-100) were added to tubes coated with Mab PC6, together with horse radish peroxidase (HRP)-conjugated Mab PE10. After reaction at 37 °C for 19 min, the tubes were washed with PBS containing 0.05% Tween 20. The relative amount of bound HRP was determined by incubation with 300 µL of 25 mM 3-(*p*-hydroxyphenyl) propionic acid, containing 0.005% H₂O₂, at 37 °C for 10 min. After stopping the reaction by addition of 1 mL of 8 mM sodium sulphite/1 mM NaOH, the fluorescence (excitation at 323 nm; emission at 410 nm) was measured in an ELISA-F750 fluorometer (Sysmex Corporation). All assays were carried out in duplicate. The standard curve was linear up to a relative fluorescence intensity of at least 360 (corresponding to an SP-A concentration of 250 ng/mL). All measurements for the samples fell within this linear range (generally with a relative fluorescence intensity between 20 and 50).

SP-D was measured by ELISA using the SP-D EIA kit Yamasa (Diagnostic Department, Yamasa Corporation, Choshi, Japan). This kit uses reagents (including the Mabs against SP-D, and recombinant SP-D as standard material), and procedures as described previously [30, 31]. In brief, capture antibody 7C6 was coated onto microtitre plates. The wells were blocked with 1.0% bovine serum albumin (BSA) in PBS, after which 100 µL of the SP-D standard solutions (ranging from 0 to 100 ng/mL) or serum samples 11 × diluted with 10 mM HEPES buffer, pH 7.4 containing 150 mM NaCl, 0.5% Triton X-100 and 1.0% BSA (HEPES-TB) were added to the wells. After overnight incubation at room temperature, the plates were washed, and incubated for 2 h at room temperature with 100 µL of HRP-conjugated F(ab')₂ fragments of Mab 6B2 diluted with HEPES-TB. Subsequently, the wells were washed and finally incubated at room temperature with 100 µL of 0.3 mM 3,3',5,5'-tetraethylbenzidine containing 0.005% H₂O₂. After 15 min the reaction was stopped by addition of 100 µL of 0.5 M sulphuric acid and the absorbance at 450 nm was measured using an ELISA plate reader. All assays were carried out in duplicate. The standard curve was linear up to an OD₄₅₀ value of 1.0. All OD readings for the samples were in this linear range (generally between 0.2 and 0.6). Previous work [31] has shown that there is a good linear relationship between the reciprocal of the dilution factor and the concentration of SP-D measured by this ELISA.

Levels of ECP were determined in sputum supernatants with an ELISA as described previously [32]. All assays were carried out in duplicate.

Statistical methods

Between-group differences were analysed with the Mann–Whitney *U*-test, within-group comparisons were done with the Wilcoxon signed-rank test. Significant differences between more than two groups were first established by the Kruskal–Wallis test, followed by the Mann–Whitney *U*-test. Relationships between normally distributed data (after log transformation where applicable) were determined by using parametric correlation (Pearson's *r*), and step-wise multiple linear regression analysis (*P*-limits: in <0.05, out >0.1). Correlations between serum concentrations of surfactant proteins and inflammatory parameters in induced sputum were determined by using rank-correlation (Spearman's, *R_s*). All *P*-values are two-tailed and *P*-levels of less than 0.05 were considered significant. The program used for the analyses was SPSS version 9.0.1 Statistics UK (Chicago, IL, USA).

Results

Baseline

Serum SP-D was significantly higher in allergic patients as compared with controls (mean serum SP-D concentration (95% confidence interval): 62.7 (55.5, 70.0) in allergic patients vs. 49.5 (36.7, 62.3) ng/mL in non-allergic controls, *P* = 0.006). There was no significant difference in serum SP-D levels between asthmatics and non-asthmatic rhinitics (Table 2), but a significantly higher serum SP-D was found in dual responders when compared with single responders (Fig. 1).

The difference between asthmatics and non-asthmatic rhinitics in numbers of eosinophils in induced sputum did not reach statistical significance (Table 2), but dual responders had significantly higher numbers of eosinophils in induced sputum as compared with single responders (Table 2). Sputum ECP concentrations were not significantly different in the subgroups of patients (Table 2).

Bronchial allergen challenge

All 50 participants tolerated the bronchial allergen challenge without complications. Short acting β_2 -agonists were not used from 8 h before to 12 h after allergen challenge. The median maximal change in FEV₁ between 0.5 and 20 min after allergen challenge was –31.0% from baseline (range: –56.1, –12.3%). The median maximal change in FEV₁ during 4–12 h after allergen challenge was –27.8% from baseline (range: –74.7, –1.0%). Eighteen asthmatics and 11 non-asthmatic rhinitics had a decline in FEV₁ of at least 20% from baseline between 4 and 12 h after allergen challenge. These patients were considered as dual responders.

The results of the measurements of SP-A and SP-D concentrations in serum and of the number of eosinophils and ECP concentration in induced sputum before and 24 h after bronchial allergen challenge are shown in Table 2. At 24 h after allergen challenge there was a significant increase in

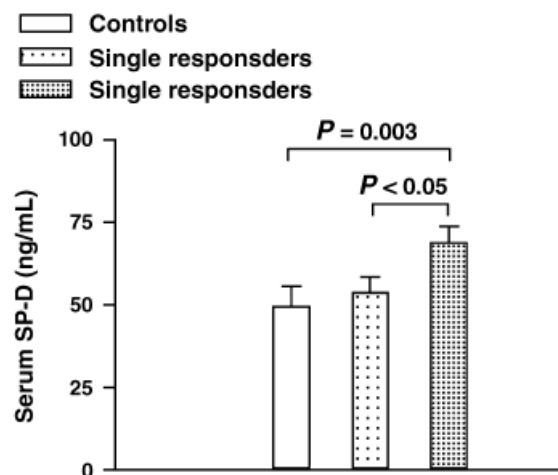


Fig. 1. Serum surfactant protein (SP)-D concentrations in non-allergic controls (*n* = 25), single (*n* = 21) and dual responders (*n* = 29). Mean values (+SEM) are shown.

Table 2. SP-A and SP-D concentrations in serum and markers of bronchial inflammation in induced sputum

	Allergic patients	Rhinitics	Asthmatics	Single responders	Dual responders
Number of patients	50	25	25	21	29
Serum SP-A (ng/mL)					
Baseline	29.1 (26.0, 32.2)	32.0* (26.9, 37.1)	26.2 (22.8, 29.6)	28.2 (24.2, 32.2)	29.8 (25.1, 34.4)
24 h after allergen challenge	30.4 (27.3, 33.6)	33.3 (28.1, 38.4)	27.6 (23.9, 31.2)	29.4 (24.7, 34.1)	31.1 (26.7, 35.6)
Serum SP-D (ng/mL)					
Baseline	62.7 (55.5, 70.0)	61.0 (50.9, 71.1)	64.5 (53.4, 75.6)	54.0** (44.3, 63.8)	69.1 (58.9, 79.3)
24 h after allergen challenge	71.6 (64.1, 79.1)	70.9 (58.9, 82.9)	72.3 (62.4, 82.2)	61.7** (50.4, 73.1)	78.7 (69.1, 88.4)
Sputum eosinophils ($\times 10^4$ /g)					
Baseline	1.9 (0.9, 3.7)	1.1 (0.4, 3.5)	3.0 (1.3, 7.0)	0.6*** (0.2, 2.3)	3.3 (1.6, 7.0)
24 h after allergen challenge	10.0 (4.6, 21.7)	7.6 (2.2, 26.7)	13.2 (4.7, 37.1)	3.8** (0.8, 18.7)	18.0 (8.2, 39.7)
Sputum ECP (ng/mL)					
Baseline	68.0 (48.6, 95.2)	53.2 (31.5, 90.0)	84.5 (53.4, 133.5)	46.9 (23.7, 96.9)	82.7 (57.2, 119.3)
24 h after allergen challenge	282.8 (178.5, 448.1)	185.7* (106.1, 325.1)	430.6 (207.1, 895.4)	208.6 (95.9, 454.2)	339.4 (184.5, 624.5)

ECP, eosinophil cationic protein; SP, surfactant protein; CI, confidence interval.

Serum SP-A and serum SP-D are expressed as mean values (and 95% CI), sputum eosinophils and sputum ECP as geometric mean values (and 95% CI).

*Non-asthmatic rhinitis vs. asthma: *P* < 0.05, **Dual vs. single responders: *P* < 0.05, ***Dual vs. single responders: *P* < 0.01.

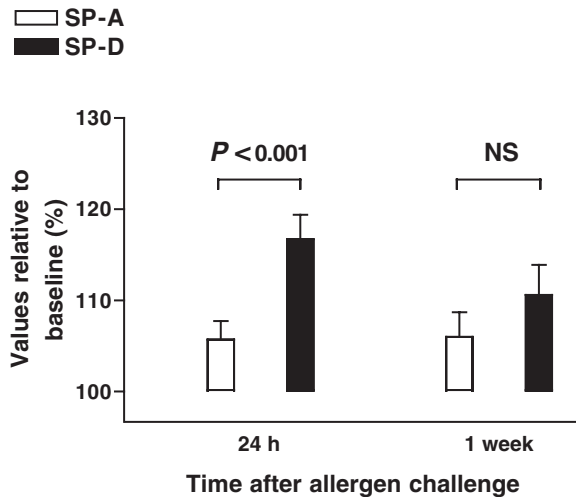


Fig. 2. Increases in serum surfactant protein (SP)-A and SP-D concentrations 24 h and 1 week after bronchial allergen challenge in 50 allergic patients. Mean values relative to baseline in percentage ($\pm$ SEM) are shown. The relative increase in serum SP-D at 24 h after allergen challenge was significantly greater than the relative increase in serum SP-A ($P < 0.001$). NS, not significant.

serum SP-D ($P < 0.001$), which was still significant at 1 week after allergen challenge ($P < 0.05$), data not shown. In serum SP-A there was a small but significant increase ($P < 0.05$) at 24 h after allergen challenge. The relative increase in serum SP-D at 24 h after allergen challenge was significantly greater than the increase in serum SP-A at 24 h post-challenge (Fig. 2). A significant correlation was found between the relative increase in serum SP-A and the relative increase in serum SP-D at 24 h after allergen challenge ($R_s = 0.41$, $P = 0.003$).

The bronchial allergen challenge led to significant increases in the number of eosinophils and the ECP concentration in induced sputum in the allergic patients as well as in the subgroups of allergic patients (Table 2, P -values < 0.05).

Correlations

No significant correlations between serum SP-D or SP-A and PC₂₀histamine were found (P -values > 0.3). However, there was a significant correlation between baseline serum SP-D and the LAR (log serum SP-D: $r = 0.40$, $P = 0.004$, Fig. 3). There were no significant correlations between serum SP-A and PC₂₀histamine or the LAR.

Significant correlations were found between serum SP-D concentrations on the one hand and inflammatory parameters (i.e. the number of eosinophils and the concentration of ECP in induced sputum) on the other (Table 3). By contrast, there were no significant correlations between serum SP-A and these inflammatory parameters (results not shown).

There was a significant correlation between baseline numbers of eosinophils in induced sputum and PC₂₀histamine ($R_s = -0.53$, $P = 0.002$), and between baseline numbers of eosinophils in induced sputum and the LAR ($R_s = -0.5$, $P = 0.004$).

Multiple linear regression

Baseline serum SP-D and PC₂₀histamine appeared to be independent predictors of the LAR, (multiple linear regres-

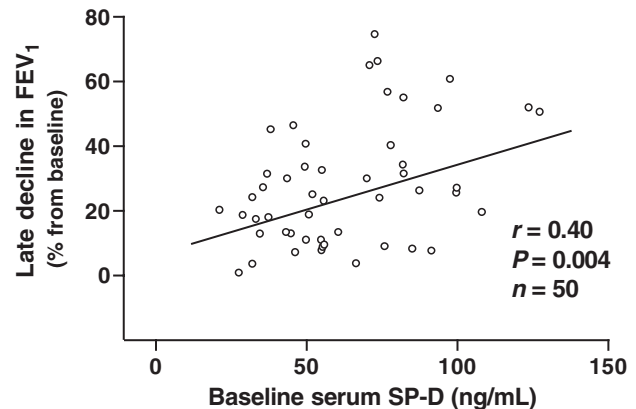


Fig. 3. Bivariate correlation between baseline serum surfactant protein (SP)-D concentration and the magnitude of the late asthmatic response.

Table 3. Associations between serum SP-D concentrations and inflammatory parameters in induced sputum, before and after allergen challenge in 50 allergic patients

	Serum SP-D baseline		Serum SP-D at 24 h	
	R_s	P -value	R_s	P -value
Eo/g sp baseline ($\times 10^4$ /g)	-0.09	0.6	0.009	0.97
Eo/g sp 24 h ($\times 10^4$ /g)	0.33	0.07	0.43	0.01*
ECP sp baseline (ng/mL)	-0.06	0.7	-0.07	0.7
ECP sp 24 h (ng/mL)	0.40	0.02*	0.47	$< 0.01^*$

SP-D, surfactant protein D; Eo/g, number of eosinophils per gram sputum; sp, sputum; ECP, eosinophil cationic protein; 24 h, 24 h after bronchial allergen challenge.

* P -value significant.

sion: model $R^2 = 0.48$, adjusted $R^2 = 0.45$, $P < 0.001$, $n = 50$. B (SE) = -30.7 (11.0), $\beta = -0.30$ and $P = 0.007$ for log serum SP-D at baseline and B (SE) = 16.0 (3.0), $\beta = 0.57$ and $P < 0.001$ for log PC₂₀histamine). When added to this model, the number of sputum eosinophils did not independently contribute to the prediction of the LAR ($\beta = -0.22$, $P = 0.17$).

Discussion

This is the first study to demonstrate significantly higher levels of serum SP-D in allergic patients when compared with non-allergic controls. In addition, we showed that the level of serum SP-D at baseline was predictive for the LAR and for sputum ECP concentrations after bronchial allergen challenge. No relationships between allergic inflammatory parameters and serum SP-A concentrations were found. These findings indicate that serum SP-D, rather than serum SP-A, is associated with bronchial inflammation in allergic patients.

The occurrence of SP-A and SP-D in very low concentrations in the vascular compartment is mainly explained by leakage from the lung into the bloodstream [16]. Serum concentrations of these proteins are therefore influenced by: (1) the local concentration in the epithelial lining fluid, which depends on production rate and local clearance, (2) the

permeability of the blood–airspace barrier for SP-A and for SP-D and (3) the rate of clearance of these proteins from the bloodstream. We will shortly discuss arguments for the occurrence of alterations in production rate and changes in permeability of the blood–airspace barrier in the present study. It seems unlikely that clearance from the blood is altered by bronchial allergic inflammation *per se* and/or after a local bronchial allergen challenge.

Our main finding that serum SP-D was elevated in allergic patients and particularly in those who had a dual asthmatic response after allergen challenge and more pronounced eosinophilic airways inflammation is in agreement with the above-mentioned evidence that bronchial inflammation leads to increased permeability of the blood–airspace barrier [17]. Increased permeation and thereby increased serum levels of SP-D in the allergic patients might be caused by the involvement of the small airways, which have a considerably larger blood–air surface than the central airways, and have been shown also to be involved in asthma. The significant correlation between the increases in serum SP-D and serum SP-A after allergen challenge also supports the idea of increased permeation as one of the mechanisms resulting in the elevated levels of serum SP-D in allergic patients. The smaller increase after allergen challenge in serum SP-A compared with SP-D is likely to be the result of the tight binding of SP-A to surfactant lipid aggregates and may confirm that SP-A cannot easily pass the blood–airspace barrier.

However, not only increased permeability, but also increased production, caused by the allergic inflammatory milieu itself, may add to the elevated serum levels of SP-D [33]. Both IL-4 [34] and IL-13 [33], i.e. cytokines that are important in the eosinophilic inflammatory response, have been reported to increase the production of SP-D. Elevated levels of IL-4 and IL-13 are therefore likely to contribute to the significantly higher serum concentrations of SP-D in allergic patients and in dual responders, as these patients also had more pronounced bronchial eosinophilic inflammation when compared with single responders. Furthermore, the increased expression [20–22] and elevated levels of SP-D found in BALF following allergen challenge [35, 36] in sensitized mice as well as the elevated levels in BALF in asthmatic patients [37] also point to the possibility of increased local production.

In contrast to the significant differences between single and dual responders, we did not find significant differences in serum SP-D between asthmatics and non-asthmatic rhinitics. This finding is in line with the lacking difference in the other inflammatory parameters between asthma and non-asthmatic rhinitis in the present study. Several papers report the existence of bronchial involvement in rhinitis and it should be noted that the degree of bronchial inflammation rather than the presence of asthmatic symptoms may have impact on the risk of developing structural bronchial changes, known as remodelling. Importantly, airway remodelling has indeed been reported in asthmatic patients as well as in rhinitic patients [38]. Identifying patients at risk, for developing structural bronchial changes, on the basis of symptoms may prove insufficient.

A striking finding in the present study was that baseline serum SP-D was associated with inflammatory parameters after allergen challenge, including the LAR. Mechanisms

underlying the LAR are incompletely understood. However, the LAR has been reported to be related to inflammatory changes in the bronchial wall [39]. Studies in BALF and bronchial biopsies obtained during the LAR indicate a cellular influx comprised predominantly of activated eosinophils and T lymphocytes [40]. These histological characteristics of the LAR resemble those found in spontaneously occurring asthma. Moreover, the LAR is associated with the induction of BHR, which is a hallmark of asthma. Hence, the LAR may represent one of the parameters reflecting bronchial disease activity in allergic patients. Determining the magnitude of the LAR is, however, time consuming and labour intensive. Studies regarding the prediction of the LAR have shown that baseline BHR provides the best predictor of the LAR [41]. The number of baseline sputum eosinophils in the present study was also associated with the LAR, but because of an association between the number of eosinophils and BHR the number of eosinophils did not independently contribute to the prediction of the LAR, as confirmed by multiple linear regression analysis. Our finding that serum SP-D was an independent predictor of the LAR suggests that the level of serum SP-D may reflect a distinct part of the bronchial inflammatory process.

We conclude that serum SP-D is elevated in allergic patients, particularly in those who exhibit a dual asthmatic response after allergen challenge. Furthermore, serum SP-D is related to levels of inflammatory parameters after allergen challenge and appeared to be an independent predictor for the magnitude of the LAR after bronchial allergen challenge. We propose that serum SP-D concentrations are associated with allergic bronchial inflammation and may give additional information, beside BHR and sputum eosinophils, about the degree of bronchial inflammation in allergic patients.

Acknowledgements

This study was financially supported by the Netherlands Asthma Foundation (grant no. 95-57). We thank Dr T. A. Out for carefully reading the manuscript and Dr K. Tsumura (Sysmex Corporation, Kobe, Japan) for performing the SP-A assays.

References

- 1 Crouch E, Parghi D, Kuan SF, Persson A. Surfactant protein D: subcellular localization in nonciliated bronchiolar epithelial cells. *Am J Physiol* 1992; 263:L60–6.
- 2 Walker SR, Williams MC, Benson B. Immunocytochemical localization of the major surfactant apoproteins in type II cells, Clara cells, and alveolar macrophages of rat lung. *J Histochem Cytochem* 1986; 34:1137–48.
- 3 Lim BL, Wang JY, Holmskov U, Hoppe HJ, Reid KB. Expression of the carbohydrate recognition domain of lung surfactant protein D and demonstration of its binding to lipopolysaccharides of gram-negative bacteria. *Biochem Biophys Res Commun* 1994; 202: 1674–80.
- 4 Pikaar JC, Voorhout WF, van Golde LM, Verhoef J, Van Strijp JA, van Iwaarden JF. Opsonic activities of surfactant proteins A and D in phagocytosis of gram-negative bacteria by alveolar macrophages. *J Infect Dis* 1995; 172:481–9.

- 5 LeVine AM, Whitsett JA, Hartshorn KL, Crouch EC, Korfhagen TR. Surfactant protein D enhances clearance of influenza A virus from the lung in vivo. *J Immunol* 2001; 167:5868–73.
- 6 Allen MJ, Harbeck R, Smith B, Voelker DR, Mason RJ. Binding of rat and human surfactant proteins A and D to *Aspergillus fumigatus* conidia. *Infect Immunol* 1999; 67:4563–9.
- 7 Allen MJ, Voelker DR, Mason RJ. Interactions of surfactant proteins A and D with *Saccharomyces cerevisiae* and *Aspergillus fumigatus*. *Infect Immunol* 2001; 69:2037–44.
- 8 Borron P, McIntosh JC, Korfhagen TR, Whitsett JA, Taylor J, Wright JR. Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo. *Am J Physiol Lung Cell Mol Physiol* 2000; 278:L840–47.
- 9 LeVine AM, Bruno MD, Huelsman KM, Ross GF, Whitsett JA, Korfhagen TR. Surfactant protein A-deficient mice are susceptible to group B streptococcal infection. *J Immunol* 1997; 158: 4336–40.
- 10 LeVine AM, Kurak KE, Bruno MD, Stark JM, Whitsett JA, Korfhagen TR. Surfactant protein-A-deficient mice are susceptible to *Pseudomonas aeruginosa* infection. *Am J Respir Cell Mol Biol* 1998; 19:700–8.
- 11 Wang JY, Kishore U, Lim BL, Strong P, Reid KB. Interaction of human lung surfactant proteins A and D with mite (*Dermatophagoides pteronyssinus*) allergens. *Clin Exp Immunol* 1996; 106: 367–73.
- 12 Malhotra R, Haurum J, Thiel S, Jensenius JC, Sim RB. Pollen grains bind to lung alveolar type II cells (A549) via lung surfactant protein A (SP-A). *Biosci Rep* 1993; 13:79–90.
- 13 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.
- 14 Cheng G, Ueda T, Nakajima H et al. Surfactant protein A exhibits inhibitory effect on eosinophils IL-8 production. *Biochem Biophys Res Commun* 2000; 270:831–5.
- 15 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.
- 16 Hermans C, Bernard A. Lung epithelium-specific proteins: characteristics and potential applications as markers. *Am J Respir Crit Care Med* 1999; 159:646–78.
- 17 Persson CG. Plasma exudation and asthma. *Lung* 1988; 166: 1–23.
- 18 Folkesson HG, Westrom BR, Pierzynowski SG, Karlsson BW. Lung to blood passage of different-sized molecules during lung inflammation in the rat. *J Appl Physiol* 1991; 71:1106–11.
- 19 Hastings RH, Grady M, Sakuma T, Matthay MA. Clearance of different-sized proteins from the alveolar space in humans and rabbits. *J Appl Physiol* 1992; 73:1310–6.
- 20 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.
- 21 Haley KJ, Ciota A, Contreras JP, Boothby MR, Perkins DL, Finn PW. Alterations in lung collectins in an adaptive allergic immune response. *Am J Physiol Lung Cell Mol Physiol* 2002; 282:L573–84.
- 22 Mishra A, Weaver TE, Beck DC, Rothenberg ME. Interleukin-5-mediated allergic airway inflammation inhibits the human surfactant protein C promoter in transgenic mice. *J Biol Chem* 2001; 276:8453–9.
- 23 International Consensus Report on Diagnosis and Treatment of Asthma. National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, Publication no. 92-3091, March 1992. *Eur Respir J* 1992; 5:601–41.
- 24 International Consensus Report on the Diagnosis and Management of Rhinitis. International Rhinitis Management Working Group. *Allergy* 1994; 49:1–34.
- 25 Sterk PJ, Fabbri LM, Quanjer PH et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J* 1993; 16 (Suppl.):53–83.
- 26 van der Veen MJ, van der Zee JS. Aerosol recovery from large-volume reservoir delivery systems is highly dependent on the static properties of the reservoir. *Eur Respir J* 1999; 13:668–72.
- 27 Cockcroft DW, Murdock KY, Kirby J, Hargreave F. Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. *Am Rev Respir Dis* 1987; 135:264–7.
- 28 Nocker RE, Out TA, Weller FR, de Riemer MJ, Jansen HM, van der Zee JS. Induced sputum and bronchoalveolar lavage as tools for evaluating the effects of inhaled corticosteroids in patients with asthma. *J Lab Clin Med* 2000; 136:39–49.
- 29 Shimizu H, Hosoda K, Mizumoto M et al. Improved immunoassay for the determination of surfactant protein A (SP-A) in human amniotic fluid. *Tohoku J Exp Med* 1989; 157:269–78.
- 30 Honda Y, Kuroki Y, Matsuura E et al. Pulmonary surfactant protein D in sera and bronchoalveolar lavage fluids. *Am J Respir Crit Care Med* 1995; 152:1860–6.
- 31 Nagae H, Takahashi H, Kuroki Y et al. Enzyme-linked immunosorbent assay using F(ab')₂ fragment for the detection of human pulmonary surfactant protein D in sera. *Clin Chim Acta* 1997; 266:157–71.
- 32 Reimert CM, Venge P, Kharazmi A, Bendtzen K. Detection of eosinophil cationic protein (ECP) by an enzyme-linked immunosorbent assay. *J Immunol Methods* 1991; 138:285–90.
- 33 Homer RJ, Zheng T, Chupp G et al. Pulmonary type II cell hypertrophy and pulmonary lipoproteinosis are features of chronic IL-13 exposure. *Am J Physiol Lung Cell Mol Physiol* 2002; 283:L52–9.
- 34 Ikegami M, Whitsett JA, Chroneos ZC et al. IL-4 increases surfactant and regulates metabolism in vivo. *Am J Physiol Lung Cell Mol Physiol* 2000; 278:L75–80.
- 35 Strong P, Reid KB, Clark H. Intranasal delivery of a truncated recombinant human SP-D is effective at down-regulating allergic hypersensitivity in mice sensitized to allergens of *Aspergillus fumigatus*. *Clin Exp Immunol* 2002; 130:19–24.
- 36 Strong P, Townsend P, Mackay R, Reid KB, Clark HW. A recombinant fragment of human SP-D reduces allergic responses in mice sensitized to house dust mite allergens. *Clin Exp Immunol* 2003; 134:181–7.
- 37 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.
- 38 Crimi E, Milanese M, Oddera S et al. Inflammatory and mechanical factors of allergen-induced bronchoconstriction in mild asthma and rhinitis. *J Appl Physiol* 2001; 91:1029–34.
- 39 O'Byrne PM, Dolovich J, Hargreave FE. Late asthmatic responses. *Am Rev Respir Dis* 1987; 136:740–51.
- 40 Bentley AM, Meng Q, Robinson DS, Hamid Q, Kay AB, Durham SR. Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am J Respir Cell Mol Biol* 1993; 8:35–42.
- 41 Van Der Veen MJ, Van Neerven RJ, De Jong EC, Aalberse RC, Jansen HM, van der Zee JS. The late asthmatic response is associated with baseline allergen-specific proliferative responsiveness of peripheral T lymphocytes in vitro and serum interleukin-5. *Clin Exp Allergy* 1999; 29:217–27.