

Allergen-induced bronchial inflammation is associated with decreased levels of surfactant proteins A and D in a murine model of asthma

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Summary

Background Increasing evidence suggests that pulmonary surfactant protein A (SP-A) and D (SP-D) participate in the lung defence against pathogens. However, the role of surfactant proteins in the pathogenesis of allergen-induced airway inflammation has not been elucidated. In this study we examined the levels and distributions of SP-A and SP-D in a dust mite (*Dermatophagoides pteronyssinus*, Der p) allergen-induced murine model of asthma.

Methods The concentration of SP-A and SP-D in the bronchoalveolar lavage fluid (BALF) and the distribution of surfactant proteins in the lung were assayed by ELISA and immunohistochemistry methods, respectively. The effect of surfactant proteins on allergen-induced pulmonary lymphocyte proliferation was also studied.

Results We demonstrated that there were marked reductions of SP-A and SP-D levels in the BALF of Der p-sensitized BALB/c mice at 48–72 h after allergen challenge (AC). Both purified SP-A and SP-D were able to suppress, in a dose dependent manner, Der p-stimulated intrapulmonary lymphocyte proliferation of naïve mice with saline or allergen challenge, or of Der p-sensitized mice with saline challenge. On the contrary, this suppressive effect was mild (< 9%) on lymphocytes from sensitized mice after AC.

Conclusion These results indicated the involvement of pulmonary surfactant proteins in the allergic bronchial inflammation of sensitized mice.

Keywords: airway allergic inflammation, surfactant proteins, bronchial asthma

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Introduction

Pulmonary surfactant is a highly surface-active complex of phospholipids and proteins located at the air–liquid interface inside the alveoli. Its major roles are to stabilize the alveolar walls and prevent the collapse of alveoli during exhalation [1]. Surfactant may have a similar effect in the small airways because surfactant deficiency might lead to

the collapse of small cylindrical airways [2,3]. *In vitro* studies also showed that surfactant exerted immunomodulatory actions [4,5]. Administration of surfactant could alleviate the immune lung injury induced by inhaled antigen [6], and aerosolized surfactant treatment would significantly improve impaired lung function in animal models [7]. Recently, Kurashima *et al.* [8,9] reported that nebulization of surfactant during an acute asthma attack would dramatically improve lung vital capacity (VC), forced expiratory volume in one second (FEV₁), and drastic changes in surfactant activity of sputum of adult asthmatics, suggesting a more complex role of airway surfactant than previously thought.

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Surfactant associated proteins A (SP-A) and D (SP-D) are members of a family of collagenous carbohydrate binding proteins (collagenous C-type lectins, now known as collectins) that are involved in the innate immunity of the lung [10,11]. Both SP-A and SP-D show specific interactions with various microorganisms and leucocytes *in vitro* [12,13]. Recent data further suggest that the change in lung collectins is part of a more generalized response of the lung to acute lung injury, and that they modulate local inflammatory/immune responses [14]. With respect to allergic diseases, Malhotra *et al.* [15] reported that SP-A bound to water-extractable, allergenic glycoproteins from pollen grains in a calcium-dependent manner, suggesting that the interaction of surfactant proteins with certain allergens may have some roles in allergic reactions. Similarly, we have demonstrated that SP-A and SP-D not only could bind to mite (*Dermatophagoides pteronyssinus*, Der p) and fungus (*Aspergillus fumigatus*) allergens in a specific way, but also could inhibit the binding of allergen-specific IgE to these allergens through competition or steric hindrance effects [16,17]. Moreover, both of these surfactant proteins were found to inhibit histamine release and allergen-induced lymphocyte proliferation, two essential steps in the pathogenesis of asthma [18]. All these results suggest that surfactant proteins may well be involved in allergen sensitization or participate in the allergic inflammatory processes that are frequently seen in asthmatic patients.

Previously, we have developed a murine model of allergic airway inflammation induced by dust mite allergen [19,20]. Intranasal provocation of specific allergen could induce pulmonary eosinophilic infiltration in sensitized mice [19]. The allergen-induced eosinophilia was CD4⁺ T cell dependent and associated with a series of inflammatory and immunological events including the development of early type hypersensitivity, tumour necrosis factor (TNF)- α production, VCAM-1 expression, and tracheal hyperreactivity [20]. To clarify the role of surfactant proteins in allergen-induced bronchial inflammation, a similar murine model of asthma, prepared and characterized as above, was designed to determine whether there is a change of SP-A and/or SP-D level in the lung during asthmatic attack.

Materials and methods

Reagents

Lyophilized house dust mite (*Dermatophagoides pteronyssinus*, Der p) was purchased from Allergon (Engelholm, Sweden). The crude mite preparation was extracted with ether. After dialysis with deionized water, the mite extract was lyophilized and stored at -20°C . The mite antigen was dissolved in pyrogen-free isotonic saline (YF Chemical,

Taipei, Taiwan) and filtered through 0.2 μm -filter (Microgen, Laguna Hills, CA) before being used. The T-cell mitogen, phytohemagglutinin (PHA), was purchased from Murex Diagnostics (Dartford, UK).

Mice, immunization and allergen challenge (AC)

Specific pathogen-free, 6–8-week-old BALB/c mice from the Laboratory Animal Center, National Cheng Kung University, were used in this study. Mice were housed in microisolator cages (Laboratory products, Maywood, NJ, USA) and provided with sterile food and water *ad libitum*. The colony was monitored regularly for the presence of murine pathogens. Results were consistently negative. All experimental animal care and treatment followed the guidelines set up by the National Science Council of the Republic of China. Groups of mice were subcutaneously immunized with 50 μL of an emulsion containing 40 μg of Der p in complete Freund's adjuvant (CFA; Difco, Detroit, MI) [19]. Fourteen days later, mice were lightly anaesthetized with intraperitoneal injection of 60 mg/kg of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL, USA), and were intratracheally infused with 50 μL of Der p (1 mg/mL). Animals were held in an upright position for 1 min to resume normal breathing. Controls included Der-p-sensitized mice challenged with saline and naïve (non-sensitized) mice challenged with Der p.

Bronchoalveolar lavage fluid sample collection and processing

Mice were euthanized with an overdose of sodium phenobarbital at various time periods after AC. Sera were collected and stored at -70°C until assay. BAL was performed and total and differential counts of BALF cells were determined as described previously [19,20]. Briefly, two separate 1-mL doses of sterile endotoxin-free saline were injected into the lung via the trachea of each mouse. About 1.8 mL of the washing solution was consistently recovered. The BAL fluids were centrifuged at 200 g at 4°C for 10 min, and the supernatants were collected and stored at -70°C until assay. Total leucocytes in BAL fluids were determined with an haemocytometer. Samples (100 μL) of BAL fluid (2×10^5 cells/mL) were cytocentrifuged on glass slides at 350 r.p.m. for 5 min using Cytospin 3 cytocentrifuge (Shandon Scientific Limited Asmoor, Runcorn, England). Slides were air-dried for 30 min before staining with Liu stain (Tonyar Diagnostics Inc., Taipei, Taiwan) for differential counting of macrophages, neutrophils, lymphocytes and eosinophils. All samples were evaluated in a double-blind manner.

Biochemical analysis of BALF

Aliquots of the cell-free supernatant of BALF were used for determination of protein concentrations according to the method of Lowery [21] and determination of phospholipid concentrations using the method of Bartlett [22] as described by Postle *et al.* [23]. The latter assay is based on a phosphorous determination carried out on the lipids extracted with chloroform/methanol. Standard curves of dimyristoylphosphatidylcholine (PC 14 : 0/14 : 0) and dipotassium hydrogen orthophosphate were constructed between 0 and 60 nmole. Phosphate concentration was calculated from the absorbance at 830 nm after addition of ammonium molybdate and Fiske and Subbarow reagent and heating at 100 °C for 10 min [22].

ELISA for SP-A and SP-D in BALF

Native human SP-A and SP-D were purified by maltose agarose affinity-chromatography from BALF obtained from alveolar proteinosis patients as described by Strong *et al.* [24]. The purity of these preparation was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining and Western blotting. No immunoglobulin contamination was detected by ELISA with antihuman IgG, IgM and IgE-peroxidase conjugates. Routine *Limulus* lysate assay (*Limulus* Amebocyte Lysate Test; Sigma) revealed that none of the preparations was contaminated with detectable endotoxin. A recombinant polypeptide composed of the neck region and CRD (N/CRD) of human SP-D was expressed in *Escherichia coli* and isolated in the form of self-associating, homotrimer as previously described [25,26]. Polyclonal antibodies to mouse SP-A and SP-D were raised in rabbit against purified recombinant fragments of mouse SP-A and SP-D, respectively. These antibodies were specific for mouse SP-A and SP-D at working dilution as assessed by ELISA and Western blotting. Concentrations of SP-A and SP-D in the lavage samples were measured using sandwich ELISAs as previously described [27]. The SP-A and SP-D assays used in this study were capable of detecting mouse SP-A in the range from 10 ng/mL to 1000 ng/mL, and SP-D ranging from 5 ng/mL to 50 ng/mL. The standard curves obtained using purified mouse SP-A, or SP-D, within this range, were linear, correlation coefficients were generally 0.90–0.95, and the diluted BALF from mice consistently yielded curves parallel to the standard curve. Sample dilution was adjusted to within the linear range. The intra-assay coefficient of variation, calculated by comparing the results of 12 standard rows on one plate, was $3.2 \pm 0.9\%$. The between-assay coefficients of variation, calculated by comparing the results of 12 standard rows on eight plates assayed in a period of 4 months, was $11.2 \pm 1.7\%$.

Immunohistochemical staining of SP-A and SP-D in the lung

Immunohistochemical staining of SP-A and SP-D in the lung was performed as described previously [19]. Briefly, the lungs of each mouse were removed and frozen in Tissue-Tek® OCT compound (Sakurafinetek, Torrance, CA, USA). Frozen sections (5 µm thick) were cut and placed on poly L-lysine (Sigma)-coated glass slides. Sections were air-dried for at least 2 h and fixed in acetone for 10 min. Non-specific binding sites were blocked by incubation with 5% (v/v) normal sheep serum in PBS containing 0.1% (wt/vol) BSA (PBS/BSA) for 30 min. The sections were then incubated with polyclonal antimouse SP-A or SP-D antibody in PBS/BSA in a humid chamber at 4 °C overnight. After washing in PBS, endogenous peroxidase activity was blocked by incubating the sections with 0.3% hydrogen peroxide in PBS for 30 min. The sections were then incubated with a 1 : 100 dilution of peroxidase-conjugated sheep antirabbit IgG Fab fragment in PBS/BSA. After further washing, staining was obtained by addition of PBS containing 3,3-diaminobenzidine (Sigma; 1 mg/mL) and hydrogen peroxide (0.2%, v/v) for 20 min. The slides were washed and counter-stained in haematoxylin (Sigma) before mounting. Control samples were incubated with PBS/BSA instead of polyclonal antibodies.

Preparation of intraparenchymal lung lymphocytes

Intraparenchymal lung lymphocytes were collected using the method as described by Abraham *et al.* [28]. In brief, mice were killed by cervical dislocation. the chest was opened, and the lungs were excised with caution to avoid the peri-tracheal lymph nodes. For each experiment lung tissues from three to six mice were pooled and minced finely. The lung tissues were then incubated in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) containing 5% (v/v) FCS, 100 µg/mL penicillin/streptomycin, 10 mM HEPES, 50 µM 2-ME, 20 mM L-glutamine, 20 U/mL collagenase (C2139, Sigma Chemical Co., St Louis, MO, USA), and 1 µg/mL DNase (type I, Sigma) for 60 min at 37 °C on a rotary agitator (approximately 60 r.p.m.). Any remaining intact tissue was disrupted by passage through a 21-gauge needle. Tissue fragments and the majority of dead cells were removed by rapid infiltration through a glass wool column, and cells collected by centrifugation.

The cell pellet was suspended in 4 mL of 40% Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 mL of 80% Percoll. After centrifugation at 600 g for 20 min at 15 °C, the cells at the interface were collected, washed twice in PBS, and counted. Viability was determined by Trypan blue exclusion that was consistently more than

98%. Cells to be used for lymphocyte proliferation assay were washed three times in cold RPMI-1640 containing penicillin, streptomycin and gentamycin. The cells were incubated on a Petri dish for 60 min at 37 °C in 5% CO₂ to remove adherent cells. Non-adherent cells, more than 90% belonged to mononuclear cells, were collected and resuspended at 2×10^6 cells per ml in RPMI 1640 supplemented with 10% (v/v) newborn calf serum (Gibco BRL). Cells were cultured at a concentration of 2×10^5 cells per well in flat-bottomed, 96-well sterile plates (Corning, Inc., Corning, NY, USA).

Cell proliferation assay

Der p extract was used to stimulate intrapulmonary lymphocytes as previously described [18]. Increasing amounts of SP-A, SP-D or SP-D(N/CRD) were added to cell culture medium with varying amounts of Der p. Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Sixty-eight hours later, each well was pulsed with 1 µCi of [³H]-thymidine (Amersham International, Oakville, Ontario, USA) for another 4 h at 37 °C and then harvested on a PHD cell harvester (Cambridge Technology, Cambridge, MA, USA). [³H]-thymidine incorporation was determined by liquid scintillation counting (Beckman Instruments, New York, USA). Cell viability was assessed by trypan blue exclusion during the course of the experiment and routinely found to be $95 \pm 3\%$. Background counts for wells containing cells without stimulation of Der p were always less than 800 cpm. Results are presented as mean $\pm$ SE for triplicate cultures. A non-specific cellular mitogen, phytohemagglutinin (PHA, Murex Diagnostics, Dartford, UK), was also included as a positive control. The percentage of inhibition by surfactant proteins was defined as the percentage of (c.p.m. of mitogen stimulated lymphocyte proliferation in the presence of surfactant protein-cpm of mitogen stimulated lymphocyte proliferation)/cpm of mitogen stimulated lymphocyte proliferation.

Statistical analysis

Results were analysed either by Student's *t*-test or by analysis of variance. Comparison of group means was done with Scheffes *F*-test when all possible pair-wise comparisons were made. Differences with $P < 0.05$ were judged significant. All experiments were repeated at least twice.

Results

Change in BALF after allergen challenge

The kinetic study showed that an intratracheal challenge of 50 µg of Der p to sensitized mice induced an increase in

total numbers and alteration of cellular distribution of BAL cells (Table 1). The sensitized mice developed typical allergen-induced airway inflammation, beginning with a neutrophilic infiltrate which was followed by lymphocytes and eosinophils. The whole process started from 24 h, peaked at 72 h to 7 d, and subsided 14 days after allergen challenge. The BAL fluid obtained after AC demonstrated only a small, insignificant change in phospholipid concentration in immunized and control mice (Table 1). While total protein concentrations in the BALF were all significantly higher in the group of sensitized mice challenged with allergen as compared with that of naïve mice challenged with saline or Der p, started from the 24 h to 7 days after AC (Table 1).

The change of SP-A and SP-D levels in the Der p-sensitized mice after AC

The BALF samples in this study were centrifuged at 200 *g* before analysis to precipitate cellular debris. To investigate whether this step resulted in significant sedimentation of surfactant proteins, standards of SP-A (100 ng/mL) and SP-D (100 ng/mL) were measured before centrifugation and in the supernatant after centrifugation. Recoveries in the supernatant were 82.3% and 92.4%, respectively. When normalized to the total phospholipid content in BALF, there was a significant decrease in SP-A (Fig. 1A) and SP-D (Fig. 1B) levels in the BALF of Der p-sensitized mice 48 h and 72 h after AC compared with those of naïve mice challenged with saline or Der p. These results were consistent when the altered concentration of SP-A and SP-D were expressed relative to total protein or volume of BALF (data not shown). The slight increase in SP-A and SP-D levels at 24 h after AC might be due to increased infiltration of cellular components of bronchial inflammation to released lung collectins from the tissue pool. Both the levels of SP-A and SP-D returned to the basal levels 7 days after AC. Immunoblot analyses also confirmed the Der p-induced changes of SP-A and SP-D concentrations in the BALF of sensitized mice (data not shown). Specifically, these surfactant proteins were recognized as single band by the antimouse SP-A or SP-D antibodies with molecular weight approximately 34 kDa and 42 kDa, respectively.

Immunohistochemistry of SP-A and SP-D

Surfactant protein containing cells in the lung tissues of naïve and Der p-sensitized mice were detected by immunohistochemical staining. In naïve mice (Fig. 2A), the immuno-staining for SP-A was observed in alveolar cells, the inner surface of the alveoli, and along the bronchiolar epithelium. The localization and morphological characteristics of these SP-A-containing cells were consistent with

Table 1. Change of total phospholipid and protein concentration, and cellular distribution in BALF, of groups of mice at various days after Der p challenge

Time AC/Groups	Total phospholipid (nmole/ml)	Total protein (mg/mL)	Cell Nos ($\times 10^4$ cell/ml)			
			Macrophages	Lymphocytes	Neutrophils	Eosinophils
<i>Before AC</i>						
Naïve (N)	70 $\pm$ 22	0.6 $\pm$ 0.5	14.5 $\pm$ 9.4	0.2 $\pm$ 5.5	0.7 $\pm$ 0.2	0
Sensitized (S)	75 $\pm$ 31	0.3 $\pm$ 0.4	16.6 $\pm$ 2.3	0.3 $\pm$ 0.5	0.8 $\pm$ 0.3	0
<i>6 h after AC</i>						
N $\pm$ saline	53 $\pm$ 21	1.2 $\pm$ 0.2	13.2 $\pm$ 1.4	0.4 $\pm$ 0.3	0.6 $\pm$ 0.9	0
N $\pm$ Der p	65 $\pm$ 18	1.3 $\pm$ 0.4	16.6 $\pm$ 1.3	0.5 $\pm$ 0.5	1.8 $\pm$ 1.3	0
S $\pm$ Der p	77 $\pm$ 23	1.6 $\pm$ 0.1	13.1 $\pm$ 3.7	1.2 $\pm$ 0.5	18.8 $\pm$ 2.9	0
<i>1 day after AC</i>						
N $\pm$ saline	66 $\pm$ 31	0.2 $\pm$ 0.2	13.9 $\pm$ 2.4	0.4 $\pm$ 0.3	0.6 $\pm$ 0.9	0
N $\pm$ Der p	63 $\pm$ 26	3.3 $\pm$ 1.4	18.6 $\pm$ 3.3	1.1 $\pm$ 0.5	27.8 $\pm$ 5.3*	0.9 $\pm$ 0.4
S $\pm$ Der p	71 $\pm$ 14	6.6 $\pm$ 2.1*	24.6 $\pm$ 4.1	3.1 $\pm$ 1.7	42.2 $\pm$ 10.2*	12.5 $\pm$ 4.2*
<i>3 days after AC</i>						
N $\pm$ saline	73 $\pm$ 15	0.2 $\pm$ 0.1	19.2 $\pm$ 8.4	0.4 $\pm$ 0.2	0.3 $\pm$ 0.2	0.2 $\pm$ 0.1
N $\pm$ Der p	65 $\pm$ 19	1.3 $\pm$ 0.4	23.6 $\pm$ 8.3	1.5 $\pm$ 0.5	2.8 $\pm$ 1.3	0
S $\pm$ Der p	58 $\pm$ 19	8.4 $\pm$ 4.1*	83.1 $\pm$ 30.7*	12.2 $\pm$ 6.2*	28.8 $\pm$ 2.9*	64.5 $\pm$ 20.2*
<i>7 days after AC</i>						
N $\pm$ saline	67 $\pm$ 25	0.1 $\pm$ 0.1	23.6 $\pm$ 9.4	0.9 $\pm$ 0.3	0.2 $\pm$ 0.1	0
N $\pm$ Der p	65 $\pm$ 22	0.3 $\pm$ 0.2	25.6 $\pm$ 8.3	0.1 $\pm$ 0.1	1.8 $\pm$ 0.3	0
S $\pm$ Der p	71 $\pm$ 26	4.6 $\pm$ 2.1*	53.1 $\pm$ 20.7*	12.2 $\pm$ 6.2*	2.8 $\pm$ 1.9	20.5 $\pm$ 10.2*
<i>14 days after AC</i>						
N $\pm$ saline	78 $\pm$ 31	0.2 $\pm$ 0.2	10.2 $\pm$ 3.4	0.4 $\pm$ 0.3	0.6 $\pm$ 0.2	0
N $\pm$ Der p	62 $\pm$ 23	0.3 $\pm$ 0.1	11.6 $\pm$ 5.3	1.1 $\pm$ 0.5	0.8 $\pm$ 0.3	0
S $\pm$ Der p	69 $\pm$ 28	0.6 $\pm$ 0.1	13.1 $\pm$ 3.7	0.8 $\pm$ 0.2	0.2 $\pm$ 0.1	0.5 $\pm$ 0.2

Groups ($n = 4$) of BALB/c mice were immunized and challenged with Der p (1 mg/mL) or saline as described in Materials and methods. Total leucocyte and differential counts of BALF were determined at various times after AC. Protein concentration in the BALF was determined by the Lowry method, and total phospholipid composition of BALF was measured as inorganic phosphorous and expressed as mean $\pm$ SD. * $P < 0.05$, when compared with control mice.

those of alveolar type II cells and non-ciliated bronchiolar epithelial (Clara) cells (Fig. 2B). Marked difference in SP-A distribution was noted between naïve and sensitized mice after AC. In sensitized mice (Fig. 2C), the SP-A staining in alveolar walls was significantly reduced compared with that in naïve mice, whereas focal intense staining of SP-A was noted at the terminal bronchiolar epithelium where eosinophil infiltration occurred (Fig. 2D). This staining for SP-A persisted for at least 7 days and gradually subsided as the cellular response diminished at day 14 after AC. In agreement with previous findings [29], SP-D immunostaining was detected in alveolar type II cells, non-ciliated bronchiolar cells, and scattered alveolar macrophages in the inner surface of the alveolar walls of naïve mice (Fig. 2E and Fig. 2F) that were treated with either saline or Der p. However, at day 3 after AC, a marked decrease in SP-D staining was noted in the alveolar spaces of sensitized mice, while positive stainings were present in the tracheal and

terminal bronchiolar spaces that were infiltrated with mononuclear cells and eosinophils (Fig. 2G). In addition, many lining epithelial cells and submucosal gland cells were positively stained for SP-D (Fig. 2H).

The inhibitory effect of SP-A and SP-D on the Der p-induced proliferation of lung lymphocytes

The inhibitory effect of SP-A (Fig. 3A) or SP-D (Fig. 3B) on Der p-stimulated lymphocyte proliferation was studied using intrapulmonary lymphocytes collected from allergen-sensitized mice at 6 h after AC. Surfactant proteins reduced [^{3} H] thymidine incorporation in a dose-dependent manner. Both SP-A and SP-D had a significantly suppressive effect on lymphocyte proliferation at the concentration above 1 μ g/mL (maximal inhibition $> 50\%$), when cocultured with 10 μ g/mL of Der p.

The inhibitory effect of surfactant protein, as calculated

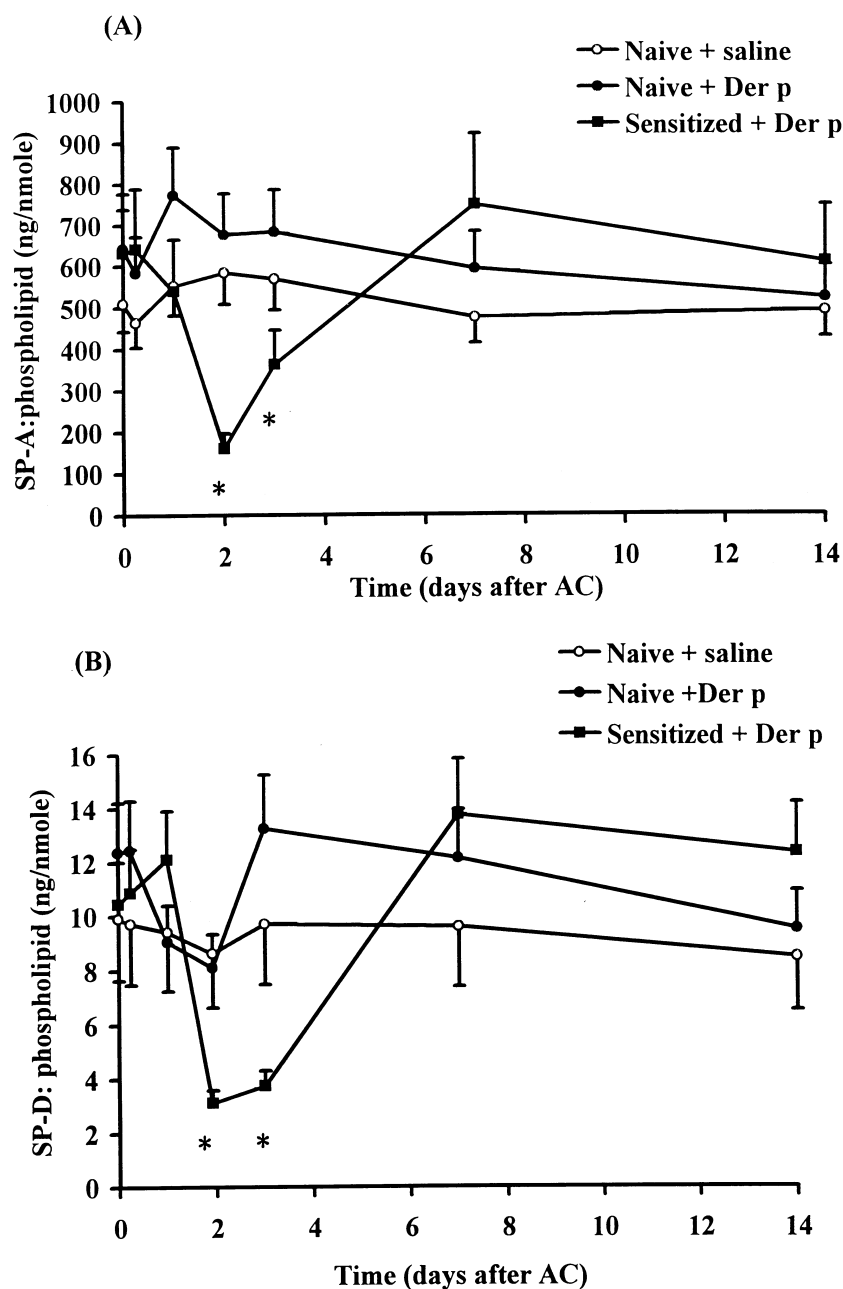
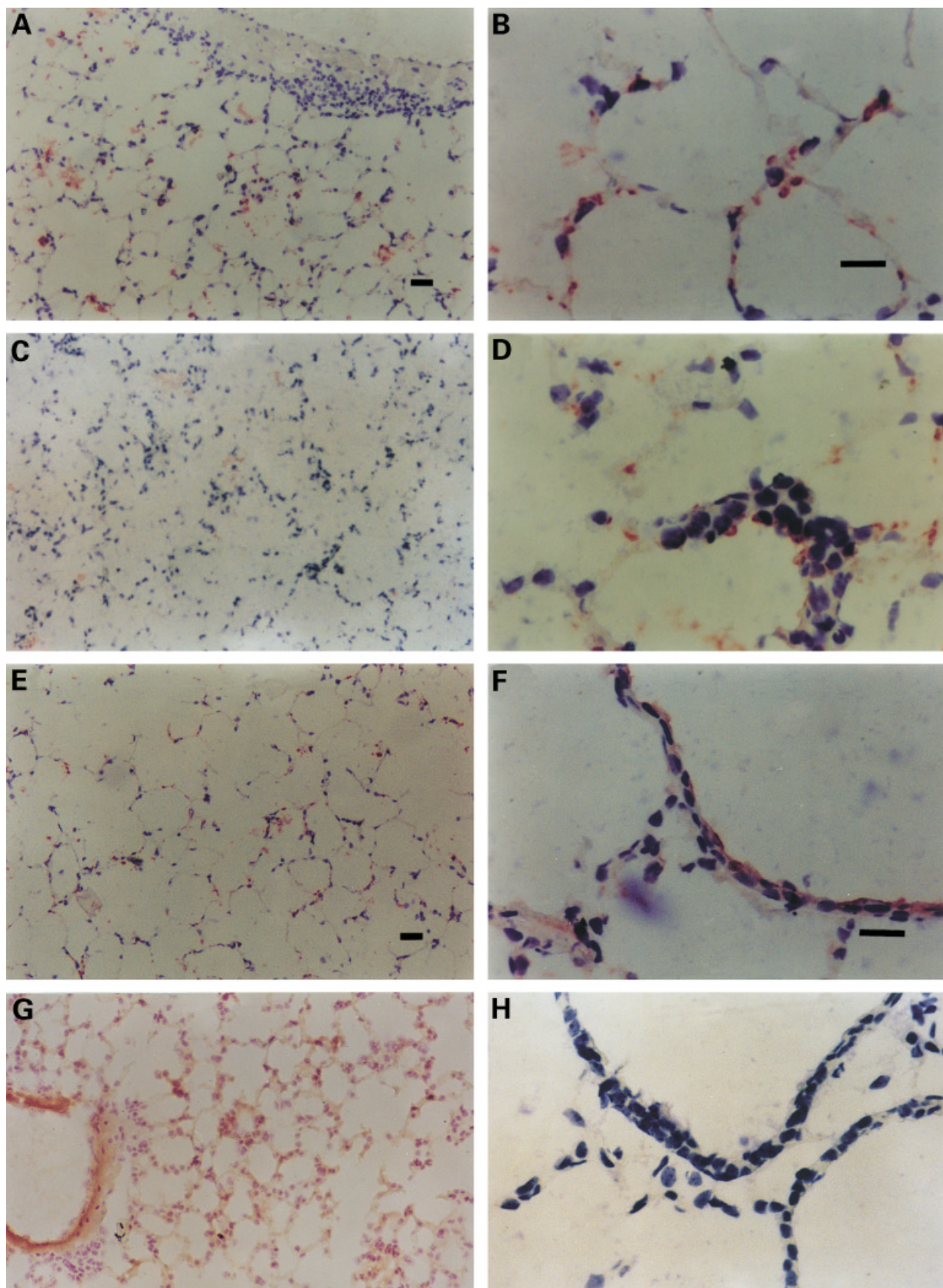


Fig. 1. The change of SP-A (Fig. 1A) and SP-D (Fig. 1B) concentrations in BALF. Groups of naïve mice were intratracheally infused with saline (open circle) or Der p (solid circle), or allergen-sensitized mice were challenged with Der p (solid square). SP-A and SP-D contents in BALF were determined as described in Materials and methods. Results represent the mean $\pm$ SD of six mice per group. * $P < 0.05$ as compared with that of naïve mice without AC.

by the percentage of decreased [^{3} H] thymidine incorporation when surfactant protein coincubated with Der p compared to Der p alone, was also studied using intrapulmonary lymphocytes collected from naïve and sensitized mice at various times after saline or Der p challenge (Fig. 4).

Isolated intrapulmonary lymphocytes were incubated with 10 μ g/mL of SP-A or SP-D for 24 h before the addition of 10 μ g/mL of Der p. In naïve mice challenged with Der p or saline, or sensitized mice challenged with saline, both SP-A (Fig. 4A) and SP-D (Fig. 4B) suppressed the Der p-induced

Fig. 2. Immunohistochemistry staining for SP-A and SP-D. SP-A (Fig. 2A to 2D) and SP-D (Fig. 2E to 2H) containing cells in the lung tissues of naïve (Fig. 2A, 2B, 2E, 2F) and Der p-sensitized mice (Fig. 2C, 2D, 2G, 2H) after AC were detected by immunohistochemical studies as described in the Materials and methods section. Original magnifications of Fig. 2 (A, C, E, G) were 100 $\times$ (bar = 20 μ m), while Fig. 2 (B, D, F, H) were 400 $\times$ (bar = 10 μ m), respectively.



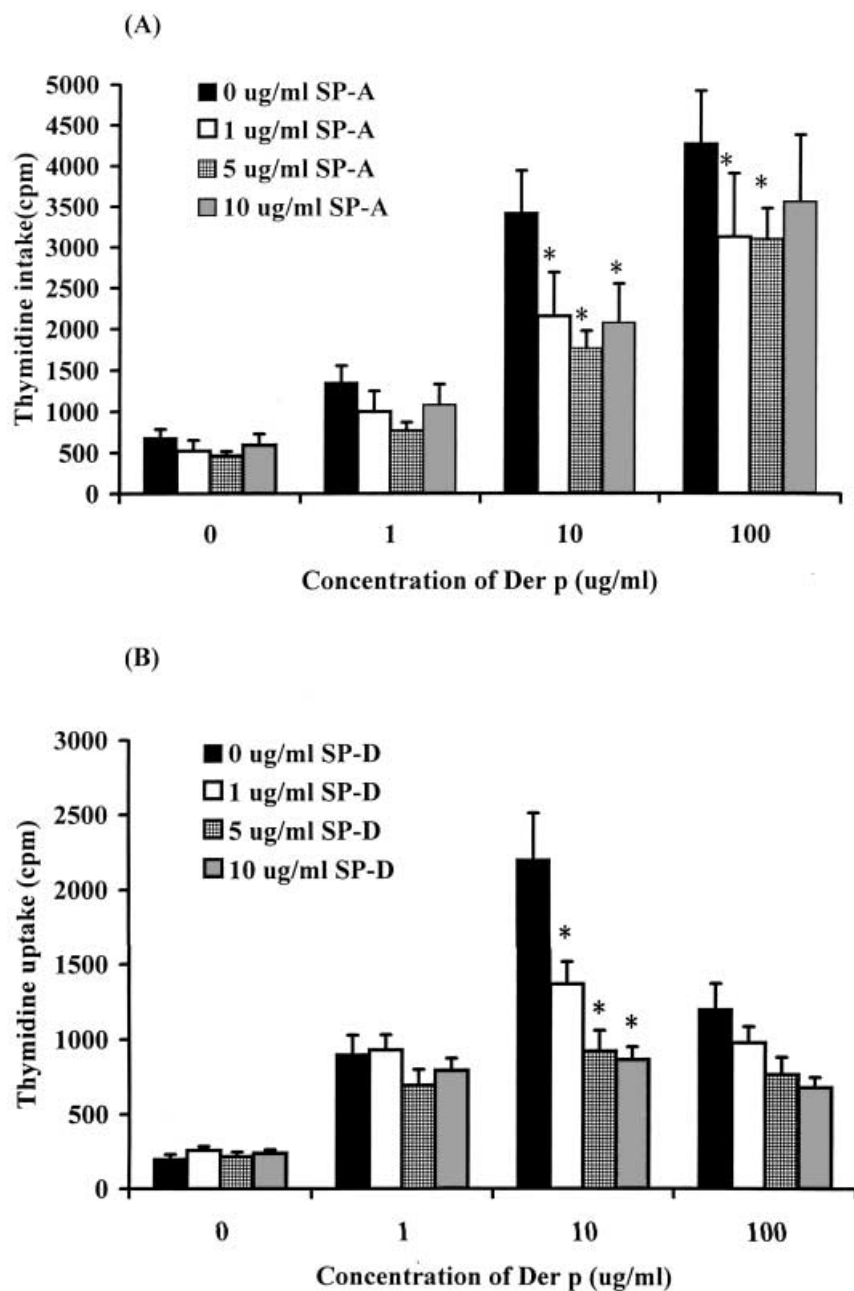


Fig. 3. Inhibitory effect of SP-A and SP-D on Der p-induced intrapulmonary lymphocyte proliferation in allergen-sensitized mice after AC. Various concentrations of SP-A (Fig. 3A) or SP-D (Fig. 3B) were added to the intrapulmonary lymphocytes (1×10^6 cells/mL) collected from sensitized mice 6 h or 48 h after AC in the presence of $10 \mu\text{g/mL}$ Der p. * $P < 0.05$, ** $P < 0.01$ compared with culture without Der p.

lymphocyte proliferation by more than 50% regardless of the time in which the lymphocytes were collected. Although both SP-A and SP-D had a significant inhibitory effect on Der-p induced lymphocyte proliferation in sensitized mice 6 h after AC, as shown in Fig. 3, they did not exhibit a suppressive effect on lymphocytes collected from sensitized mice at 48 or 72 h after AC (Fig. 4). The inhibitory effect of SP-D was less potent on lymphocytes collected from sensitized mice 24 h after AC (decreased only 28.5% compared with base level of Der p alone)

(Fig. 4B). The addition of sugar or EDTA into culture medium could abolish this inhibitory effect of surfactant proteins on lymphocyte proliferation (data not shown).

Discussion

In this study, we demonstrated for the first time that the levels of SP-A and SP-D fluctuated following the course of the allergen-induced bronchial inflammation in mice. Specifically, the SP-A and SP-D levels were decreased at

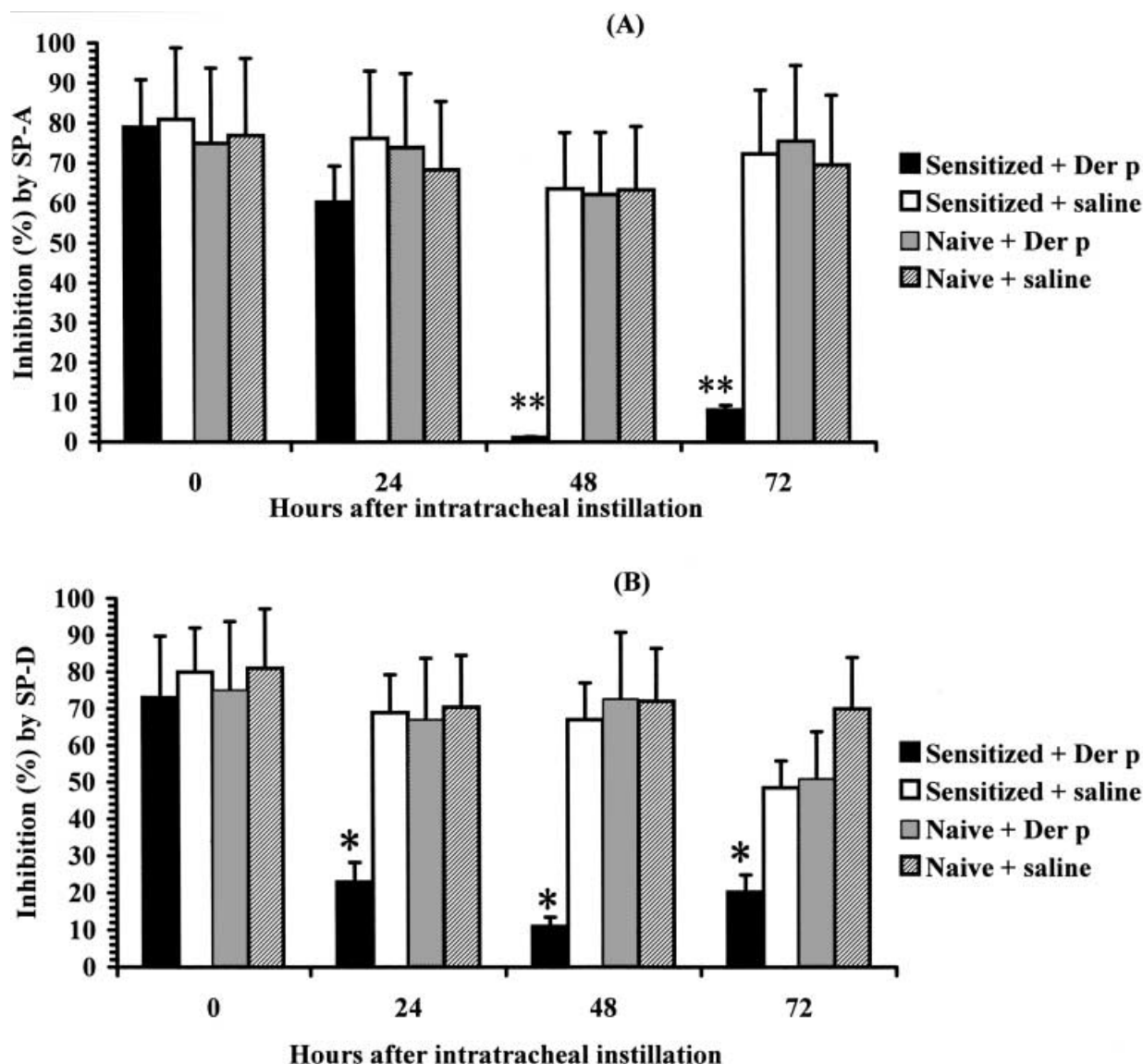


Fig. 4. Surfactant proteins exert no inhibitory effect on allergen-activated intrapulmonary lymphocytes of sensitized mice. Intrapulmonary lymphocytes (1×10^6 cells/mL) obtained from naïve or sensitized mice 0, 24, 48 and 72 h after challenge with saline or Der p were pre-incubated with 10 μ g/mL of SP-A (Fig. 4A) or SP-D (Fig. 4B) for 24 h before the addition of 10 μ g/mL of Der p. The percentage of inhibition of lymphocyte proliferation is defined in the Materials and methods section. Results represent the mean $\pm$ SD of six mice per group. * $P < 0.05$; ** $P < 0.01$ compared with naïve mice without AC.

the peak of inflammatory response of asthma attack, and then gradually returned to baseline as the inflammatory process subsided. Immuno-histochemically, SP-A and SP-D diminished along the cellular walls of alveolar spaces, whereas increased levels of these proteins were demonstrated on the epithelium surface of the inflammatory terminal airways.

The mechanism underlying the impairment and diminishing of surfactant proteins in our murine model of asthma remains to be defined. One recent study suggested that

dysfunction of type II cells might be responsible for decreased SP-A levels and the low SP-A/phosphatidylcholine ratios in patients with asthma [30]. In a preliminary study, Hockey *et al.* showed that the levels of SP-A and SP-D in BALF of asthmatic patients were significantly lower than those of normal subjects (4.6 ± 1.9 vs. 7.2 ± 1.1 μ g/mL in SP-A, and 519 ± 41 vs. 810 ± 98 μ g/mL in SP-D) [31]. The protein concentrations and total phospholipids in lavage fluid were not increased in these mild asthmatic subjects and therefore were unlikely to have caused an

inhibitory effect on the synthesis of SP-A and SP-D. It is possible that a protein-rich, inflammatory exudate may inhibit surfactant protein synthesis during an acute asthmatic attack. Previously, it was found that allergen-induced airway inflammatory response would lead to increased endothelial and epithelial permeability, followed by an influx of fluid and plasma protein into the airways in sensitized animals. The oedematous response becomes worse as surfactant degrades during the inflammatory process [32]. This vicious circle of oedema formation and surfactant dysfunction is a major contributor to the formation of liquid plugs within the bronchioles, trapping of air in the alveoli, and the rapid increase in airway resistance during an asthma attack.

Previous studies with sensitized guinea-pigs exposed to aerosolized allergen have shown that the surface activity of the BALF of these animals was significantly reduced, while neither the concentration nor the composition of the surfactant phospholipids was changed [33,34]. Surfactant depletion has been shown to promote the cellular inflammatory response in sensitized animals after challenge [6]. On the other hand, pre-treatment with surfactant not only significantly decreased the extent of the impaired lung function, but also alleviated airway resistance in sensitized guinea-pigs after challenge [34,35]. Furthermore, their studies also indicated surfactant dysfunction as well as *in vitro* impairment of surfactant synthesis by type II pneumocytes after repeated, mild asthma attacks. Although SP-A and SP-D do not relate to surfactant function in preventing alveolar collapse, the impaired surface activity of lavage fluids was noted in sensitized animals 48–72 h after AC, the time period correlated with the lowest levels of SP-A and SP-D. Taken together, our studies and others all have indicated that a dysfunction of the pulmonary surfactant system, either structural or functional, contributes to the pathogenesis of asthma.

Surfactant proteins exert immunomodulatory effects that may contribute to the hyporesponsiveness of the lungs [36,37]. In a previous study we demonstrated that SP-A and SP-D were able to inhibit specific allergen-induced lymphocyte proliferation and suppress histamine release in symptom-free asthmatic children, while these suppressive effects of surfactant proteins were diminished in asthmatic children with an acute attack [18]. In this study, we further demonstrated that SP-A, SP-D and recombinant SP-D (N/CRD) were able to suppress Der p-induced proliferation of pulmonary lymphocytes from naïve mice or sensitized mice without specific allergen challenge. On the other hand, the suppressive effect was less prominent or almost abolished on lymphocytes taken from sensitized mice 24 and 48 h after AC, respectively. Therefore, reduced SP-A and SP-D levels in asthmatics reflect not only a disturbance in the turnover of the surfactant system, but also an

impaired activity of surfactant proteins in the induction and maintenance of hyporesponsiveness against pulmonary inflammation.

In conclusion, we demonstrated that there were marked reductions of SP-A and SP-D levels in BALF of Der p sensitized BALB/c mice during allergen-induced lung inflammation. The reduced SP-A and SP-D contents of BALF might be responsible for the increased airway resistance and pulmonary lymphocyte activation frequently seen in asthma attacks. Therefore, we believe that pulmonary surfactant has a preventive role against allergen sensitization and that increased synthesis, or supplement with, surfactant proteins may provide a new treatment for bronchial asthma. Serious consideration should now be given to exploring in more detail both the extent of any surfactant dysfunction in asthma, and the influence of both normal and abnormal lung surfactant on the inflammatory reaction characteristic of asthma.

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