

Surfactant dysfunction develops when the immunized guinea-pig is challenged with ovalbumin aerosol

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Summary

Background The cause of the airway resistance developing during an asthma attack is not completely understood. Besides bronchospasm and airway oedema a surfactant dysfunction has been suggested as a reason for an increased airway resistance.

Objective This paper aims at examining if indeed surfactant dysfunction develops when an asthma attack is induced in guinea-pigs.

Methods Guinea-pigs, immunized against ovalbumin and then challenged (by inhaling the antigen) underwent lung function tests ($n=7$) and were compared with seven animals challenged, but not immunized. Lung lavage was carried out in three groups of guinea-pigs: controls, never immunized nor challenged ($n=7$), not immunized but challenged ($n=6$), immunized and challenged, no lung function test ($n=6$). After concentrating the lavage fluid 10 times the surface activity was evaluated with the pulsating bubble surfactometer. The fluid's concentration of phospholipids and proteins was determined as was the phospholipid composition.

Results The 19 immunized and challenged animals all developed severe respiratory distress, six so seriously that they died. Lung function tests showed significantly increased airway resistance and decreased tidal volume, minute volume, and dynamic compliance. Surface activity of lavage fluid from immunized and challenged animals was significantly reduced when compared with fluid from control animals ($P<0.01$). Immunization and challenge had no effect on the lavage fluid's phospholipid concentration or composition, but the proteins were at a higher concentration than in the fluid of the controls ($P<0.01$).

Conclusion Proteins leaking into the airways inhibited the surfactant. This, in turn might have caused conducting airways to become blocked by liquid columns, which would increase airway resistance.

Keywords: airway challenge, airway inflammation, airway resistance, surfactant dysfunction, asthma

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Introduction

Asthma is a chronic pulmonary disease characterized by a hyperresponsiveness to various situations and agents that cause a change in airway resistance. Its pathophysiology is generally considered to include an inflammatory

reaction with oedema, mucociliary dysfunction, and spasm of the smooth muscles of the airways [1]. This overreaction of smooth musculature, generally known as bronchospasm, is often deemed to be the main reason for the increased airway resistance, mucus plugging and oedema in the airway wall being other widely accepted reasons.

This paper considers the possibility that dysfunction of pulmonary surfactant might be yet another contributing factor to airway resistance. It was recently demonstrated

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that a small quantity of liquid, blocking the narrow section of a glass tube, will not return to the narrow section after it has been extruded, provided the liquid contains well functioning pulmonary surfactant [2,3]. If, on the other hand the surfactant is not functioning well, because it is inadequate in quantity or quality, the liquid will return and block the glass tube's narrow section. If conditions are similar in the lungs' airways, patency would partly hinge on the surfactant function. If the surfactant is disturbed in its function many terminal conducting airways might become blocked during expiration, and that would cause airway resistance to increase.

There may be several reasons why a person with asthma might develop a surfactant dysfunction. It could be that the immunization has caused surfactant synthesis to slow down. It could be that the essential phospholipids or apoproteins have become inactivated by hydrolyzing enzymes from mast cells and eosinophils. There is also a possibility that plasma proteins invading the airways have inhibited the normal surface activity. This study was designed to investigate the possibility that guinea-pigs, immunized against ovalbumin, are suffering from a surfactant dysfunction when, following inhalation of the antigen, serious breathing problems have developed.

Materials and methods

Animals

Each of the 39 guinea-pigs (Buckberg Laboratories, Liberty, NY, USA) used for this study weighed approximately 400 g. The experiments were approved by the University Animal Care Committee. Seven animals, never exposed to the antigen, formed a group of controls. They received a lethal intraperitoneal (i.p.) dose of sodium pentobarbital so that bronchoalveolar lavage (BAL) could be carried out. Of the remaining 32 guinea-pigs 19 were immunized (I).

Immunization

Immunization was according to the method of Popa *et al.* [4] and Dunn *et al.* [5]. Briefly, into each hind limb the guinea-pigs were given an intramuscular injection of ovalbumin (Grade III, Sigma, St Louis, MO, USA) dissolved in saline, 0.35 mL 5% (w/v). The procedure was repeated after 7 days.

Challenge

The 32 guinea-pigs not serving as controls were all

challenged (Ch), i.e. they were forced to inhale the ovalbumin that some of them previously had been immunized against. With a DeVilbiss 65 ultrasonic nebulizer (DeVilbiss, Somerset, PA, USA) an 0.08% (w/v) ovalbumin solution was nebulized. Of the challenged animals 19 had been immunized (I Ch) and 11 of them were given an anesthetic dose of sodium pentobarbital i.p. before the challenge so that lung function tests could be carried out (I Ch LF). Originally, we planned to lavage the lungs after the lung function tests. However, preliminary results indicated that following those tests, requiring prolonged anesthesia, bleeding was often noted and the lavage fluid was bloody. For this reason eight animals were exposed to challenge only, and after 30 min they were given a lethal i.p. dose of sodium pentobarbital so that BAL could be executed (I Ch BAL).

The 13 guinea-pigs not immunized but challenged (NI Ch) were also divided into two groups, NI Ch LF ($n = 7$) and NI Ch BAL ($n = 6$). They were examined with the same technique as the immunized animals were.

Pulmonary function tests

To evaluate the airway resistance, anesthetized guinea-pigs of groups I Ch LF ($n = 11$) and NI Ch LF ($n = 7$), were challenged (Ch) with ovalbumin spray and, 10 min before, immediately after, and 30 min after the challenge they were studied with lung function (LF) tests. Sodium pentobarbital was given i.p. and a catheter (PE 60) was fed into the oesophagus. It was closed at the tip but had several side holes in a short section which was covered by a thin-walled balloon. The catheter, connected with a pressure transducer, was initially inserted as far as to the stomach, but was then withdrawn until the pressure recording clearly indicated that it was in the thoracic cavity. The trachea was then exposed and a tube, PE 240, was inserted and connected with a Fleisch pneumotachograph (Gould, Hato Rey, Puerto Rico). The flow and pressure signals were recorded with a polygraph (Model 79D, Grass, Quincy, MA, USA). Flow was integrated to tidal volume and minute ventilation was calculated from tidal volume and frequency of respiration. Dynamic compliance, C_{dyn} , was calculated by dividing the expiratory volume with the change in esophageal pressure, and total resistance of the lung, R_L , was estimated by dividing change in esophageal pressure with expiratory flow rate.

BAL

The procedure for BAL of the newly killed animal was as follows. Into the trachea a short polyethylene tube, PE-

Table 1. Weights and biochemical data as affected by immunization and challenge

	Not immunized, Not challenged, BAL Controls	Immunized, challenged, BAL I Ch BAL	Not immunized, challenged, BAL NI Ch BAL
Number of animals	7	(8) 6	6
Body weight, kg	0.427 ± 0.04	0.427 ± 0.005	0.433 ± 0.004
Dry lung weight, g	0.487 ± 0.01	0.627 ± 0.025	0.495 ± 0.021**# #
Dry lung/Body weight, g/kg	1.14 ± 0.03	1.47 ± 0.06	1.14 ± 0.05**# #
Recovery (ml) of the 14 ml used for lavage	10.9 ± 0.2	8.0 ± 1.1	11.7 ± 0.2*#
Total phospholipids, mg/kg body weight	4.67 ± 0.25	4.56 ± 0.27	4.84 ± 0.34
Total proteins, mg/kg body weight	6.30 ± 0.45	28.45 ± 6.54	10.40 ± 1.07*#(@ @)
Proteins/Phospholipids	1.35 ± 0.07	6.35 ± 1.37	2.14 ± 0.17*#(@ @)

All the values are means ± SEM.

* $P < 0.05$ (I Ch BAL vs Controls).

** $P < 0.01$ (I Ch BAL vs Controls).

$P < 0.05$ (I Ch BAL vs NI Ch BAL).

$P < 0.01$ (I Ch BAL vs NI Ch BAL).

@ @ $P < 0.01$ (NI Ch BAL vs Controls).

The phospholipids and proteins are the quantities calculated to exist in 14 mL lavage fluid.

Eight guinea-pigs were assigned to the I CH BAL group, but only six survived and could be examined with BAL.

240, was inserted and via a liquid filled PE-60 tube it communicated with a container with 14 mL saline solution. That volume would just expand the guinea-pig's airways when the container was raised to 20 cm above the animal. When it was lowered again, to 20 cm below the animal, some of the liquid returned. Raising and lowering pressure in this way, a gentle lung lavage was carried out. It was repeated six times with the same liquid, whereby the liquid returning to the container and that left in the lungs' airways could be assumed to have a very similar composition. With the procedure described, the surfactant components became more concentrated than they would have been with a conventional BAL. When the container had been lowered the sixth time, the collected lavage fluid, usually around 10 mL (Table 1), was measured and it was centrifuged at 80 g for 6 min. The pellet was discarded and the supernatant was used for biophysical and biochemical studies. The lavaged lungs were removed from the thorax and dried at 70 °C for at least 72 h, following which they were weighed on a Mettler balance #H20.

Biophysical analysis of the BAL fluid

One millilitre of the supernatant, obtained after the

lavage fluid's mild centrifugation, was transferred to a glass test tube (ID 12 mm, length 75 mm). The tube was closed with a rubber stopper pierced with two hypodermic needles. One was a 22 G needle which, when extended with a small bore plastic tubing, gave communication from ambient air to a point close to the surface of the liquid which was to be concentrated. The other needle piercing the rubber stopper was wider, 18 G, and was connected to a vacuum pump. A low pressure could thus be produced in the test tube which was not significantly altered by the relatively low flow of room air entering through the narrow tubing. The evaporation that took place caused a lowering of the liquid's temperature to around 10 °C, and within 2 h there was only a small remnant of fluid. The test tube, which had been weighed empty, was weighed again with the residual of the sample. This made it possible to calculate how much distilled water should be added to bring the weight up to 100 µg. In this way the BAL was concentrated ten times without the relationship between phospholipids and proteins being affected.

A pulsating bubble surfactometer (PBS, Electronetics, Amherst, NY, USA) was used for studying the lavage liquid's dynamic surface properties [6]. A bubble, which is perceived as a large model of an alveolus, is expanded

in 20 μL of the sample liquid, and through a vertical capillary it communicates with ambient air. The bubble is pulsating, usually at a rate of 20 cycles per minute, whereby its radius alternates from a maximum of 0.55 mm to a minimum of 0.4 mm. This means that the area of the surfactant film, formed at the air-liquid interphase of the bubble, will be compressed to 50% of its maximal size. Since the pressure difference across the bubble surface, ΔP , is continuously recorded and the bubble radius, r , changes according to a fixed pattern, surface tension, γ , can be calculated according to the law of Laplace, $\Delta P = 2\gamma/r$. A microprocessor carries out that calculation and gives an analog tracing of γ . Digital printouts are also obtained, one at minimal bubble size when the surface film is maximally compressed. For this study the value at minimal bubble size after 15 min of pulsation was assessed. It should be 5 mN/m or less to indicate that the surfactant was functioning well.

Biochemical analysis of the BAL fluid

Of the supernatant obtained with the mild centrifugation, 1 mL was used for an assessment of surface tension. The rest, 5–10 mL, was used for analysis of phospholipids and proteins. The lipids were extracted according to the method of Bligh and Dyer [7]. For an assessment of the lipid phosphorus content the micromethod of Chen *et al.* was used [8], and the composition of the phospholipids was determined with thin-layer chromatography (TLC) using the solvent system of Touchstone *et al.* [9]. Protein content was determined by the method of Lowry *et al.* [10] with a sodium dodecyl sulfate (SDS) modification to permit analysis in the presence of lipids. The BAL, which was carried out six times with the same liquid, was assumed to give concentrations of phospholipids and proteins, which were the same in the liquid returning as in the liquid remaining in the airways. Those two fractions would add up to 14 mL provided there was

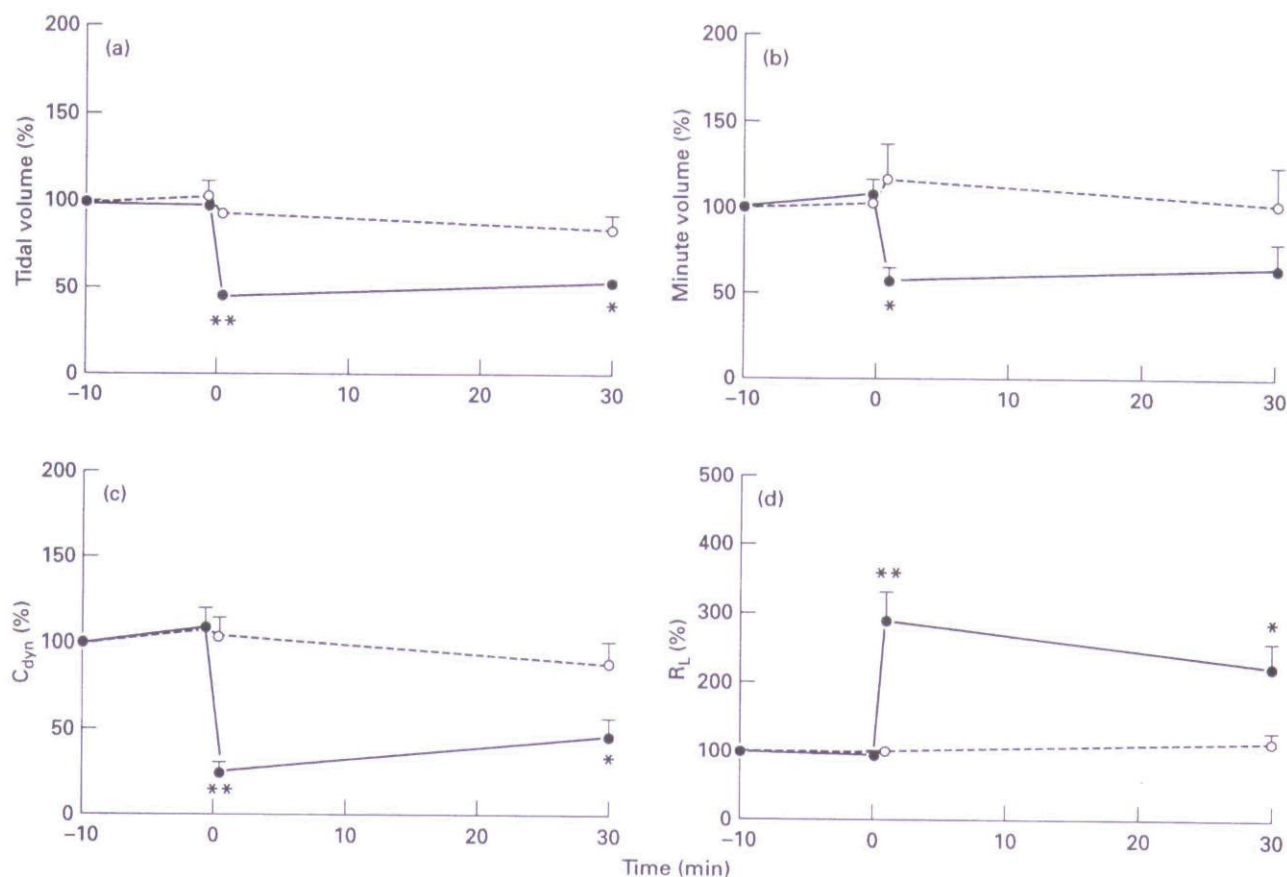


Fig. 1. When animals, immunized against ovalbumin (I Ch LF [●—●], $n = 7$) were challenged tidal volume, minute volume, and dynamic compliance (C_{dyn}) decreased as airway resistance (R_L) increased. The non-immunized animals studied with lung function tests (NI Ch LF [○—○], $n = 7$) did not react when exposed to the same challenge. Values in this and following figures are means $\pm$ SEM. * $P < 0.05$ ** $P < 0.01$.

no liquid in the airways before the BAL and no liquid had left the airways. The concentrations could thus be calculated and reported as mg/kg bodyweight.

Statistics

The two-tailed Student *t*-test for unpaired samples was used for analysis of differences between the means of two variables. One-way analysis of variance (ANOVA) without replications was used as a test of significant differences among the means of more than two groups of data. Linear regression analysis was carried out to couple the relationship between the samples' surface tension at minimal bubble size, and their protein/phospholipid ratio. A *P*-value of <0.05 was considered significant.

Results

When the immunized guinea-pigs were exposed to an aerosol spray of ovalbumin, which was the antigen they had received *i.m.* 5 and 4 weeks earlier, they all reacted with respiratory symptoms similar to those previously described [11–13]. Each animal developed coughing and respiratory distress within 1 min, albeit with varying severeness. Within 2–5 min after the challenge, convulsions were frequently seen and in six of the 19 immunized animals, death occurred in what appeared to be anaphylactic shock. Four animals died in the I Ch LF group and two in the I Ch BAL group. Immediately following the aerosol challenge, artificial ventilation had to be given intermittently to all animals in group I Ch LF. However, once the acute crisis was over, spontaneous regular breathing returned within 15 min. Not one of the non-immunized animals showed any sign of respiratory distress when they inhaled aerosolized ovalbumin.

Pulmonary function tests

In each of the groups I Ch LF and NI Ch LF seven animals could be examined with pulmonary function tests. The baseline values, *i.e.* those obtained 10 min before the challenge, were not different in the two groups. As seen in Fig. 1 values for tidal volume, minute volume, dynamic compliance (C_{dyn}), and total airway resistance of the lung (R_L) were about the same in the two groups before the challenge. However, following the challenge those values changed dramatically among the immunized animals, I Ch LF; tidal volume, minute volume, and dynamic compliance were significantly reduced as airway resistance increased already within 2 min. However, 30 min after the challenge the values had improved. The pulmonary function tests were pursued for only 30 min, since that period covered the

acute stage when changes in the surfactant system, if they occurred, were likely to be most pronounced. On the other hand, among the animals previously not immunized, NI Ch LF, lung function was not affected by the challenge.

Bodyweight and dry lung weight

The animals used for biophysical and biochemical studies of the BAL fluid were those not undergoing lung function tests, *i.e.* controls and groups I Ch BAL and NI Ch BAL. As seen from Table 1, bodyweight for the three groups was about the same, but dry lung weight was clearly higher in the group of immunized animals, I Ch BAL, and the ratio of dry lung weight to bodyweight in that group was 29% higher than for controls and NI Ch BAL ($P < 0.01$). This indicated that in group I Ch BAL, blood components had been retained by an infiltration of the lungs' extravascular tissue or by an expansion of blood vessels.

Surface tension measurements

As seen in Fig. 2 the concentrated BAL fluid from the group of immunized and challenged animals, I Ch BAL, had a surface tension at minimal bubble size which was higher shortly after starting the pulsation than it was in the groups NI Ch BAL ($P < 0.05$) and controls

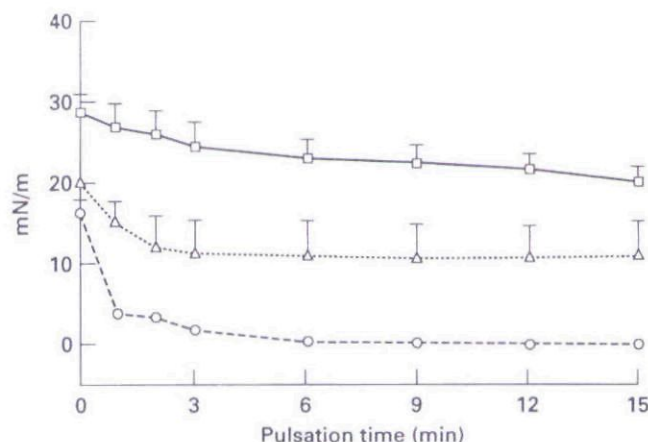


Fig. 2. Surface tension of the concentrated lavage fluid from groups I Ch BAL ($\square$), NI Ch BAL ($\triangle$), and Controls ($\circ$). The values, in mN/m (mean $\pm$ SEM), were those existing when the bubble was of minimal size. They declined with time as a film was formed at the bubble's surface, and were significantly higher in the group of immunized and challenged animals, I Ch BAL. Each of the values shown in this group was significantly higher than the corresponding value in groups NI Ch BAL ($P < 0.05$) and controls ($P < 0.01$). For the controls the standard errors are not large enough to be demonstrated with error bars.

($P < 0.01$), and it remained higher during the 15 min of pulsation. Also in the group of animals, not immunized and exposed to challenge only, NI Ch BAL, surface tension at minimal bubble size was seemingly higher than it was among the controls, but the difference was not statistically significant. After 15 min of pulsation, surface tension at minimal bubble size was 20.3 ± 1.8 mN/m (mean $\pm$ SEM) in group I Ch BAL, 11.2 ± 4.1 mN/m in group NI Ch BAL, and 0 mN/m in the group of controls (Fig. 2).

Phospholipids and proteins in the BAL fluid

Because the lungs of animals in group I Ch BAL had increased so much in weight, the phospholipids and proteins were related to bodyweight rather than dry lung weight. Whereas total phospholipids were approximately the same in all groups, proteins of the lavage fluid were 65% greater in group NI Ch BAL than in controls, a difference that was statistically significant ($P < 0.01$). However, among the immunized, I Ch BAL, proteins on average had increased as much as 350% ($P < 0.05$, Table 1). Since, compared with the controls, the phospholipid concentration was unaffected in groups NI Ch BAL and I Ch BAL, the ratio of proteins to phospholipids was raised 60% ($P < 0.01$) and 370% ($P < 0.05$) in the two groups, respectively (Table 1). Also the group I Ch BAL was significantly different from NI Ch BAL as regards protein concentration and protein to phospholipid ratio ($P < 0.05$). The TLC studies demonstrated that the composition of phospholipids in BAL fluid was about the same in the three groups: the controls and the two groups of challenged animals, those who had been and those who had not been immunized. In all three groups the content of phosphatidylcholine was about 70% and that of lysophosphatidylcholine was very minimal, indicating that PLA2 hydrolysis had been limited.

Surface activity affected by proteins

In Fig. 3 surface tension at minimal bubble size after 15 min of pulsation has been plotted against the ratio of proteins to phospholipids. There was a highly significant correlation between these values ($r = 0.822$, $P < 0.0001$).

Discussion

The immunized guinea-pig has often been used as a model of human asthma since the animal develops similar symptoms in response to asthma inducing agents and, furthermore, the chemical mediators are alike [5,11–13]. For these reasons we felt the guinea-pig would be an appropriate animal model although its

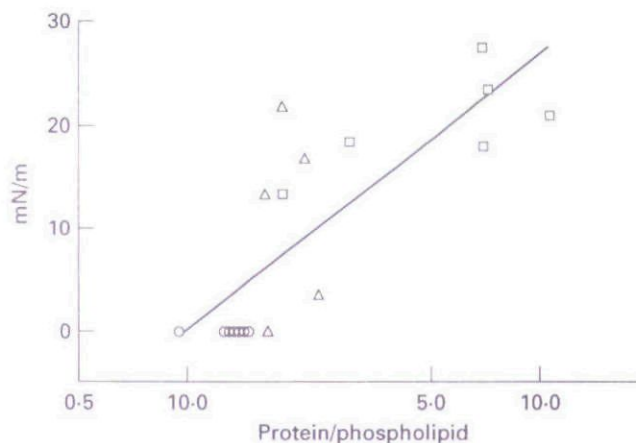


Fig 3. Surface tension of concentrated BAL fluid at minimal bubble size following 15 min of pulsation, plotted against the ratio of proteins to phospholipids. □, I Ch BAL. △, Ni Ch BAL. ○, Controls.

antigen response is often very severe. In this study we evaluated pulmonary function before and after challenge with aerosolized ovalbumin to make sure that in our hands the system worked well and affected airway resistance the way others have reported. The rapid increase in R_L and the decrease in tidal volume, minute volume, and C_{dyn} that we observed after challenge among the immunized animals, were responses similar to those previously reported [5,11–13].

The prompt antigen response suggests smooth muscle spasm, but it is also conceivable that an increased resistance to airflow might have developed because many of the most peripheral airways lost their ability to retain patency and instead became blocked by columns of liquid. *In vitro* experiments [2,3] have demonstrated that if a surfactant suspension of high quality is instilled into the narrow section of a glass capillary, the suspension will not return to the narrow section once it has been extruded from there, i.e. the capillary remains patent. If, on the other hand, the surfactant is of poor quality, the glass tube will not retain patency; instead the liquid will return to the narrow section of the tube as soon as it has been pushed out from there. A recent study offered strong indication [14] that the airway of a lung will also become blocked by liquid columns, when the surfactant lining the airway is of poor quality. Studies on airways of rats demonstrated that there was a better chance for a free flow of air through narrow conducting airways if the lining surfactant was of high quality.

In view of the studies just described it appeared essential to clarify how the surfactant quality was affected when a condition had been induced which like asthma caused an increased airway resistance. For this

reason we collected the BAL fluid with a standardized technique which yielded a high concentration of pulmonary surfactant. However, for a study with the pulsating bubble surfactometer the concentration had to be even higher. This was our motivation for arranging a fast evaporation of water, which concentrated the surfactant ten times. Obviously, the water evaporation would also raise the salt concentration, which only had a marginal effect on the surface tension readings. Other ways to increase the concentration, such as centrifugation, were contemplated, but were considered inappropriate since the ratio between surfactant and inhibiting agents would then have been altered.

The surfactant present in the BAL fluid we studied originated from alveolar cells type II. Once released from those cells it would eventually form a film at the alveolar air-liquid interfaces, thereby offering stability to alveoli. Most likely that film, when compressed to a diminished area during expiration, would partly be extruded into adjacent conducting airways and a surfactant film with that location, an extension of the alveolar film, has been demonstrated with electron microscopy [15,16].

An unexpected observation was that the NI Ch BAL group of animals, those not immunized but challenged, had an increased concentration of proteins in their BAL fluid. The amount of protein inhaled was probably too small to explain this observation. More likely, the inhaled proteins caused an irritation, and as part of the inflammatory reaction that followed, plasma proteins leaked into the airways.

Asthma is now recognized as being an inflammatory disorder, with oedema and airway leakage of plasma proteins [1,17–21]. A recent clinical study [22] offered evidence that it is mainly during the acute asthma attack that the bronchial mucosa has an increased permeability and will allow proteins to leak into the airway. Our study, during the acute phase of an asthma simulating condition, has confirmed that the airways become invaded by proteins, and they probably are the cause of a surfactant inhibition. However, our study says nothing about chronic asthma.

Several plasma proteins may inhibit pulmonary surfactant [23–27] and it is particularly the adsorption rate that is affected by albumin, gammaglobulin and, specifically, fibrinogen. Increasing the surfactant concentration in relation to the inhibiting proteins will improve the surface properties [26,27] and this might explain the results of a Japanese clinical trial which showed that the lung function improved when asthmatic subjects inhaled aerosolized surfactant [28]. In an experimental study [29] albumin sensitized guinea-pigs were challenged after they had received a tracheal instillation of surfactant. The prophylactic treatment was successful in that it pre-

vented the challenge from decreasing tidal volume the way it otherwise did. The surfactant was administered, not to affect the surface properties but as an agent that would prevent the antigen from contacting cell-surface receptors.

A delayed adsorption, caused by serum proteins invading the airways, might be of particular importance for the function of conducting airways. If, because of an inflammatory reaction, they are more narrow and are lined by surfactant films that are of marginal efficiency, they might become blocked by liquid columns during each expiration when the airway diminishes in width, but they may open up again during the following inspiration. An extremely fast adsorption is probably required to prevent the columns from returning when the airway lumen diminishes during the next expiration. The adsorption promoting apoproteins SP-A and SP-B have been detected in bronchiolar epithelial cells that are non-ciliated [30], and that may be a strategically important site of origin.

It can be concluded from this study that when the immunized guinea-pig is challenged, severe surfactant dysfunction develops, probably because plasma proteins have been leaking into the airways. Pulmonary surfactant is important for alveolar stability and function, but it probably also has the important function of maintaining patency of narrow conducting airways. If a surfactant dysfunction were to develop, many of those peripheral airways might become blocked by liquid columns which would increase airway resistance.

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