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Lung protein leakage in respiratory failure induced by a hybridoma making monoclonal antibody to the hydrophobic surfactant-associated polypeptide SP-B

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Summary. Adult mice were inoculated intraperitoneally with a hybridoma (8B5E) making monoclonal antibody to the porcine surfactant-associated polypeptide SP-B; this antibody cross-reacts with the corresponding polypeptide in the mouse surfactant system. Respiratory failure, developing 7–9 days after inoculation, was associated with a decrease in lung–thorax compliance determined during artificial ventilation, and an increase in the amount of protein including the specific antibody in lung lavage fluid. There was a statistically significant negative correlation between the compliance and the amount of protein as well as antibody recovered by lung lavage: r (log scale) $= -0.69$ and -0.82, respectively ($P < 0.01$, both), but no decrease in the amount of phospholipids in lung lavage from animals inoculated with the hybridoma. Treatment with a large dose of porcine surfactant (about 320 mg phospholipids/kg body weight) had no positive effect on lung–thorax compliance during artificial ventilation; on the contrary, surfactant-treated animals showed a decrease in compliance similar to that seen in control animals after instillation of a similar volume of saline into the airways. We conclude that respiratory failure developing after inoculation with this hybridoma is probably at least in part mediated by flooding of the airspaces with antibody interfering with surfactant function.

Keywords: hybridomas, monoclonal antibodies, respiratory insufficiency, pulmonary surfactants, SP-B

Two hydrophobic polypeptides with molecular weights of 8.7 (Curstedt *et al.* 1988) and 4.2 kDa (Curstedt *et al.* 1990) are probably essential components of the pulmonary surfactant system. These polypeptides, currently known as SP-B and SP-C, respectively, accelerate the surface adsorption of the surfactant phospholipids (Curstedt *et al.* 1987; Notter *et al.* 1987; Revak *et al.* 1988; Suzuki *et al.* 1986a; Takahaski & Fujiwara 1986; Tanaka *et al.* 1986; Whitsett *et al.* 1986; Yu & Possmayer 1986) and may

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enhance uptake and recycling of these lipids by the alveolar epithelium (Claypool *et al.* 1984). We have shown previously that respiratory failure can be induced in adult mice by a hybridoma (8B5E) making an antibody to a 15 kDa surfactant apoprotein, now identified as SP-B (see below). Within 9 days of intraperitoneal inoculation with this hybridoma, the animals developed severe lung disease characterized by reduced compliance, congestion, alveolar collapse, haemorrhagic oedema, and hyaline membranes (Suzuki *et al.* 1988). A similar spectrum of lung parenchymal damage was induced in mice by repeated intraperitoneal injection of isolated antibody produced by the same hybridoma (Fujita *et al.* 1988). The exudative, haemorrhagic lung lesions induced in this experimental model are of the same type as those seen in human adult respiratory distress syndrome (ARDS).

The present study was designed to clarify whether respiratory failure, developing after inoculation with this hybridoma cell line, is associated with vascular-to-alveolar leakage of protein including the monoclonal antibody and/or a reduction of the intra-alveolar pool of surfactant phospholipids (due to reduced synthesis or increased removal across the damaged alveolar epithelium). We also wanted to find out whether this particular form of experimental ARDS is amenable to surfactant replacement therapy.

Methods

Hybridoma cell line

Hybridoma cells were obtained by fusing spleen cells from BALB/c mice immunized against porcine surfactant-associated protein fractions (Suzuki *et al.* 1982), with a myeloma cell line (Suzuki *et al.* 1986b). One hybridoma cell line (8B5E) produced an antibody to porcine 15kDa surfactant apoprotein. The antibody cross-reacted with the corresponding protein fraction of mouse surfactant (Fujita *et al.* 1988). The specificity of this antibody was assessed by immunoblotting of porcine hydrophobic surfactant-associated protein fractions, separated by chromatography on Sephadex LH-60 (see below).

Animal experiments

Ninety young adult mice were inoculated intraperitoneally with the hybridoma 8B5E (10^7 cells per animal) and were examined after 7 days ($n=24$), 8 days ($n=23$), or 9 days ($n=23$). Twenty mice served as non-inoculated controls. The animals were tracheotomized, relaxed with intraperitoneal pancuronium bromide, placed in a system of body plethysmographs and subjected in parallel (up to ten animals in each experiment) to pressure-constant ventilation with 100% oxygen, a frequency of 40/min, and 33% inspiration time. We applied a standardized sequence of peak insufflation pressures, viz 10, 20, 30, and 10 cmH_2O, without positive end-expiratory pressure (Suzuki *et al.* 1988). Tidal volumes were measured with a pneumotachograph connected to the plethysmograph box; recordings were obtained after about 5 min of ventilation at each pressure level. Lung–thorax compliance was calculated by dividing tidal volume by peak insufflation pressure.

After the second recording at insufflation pressure 10 cmH_2O, approximately one-third of the experimental and control animals were randomized to receive 0.1 ml of porcine surfactant (80 mg phospholipids/ml, corresponding to about 320 mg phospholipids/kg body weight) via the tracheal cannula; this surfactant had been prepared from minced lungs by a combination of chloroform : methanol extraction and liquid-gel chromatography (Robertson *et al.* 1990). The dose of surfactant administered in the present experiments is similar to that suggested by Lachmann (1989) for treatment of viral pneumonia in mice. Surfactant-treated mice were reconnected to the ventilator and all animals, including non-treated controls, were again ventilated in parallel with two pressure levels, first 30 cmH_2O for 5 min, and

then 10 cmH_2O for 5 min. Tidal volumes were recorded at the end of each of these intervals (in pilot experiments there was no further change in tidal volumes when measurements at both pressure levels were repeated after 60 min).

The animals were then killed and their lungs lavaged via the tracheal cannula with 1 ml normal saline. This volume was flushed in and out of the lungs twice, and the procedure was repeated with fresh saline four times. The lavage fluid from each animal was pooled. The protein content of the lavage fluid was determined (Lowry *et al.* 1951), and the amount of specific antibody assessed by immunodiffusion (see below). The phospholipids in the wash were extracted with chloroform: methanol (Folch *et al.* 1951) and quantified according to Bartlett (1959).

Immunodiffusion studies for quantification of antibody in lung lavage

Pig pulmonary surfactant isolated by sucrose density gradient ultracentrifugation was delipidated three times with ethanol:ether (3:2) and once with ether at −10°C. The dried residue was solubilized in 10 mM phosphate buffer (pH 7.2) containing 9 mg/ml of NaCl and 1% Tween 20, and mixed with hot agarose solution. This was then layered on plastic plates; the final concentrations of agarose, protein, and Tween 20 were 1, 0.02, and 0.1%, respectively. Standard samples of antibody and lung lavage fluid were filled into wells made in the substrate and were left for 48 h at room temperature. After drying, proteins were stained with Coomassie Brilliant Blue and the concentration of antibody in the lavage fluid was determined by comparing the diameter of the stained rings for the samples and the standards. Lavage fluid samples showing no reaction were re-examined at a tenfold higher concentration.

Histological examination

After the lavage procedure, the lungs were fixed in 4% neutral formalin. Paraffin sections from both lungs, stained with haematoxylin and eosin, were examined by conventional light microscopy with particular reference to evidence of exudative inflammatory parenchymal lesions.

Statistical analysis

All values are given as mean and s.e.m. Differences between the groups were evaluated with the Wilcoxon two-sample test (two-tailed). For linear regression analysis, we used the method of least squares.

Results

Characterization of antibody made by hybridoma 8B5E

The hydrophobic proteins in porcine surfactant were separated by chromatography on Sephadex LH-60 (Curstedt *et al.* 1987). All fractions containing SP-B were stained with antibody. The first fraction of the SP-C peak was also stained, probably reflecting tailing of SP-B. Subsequent fractions, containing only SP-C, did not react with the antibody (Fig. 1). From these results, we conclude that the antibody made by the hybridoma is specific for SP-B.

Compliance measurements; effect of surfactant replacement

Lung–thorax compliance was clearly reduced in animals inoculated with the hybridoma (Fig. 2). This difference was most obvious for animals studied 9 days after inoculation and especially in the recordings obtained after the second period of ventilation with insufflation pressure 10 cmH_2O. Treatment with surfactant resulted in a reduction of lung–thorax compliance in all groups of hybridoma-inoculated animals similar to the effect seen after instillation of surfactant or saline into the airways of control animals (Table 1).

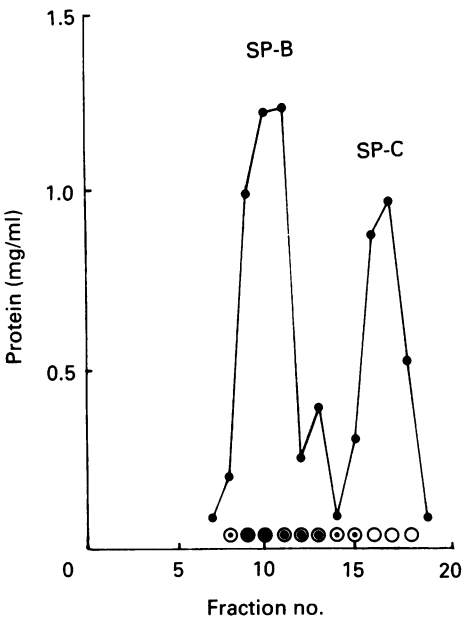

Fig. 1. Spot blot staining reaction of the hydrophobic proteins SP-B (8.7 kDa) and SP-C (4.2 kDa), isolated from porcine surfactant by chromatography on Sephadex LH-60 (Curstedt *et al.* 1987), to antibody made by hybridoma 8B5E. The symbols below indicate the intensity of the reaction: ●, strong; ◉, moderate; ⊙, weak; ○, negative.

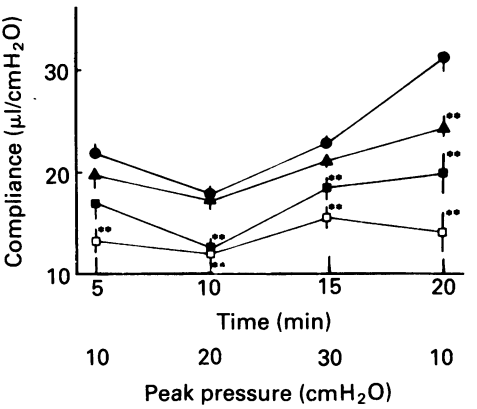

Fig. 2. Lung–thorax compliance (mean, s.e.m) in animals inoculated with hybridoma 8B5E (making antibody to porcine SP-B, cross-reacting with mouse SP-B) and in controls. The recordings were obtained after different periods of inoculation during artificial ventilation with a standardized sequence of insufflation pressures. $^{**}P$ *vs* controls < 0.01. ●, Controls; ▲, 8B5E 7 days; ■, 8B5E 8 days; □, 8B5E 9 days.

Phospholipids, proteins, and antibody levels in lung lavage fluid; correlations with compliance measurements

The phospholipid content in lung lavage fluid was increased by 5–11 times (average 8 times) in animals receiving surfactant ($P < 0.005$) (Table 2). However, on the average only about 26% of the instilled surfactant lipids could be recovered by the lavage procedure. The recovery of instilled surfactant was lower in each of the three groups of hybridoma-inoculated animals than in the control group ($P < 0.01$). In general, average values for total phospholipids were higher in hybridoma-inoculated mice than in controls, but this difference was statistically significant only for animals examined on day 7 ($P < 0.01$).

Substantial amounts of proteins were detected in lung lavage fluid from all groups of animals including the controls. The protein levels from animals inoculated with the hybridoma increased from day 7 to day 9 after inoculation ($P = 0.05$). The same holds for the levels of specific antibody, showing a more than fivefold increase from day 7 to day 9 ($P < 0.01$) (Table 3).

Correlations between compliance values obtained after the second period of ventilation with insufflation pressure 10 cmH_2O and the contents of proteins, specific antibody, and phospholipids in lung lavage fluid are shown in Table 4. There was a statistically significant negative correlation between compliance and protein content, and especially between compliance and antibody levels in the lavage fluid. Both these correlations were amplified when log-scale measurements were subjected to analysis. Values for protein content and antibody levels showed a close correlation ($r = 0.88$; $P < 0.01$). There was no statistically significant correlation between phospholipid content and compliance.

Table 1. Lung–thorax compliance in animals inoculated with hybridoma 8B5E and in controls, before and after tracheal instillation of surfactant or saline. Values obtained at insufflation pressure 10 cmH_2O are given as mean (s.e.m.)

Inoculation	Time after inoculation (days)	Treatment	*n*	Compliance (μl/cmH_2O) Before treatment	After treatment	*P*
Hybridoma 8B5E*	7	surfactant	7	30.7 (1.6)	13.9 (2.4)	<0.01
Hybridoma 8B5E*	8	surfactant	7	20.9 (3.8)	6.9 (1.2)	<0.05
Hybridoma 8B5E*	9	surfactant	7	12.1 (3.7)	4.9 (1.2)	n.s.
Controls	—	surfactant	7	34.9 (2.5)	11.4 (1.4)	<0.01
Controls	—	saline	4	37.5 (0.9)	14.3 (1.3)	=0.05
Controls	—	—	3	30.7 (3.3)	30.3 (3.2)	n.s.

* Making antibody to porcine SP-B, cross-reacting with mouse SP-B.

Histological findings

Lungs from animals inoculated with hybridoma 8B5E showed the same type of interstitial inflammatory reaction as observed in previous experiments (Fujita *et al.* 1988). The severity of these changes increased with time after inoculation and in some animals killed on day 9 the interstitial inflammation was associated with exudative lesions including hyaline membranes in terminal airspaces. The lungs of non-inoculated animals were unremarkable. Since all lungs were washed via the airways prior to fixation and the lavage procedure might have modified the histological appearance of the airspaces, we made no further efforts to characterize or quantify the histological findings,

Table 2. Total phospholipids in lung lavage fluid from hybridoma-inoculated animals and controls; values are given as mean (s.e.m.)

Inoculation	Time after inoculation (days)	Total phospholipids (mg) Surfactant-treated	Non-treated
Hybridoma 8B5E†	7	1.69 (0.13)† (*n*=7)	0.33 (0.02)* (*n*=17)
Hybridoma 8B5E†	8	1.95 (0.12)† (*n*=7)	0.28 (0.03) (*n*=16)
Hybridoma 8B5E†	9	2.05 (0.09)† (*n*=7)	0.31 (0.04) (*n*=16)
Controls	—	2.78 (0.10) (*n*=7)	0.25 (0.01) (*n*=13)

* *P vs* controls <0.01.

† Making antibody to porcine SP-B, cross-reacting with mouse SP-B.

Table 3. Total protein content and the amount of specific antibody to SP-B in lung lavage fluid; values are given as mean (s.e.m.)

Inoculation	Time after inoculation (days)	*n*	Protein content (mg)	Antibody content (mg)
Hybridoma 8B5E†	7	24	1.09 (0.10)	0.09 (0.02)
Hybridoma 8B5E†	8	23	2.94 (0.98)	0.27 (0.08)*
Hybridoma 8B5E†	9	23	4.12 (1.43)*	0.51 (0.14)**
Controls	—	20	1.58 (0.34)	0 (0)

* *P vs* day 7 $=0.05$; ** *P vs* day 7 <0.01 (further statistical analysis is given in the text).

† Making antibody to porcine SP-B, cross-reacting with mouse SP-B.

nor did we evaluate the effect of surfactant replacement on alveolar expansion.

Discussion

The present data confirm that inoculation with a hybridoma making antibody to the 15 kDa surfactant apoprotein (Suzuki et al. 1986b), now identified as SP-B, leads to respiratory failure with a striking fall in lung–thorax compliance. In accordance with our earlier observations, differences between inoculated animals and controls were most prominent after the second period of ventilation with insufflation pressure 10 cmH_2O. This is because, in all groups, new alveoli are probably recruited during ventilation with pressure 30 cmH_2O; when the insufflation pressure is again set at 10 cm H_2O, these alveoli remain stable in control animals but collapse in the mice inoculated with the hybridoma, especially in those studied on day 9.

Our data furthermore indicate that the respiratory failure is not caused by a general 'wash-out' of phospholipids from the lungs. Although recovery of intra-alveolar surfactant phospholipids by lung lavage is notoriously incomplete (Kobayashi *et al.* 1989), there seemed to be no decrease in total lung

Table 4 Correlations between parameters of protein leakage, phospholipid content in lavage fluid, and final lung–thorax compliance recorded at insufflation pressure 10 cm H_2O; the values were obtained from animals inoculated with hybridoma 8B5E (making antibody to porcine SP-B, cross-reacting with mouse SP-B)

Parameters	*r*	*r* (log-scale)	*P*
Total proteins in lavage fluid *vs* compliance	−0.46	−0.69	<0.01
Specific antibody content of lavage fluid *vs* compliance	−0.66	−0.82	<0.01
Total phospholipids in lavage fluid *vs* compliance*	−0.28	0.01	n.s.
Total proteins *vs* specific antibody content	0.88	—	<0.01

* Only animals *not* treated with surfactant.

lavage phospholipids during the development of respiratory failure from day 7 to 9 after inoculation with the hybridoma; on the contrary, the average values for total phospholipids in animals not receiving surfactant were somewhat higher in the hybridoma-inoculated mice than in controls. As mentioned above, surfactant-associated hydrophobic proteins may augment uptake of surfactant lipids for recycling by alveolar type II cells (Claypool *et al.* 1984). If so, the antibody to SP-B could interfere with this process, thereby contributing to accumulation of phospholipids in the air spaces.

There was no correlation between phospholipid content and compliance values. Although this does not rule out the possibility that changes in the composition of the intra-alveolar phospholipids may have contributed to the observed deterioration of lung function (this aspect was not further examined in the present study protocol), we believe the reduced compliance largely results from increased permeability of the alveolar walls leading to flooding of the airspaces with proteinaceous oedema (Suzuki *et al.* 1988). The damage to the alveolar epithelium, documented by electron microscopy (Fujita *et al.* 1988), is probably in part mediated by immune complexes causing an activation of complement with accumulation of C_3 in the airspaces (Fujita *et al.* 1988), recruitment of neutrophils and macrophages, and release of lysosomal enzymes from inflammatory cells. Endothelial cell damage is not a prominent ultrastructural finding in this experimental model (Y. Fujita, K. Kogishi and Y. Suzuki, unpublished observations).

The protein content of lung lavage fluid increased with advancing disease from day 7 after inoculation with hybridoma 8B5E, showing a nearly fourfold higher level on day 9. In the same time span, the average content of specific antibody in the lavage fluid showed a more than fivefold increase. Since a substantial amount of protein (without specific antibody) was also recovered in lung wash from control animals, it seems that the experimental procedure (tracheotomy, artificial ventilation, lung lavage) in itself induced a 'baseline' leakage of serum components into the airspaces.

Several serum proteins are potent inhibitors of surfactant function (Fuchimukai *et al.* 1987; Ikegami *et al.* 1984, 1986). However, in the present model the leaking proteins also include an antibody that may cause in-situ inactivation of an essential component of the surfactant system, SP-B. Previous electron microscopic observations (Fujita *et al.* 1988) are in keeping with this interpretation: in animals inoculated with hybridoma 8B5E, there was not only necrosis and desquamation of alveolar epithelium but also accumulation of 'unexpanded' lamellar bodies in the intra-alveolar oedema fluid, suggesting interference with the normal transformation of lamellar bodies into tubular myelin and a surface film. The strong negative correlation between the antibody levels in lung lavage fluid and lung–thorax compliance lends further support to the concept of direct inactivation of surfactant as a mechanism involved in the development of respiratory failure.

Treatment with porcine surfactant seems to have no immediate beneficial effect in this experimental model, not even during the early stage of respiratory failure (7–8 days after inoculation). To some extent this can be explained simply with inactivation of the exogenous material. However, the decrease in compliance following surfactant administration was similar to that seen after instillation of a similar volume of surfactant or saline into the airways of control animals, suggesting that the mice were unable to handle the fluid volume load under the present conditions. Our data from control animals confirm earlier observations on artificially ventilated adult guinea-pigs, showing significant reduction in lung–thorax compliance after tracheal instillation of a corresponding volume of saline (Lachmann 1985). Further studies are required to find out whether treatment would be more effective with some other type of surfactant, not

recognized by the specific antibody, or with ventilation using high positive end-expiratory pressure, which is known to potentiate the effect of surfactant replacement therapy in respiratory failure induced by repeated lung lavage (Kobayashi *et al.* 1984).

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