Lung surfactant in a cystic fibrosis animal model: increased alveolar phospholipid pool size without altered composition and surface tension function in *cftr*$^{m1HGU/m1HGU}$ mice

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Abstract

***Background* – Progressive pulmonary dysfunction is a characteristic symptom of cystic fibrosis (CF) and is associated with functional impairment and biochemical alterations of surfactant phospholipids in the airways. However, the fundamental question of whether surfactant alterations in the CF lung are secondary to the pulmonary damage or are present before initiation of chronic infection and inflammation has yet to be resolved in patients with cystic fibrosis but can now be addressed in CF mice that exhibit the basic defect in the airways. A study was therefore undertaken to investigate the pool sizes, composition, and function of lung surfactant in the non-infected *cftr*$^{m1HGU/m1HGU}$ mouse.**

***Methods* – The amount and composition of phospholipid classes and phosphatidylcholine molecular species were determined in bronchoalveolar lavage (BAL) fluid and lavaged lungs by high performance liquid chromatography (HPLC). Surfactant protein A (SP-A) levels in BAL fluid were determined by ELISA and surfactant for functional measurements was isolated from BAL fluid by differential ultracentrifugation. Equilibrium and minimal surface tension of surfactant was assessed by the pulsating bubble surfactometer technique. MF1, BALB/c, C57/BL6, and C3H/He mice served as controls.**

***Results* – BAL fluid of *cftr*$^{m1HGU/m1HGU}$ mice contained 1.02 (95% confidence interval (CI) 0.89 to 1.16) µmol phospholipid and 259 (239 to 279) ng SP-A. BAL fluid of MF1, BALB/c, C57BL/6, and C3H/He mice contained 0.69 (0.63 to 0.75), 0.50 (0.42 to 0.57), 0.52 (0.40 to 0.64), and 0.45 (0.27 to 0.63) µmol phospholipid, respectively. After correction for the different body weights of mouse strains, phospholipid levels in BAL fluid of *cftr*$^{m1HGU/m1HGU}$ mice were increased by 64 (52 to 76)%, 60 (39 to 89)%, 72 (45 to 113)%, and 92 (49 to 163)%, respectively, compared with controls. The amount of SP-A in BAL fluid and the composition of phospholipid as well as phosphatidylcholine molecular species in BAL fluid and lung tissue was unchanged in *cftr*$^{m1HGU/m1HGU}$ mice compared with controls. The increase in phospholipids in BAL fluid of *cftr*$^{m1HGU/m1HGU}$ mice resulted from an increased fraction of large aggregates which exhibited normal surface tension function.**

***Conclusion* – In *cftr*$^{m1HGU/m1HGU}$ mice surfactant homeostasis is perturbed by an increased phospholipid pool in the alveolar compartment.**

(*Thorax* 1997;**52**:723–730)

Keywords: cystic fibrosis, surfactant pool size, *cftr*$^{m1HGU/m1HGU}$ mouse.

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Received 19 December 1996
Returned to authors
7 February 1997
Revised version received
17 April 1997
Accepted for publication
29 April 1997

Cystic fibrosis is an inherited disease with a defect in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. This gene codes for an integrated plasma membrane protein which is responsible for cAMP regulated chloride transport through the plasma membrane.[1 2] Mucus viscosity is increased in bronchial secretions from patients with cystic fibrosis.[3 4] Additionally, airway surfactant from patients with cystic fibrosis chronically infected with *Pseudomonas aeruginosa* shows impaired function and altered phospholipid composition, possibly due to enzymatic destruction and contamination of surfactant secondary to infection.[5] However, a primary alteration of surfactant metabolism in cystic fibrosis may additionally exist since the *CFTR* gene is expressed in Clara cells and type II pneumocytes.[6 7] Type II pneumocytes synthesise and secrete all the phospholipid and apoprotein components of surfactant, while Clara cells release surfactant apoproteins SP-A, SP-B, and SP-D into the airways.[8 9] Surfactant phospholipid comprises about 80% phosphatidylcholine (PC), with dipalmitoyl phosphatidylcholine (PC16:0/16:0) being the most important molecular species, and about 10% phosphatidylglycerol (PG). Phospholipid homeostasis may be generally altered in cystic fibrosis since the rate of choline incorporation into PC is increased in isolated blood platelets and fibroblasts from patients with cystic fibrosis.[10] It is possible that defective *CFTR* gene expression may alter surfactant phospholipid homeostasis before colonisation of the lungs with opportunistic bacteria. Since the concentration of surfactant phospholipids and the ratio between individual components, particularly between SP-A and phospholipids, can influence the anti-

body-independent defence functions of surfactant,[11] altered phospholipid homeostasis may consequently affect pulmonary integrity. To investigate any such primary alterations in surfactant homoeostasis we analysed the concentration of surfactant phospholipids and SP-A in lungs of *cftr*$^{m1HGU/m1HGU}$ mice.[12 13] We further investigated in this mouse model the composition of surfactant phospholipid classes as well as phosphatidylcholine molecular species and, finally, the surface tension function of lung surfactant in comparison with healthy controls. In *cftr*$^{m1HGU/m1HGU}$ mice a *cftr* gene defect was generated by insertional mutagenesis into exon 10 of the *cftr* gene[12] in which aberrant skipping results in the synthesis of 5–10% wild type transcript and therefore relatively mild symptoms of *cftr* deficiency.[13] It was our aim to determine whether there was any primary deviation in pulmonary phospholipids of *cftr*$^{m1HGU/m1HGU}$ mice in the absence of chronic pulmonary infection.[5]

Methods

MATERIALS

Specific pathogen-free *cftr*$^{m1HGU/m1HGU}$ mice supplied from Edinburgh[12] as well as Ztm:MF1, BALB/cZtm, C57/BL6Ztm, and C3H/HeZtm mice were bred at our local animal house. *cftr*$^{m1HGU/m1HGU}$ mice originated from five different breeding lines. Mice were kept in a flexible film isolator under specified pathogen-free conditions. The hygienic status was tested monthly according to Kunstyr[14] and the mice were free from murine pathogens. The temperature within the isolator was maintained at 20–24°C with 40–50% relative humidity. Animals were fed an irradiated (5 Mrad) standard diet (Altromin 1314) and autoclaved water (134°C, 50 minutes) was given ad libitum. Mice subjected to analysis were 5–7 weeks old and the numbers of experiments were not based on any pre-study power calculations. High performance liquid chromatography (HPLC) grade solvents were supplied by Baker (Deventer, Holland). All other solvents and chemicals were of analytical grade and from various commercial sources.

HISTOLOGICAL EXAMINATION

For histological examination *cftr*$^{m1HGU/m1HGU}$ mice (n = 6, 6–10 weeks old) and MF1 mice (n = 6) were sacrificed by cervical dislocation and bled by incision of the abdominal aorta. The thorax was opened and, after perfusing the lung with 1 ml cold phosphate buffered saline via the right ventricle, the trachea was cannulated with a small catheter. The lung was then filled with 1 ml OCT embedding medium (Miles Inc, Elkhart, Indiana, USA) diluted 1:4 with 4% buffered formalin and further immersed in 4% buffered formalin for fixation. Paraffin-embedded sections (5 μm) were prepared and stained with haematoxylin and eosin.

HARVESTING OF BRONCHOALVEOLAR LAVAGE FLUID AND LAVAGED LUNG TISSUE

Animals were anaesthetised with an intraperitoneal injection of 100 mg ketamine hydrochloride (Ketanest, WDT Corporation, Garbsen, Germany) and 4 mg xylocaine hydrochloride (Rompun, Bayer AG, Leverkusen, Germany) per kg body weight prior to sacrifice. This standard procedure was used since it does not cause respiratory depression during anaesthesia. The trachea was cannulated with a small catheter, the abdomen opened, and the animal bled by dissecting the abdominal aorta. The thorax was then opened, the lungs ventilated once with 1 ml of ambient air, and the lung perfused with 154 mM saline at room temperature at a pressure of 15 cm H_2O via the right ventricle. The lung was then flushed five times via the tracheal catheter with 1 ml 154 mM saline and the pooled bronchoalveolar lavage (BAL) fluid was centrifuged for 10 minutes at 150*g* and 4°C to remove cells. The total recovery of BAL fluid was 4.5 ml and did not differ between the experimental groups. BAL fluid and lavaged lungs were frozen at −80°C until further analysis.

PHOSPHOLIPID EXTRACTION

For phospholipid quantitation in cell-free BAL fluid two aliquots of 300 μl were placed into phosphate-free glass tubes, the volumes adjusted to 1 ml with 154 mM saline, and this material was extracted with chloroform/methanol according to the method of Bligh and Dyer.[15] The chloroform extract was dried under a stream of nitrogen and the glass tube directly used for phospholipid quantitation. To quantify phospholipids in subfractions of BAL fluid aliquots of P60000 and S60000 were extracted with chloroform/methanol as outlined above. Lavaged lungs were extracted according to Folch *et al*,[16] the extract adjusted to 10 ml with chloroform/methanol 9:1 (v/v) and two aliquots of 100 μl used for phospholipid quantitation. Phospholipid phosphorus was determined by the method of Bartlett[17] after digesting the organic compounds at 190°C for 35 minutes in the presence of 500 μl 70% perchloric acid (w/v) and 200 μl 30% hydrogen peroxide (w/v). The amount of phospholipid in total samples was calculated from phospholipid phosphorus in the measured sample aliquots and the original sample volume (BAL fluid, P60000, S60000) or extract volume (total lung extract).

ANALYSIS OF PHOSPHOLIPID CLASSES

The distribution of total phospholipid classes in lipid extracts was determined by normal phase HPLC as described previously.[18] In brief, aliquots containing 80–100 nmol phospholipid dissolved in chloroform/methanol (1:4 v/v) were resolved isocratically on an aminopropyl silica column (Nucleosil NH_2, 5 mm, Schambeck, Germany) of 235 × 4.6 mm internal diameter. The mobile phase was delivered at a flow rate of 1 ml/min and comprised acetonitrile/methanol/water/methylphosphonic

acid (1460:500:30:0.3 v/v/v/v), adjusted to pH 6.3 by titration with NH_4OH (25% v/v in water). Eluted phospholipids were quantitated by fluorescence (excitation wavelength 340 nm, emission wavelength 440 nm) after post-column formation of mixed micelles in the presence of 1,6-diphenyl-1,3,5-hexatriene (DPH).

ANALYSIS OF PHOSPHATIDYLCHOLINE MOLECULAR SPECIES

The composition of individual molecular species of phosphatidylcholine (PC) was determined as previously described by Postle.[19] A PC fraction was isolated from the total lipid extract on a 100 mg Varian Bondelut NH_2 disposable cartridge (Jones Chromatography, Hengoed, Mid Glamorgan, UK). PC molecular species were then resolved on a Spherisorb ODS II column (Schambeck SFD, Bad Honnef, Germany) of internal diameter 250 × 4.6 mm at 50°C using a mobile phase of methanol/water (92.5:7.5 v/v) containing 40 mM choline chloride at a flow rate of 1 ml/min. Eluted PC molecular species were quantified by post-column fluorescence derivative formation as outlined above.

DETERMINATION OF TOTAL PROTEIN AND SP-A

Total protein was determined according to the method of Lowry *et al.*[20] SP-A was determined by an ELISA technique using polyclonal anti-human SP-A antibodies which cross-reacted with murine SP-A.[21] Briefly, polyclonal anti-human antibodies were prepared in chickens and New Zealand rabbits by repeated injection of approximately 1 mg purified human SP-A in Freund's complete adjuvant. The chicken and rabbit antisera were then used as the first and second antibodies in a capture ELISA. The microtitre plates (Limbro, ICN Biomedicals Ltd, High Wycombe, Bucks, UK) were coated with chicken anti-human SP-A IgY (10 μg/ml in 35 mM Na_2CO_3, pH 9.6) at 4°C overnight and the non-specific binding sites were blocked with TBS-NTC (50 mM Tris, 50 mM NaCl, 2 mM $CaCl_2$, 0.05% (v/v) Tween-20, 0.05% (w/v) NaN_3, pH 7.4) containing 1 mg/ml bovine serum albumin for one hour at 37°C. Purified human SP-A (10–1000 ng/ml) to be used as standards and BAL fluid samples were duplicated in four serial dilutions in TBS-NTC buffer. The final amounts of SP-A were calculated using these four dilutions. After extensive washes of the wells with TBS-NTC buffer, the samples and standards (100 μl) were added to each well and incubated for three hours at 37°C. After a series of washes with buffer the plates were then incubated with biotinylated rabbit anti-human IgG (50 μg/ml, 100 μl/well) for two hours at 37°C. Finally, streptavidin-alkaline phosphatase conjugate (Sigma Chemical Co. Ltd, Poole, Dorset, UK) diluted 1:10000 in TBS-NTC containing 1 mg/ml BSA was added and allowed to bind for one hour at room temperature. After washing the plates with TBS-NTC buffer, *p*-nitrophenyl phosphate (Sigma) was used as a substrate for the phosphatase reaction and incubated for 30 minutes at 37°C. The reaction was stopped by addition of 1 N NaOH and the plate was read at 405 nm. Correlation coefficients of the standard curve were 0.90–0.95. Sample dilutions were adjusted to within the linear range. Total amounts of SP-A in the BAL fluid were calculated from the apparent SP-A concentrations per ml BAL fluid.

PREPARATION OF SURFACTANT FROM BAL FLUID

Surfactant was prepared from cell-free BAL fluid by centrifugation at 60 000*g* and 4°C for one hour.[22] The 60 000*g* supernatant (S60000) was harvested and frozen at −80°C for further lipid analysis. The 60 000*g* pellet (P60000) was resuspended in 60 μl 154 mM saline supplemented with 1.5 mM calcium chloride and the phospholipid concentration determined from an aliquot and then adjusted to the desired concentration for functional analysis (see below).

FUNCTIONAL SURFACTANT ANALYSIS

For functional analysis P60000 was adjusted to 1.33 μmol phospholipid/ml with 154 mM saline/1.5 mM calcium chloride. Equilibrium (γequil) and minimal (γmin) surface tensions were then determined using a pulsating bubble surfactometer (Electronetics Co, Amherst, New York, USA).[23] Briefly, a bubble was created in a surfactant suspension at 37°C and γequil was determined as surface tension 10 seconds after formation of the bubble. The bubble was then pulsated for five minutes at a frequency of 20 oscillations per min between a minimal bubble radius of 0.4 mm and a maximal radius of 0.55 mm. The pressure across the bubble was measured by a pressure transducer. γequil and γmin were calculated using the LaPlace equation.[24]

DATA ANALYSIS

Data are expressed as means with 95% confidence intervals (CI) in parentheses. Statistical analyses were performed by one factor analysis of variance (ANOVA) and the two tailed Student's *t* test using commercial software (GraphPad InStat Version 1.1, San Diego, USA). Statistical values were corrected for multigroup comparisons using the Bonferroni method and statistical values of regression coefficients were calculated using the same software.

Results

HISTOLOGICAL EXAMINATION

The morphology of *cftr*$^{m1HGU/m1HGU}$ mice showed a regular structure of lung tissue (fig 1). No signs of inflammation as leucocytic infiltration or interstitial oedema were found in any of the animals examined. Comparison of the number and distribution of Clara cells and type II alveolar epithelial cells revealed no detectable differences between MF1 and *cftr*$^{m1HGU/m1HGU}$ mice.

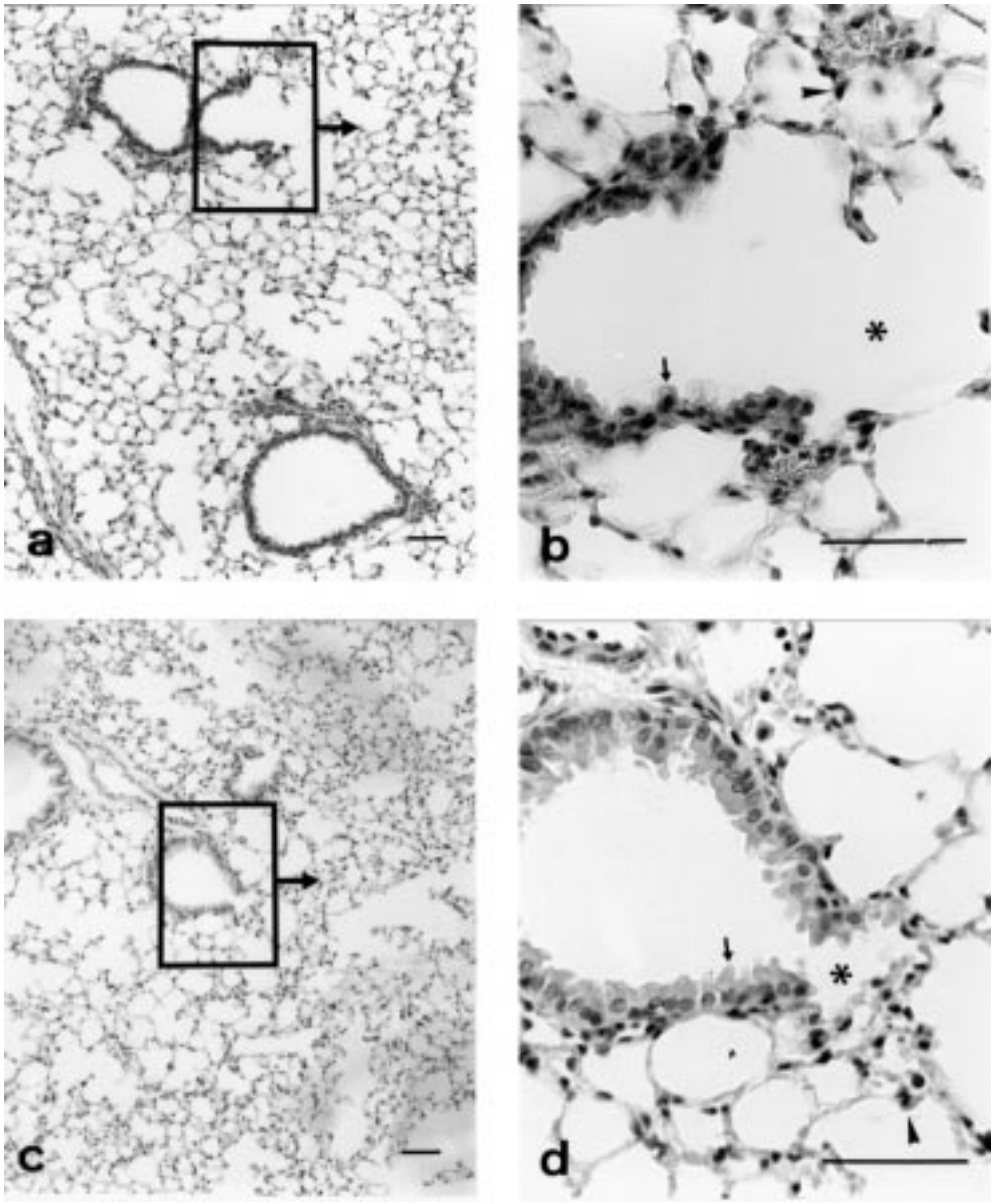

*Figure 1 Histological photographs of cftr$^{m1HGU/m1HGU}$ and control mouse lungs. Microphotographs were produced from haematoxylin and eosin stained sections of lung tissue (5 µm) from a cftr$^{m1HGU/m1HGU}$ mouse (a, b) and from an MF1 control mouse (c, d). The morphology in both lungs revealed no histopathological findings such as leucocytic infiltration or altered numbers of Clara and type II alveolar epithelial cells. The morphology was similar in all examined animals. * = alveolar duct; ↓ = Clara cell; ▲ = type II alveolar epithelial cell. Bar represents 50 µm.*

AMOUNT OF PHOSPHOLIPIDS AND SP-A

In BAL fluid from *cftr*$^{m1HGU/m1HGU}$ mice the amount of phospholipid was 1.02 (95% CI 0.89 to 1.16) µmol/total BAL fluid but it was significantly lower in controls (table 1). In BAL fluid from BALB/c, C57BL/6, and C3H/He mice the concentrations of phospholipid were 0.50 (0.42 to 0.57), 0.52 (0.40 to 0.64), and 0.45 (0.27 to 0.63) µmol/total BAL fluid, respectively, and in BAL fluid from MF1 mice it was between the values of these controls and *cftr*$^{m1HGU/m1HGU}$ mice (0.69 (0.63 to 0.75) µmol/total BAL fluid). Assuming that different body weights of the individual mouse strains contributed to the amount of phospholipid in the BAL fluid, we calculated the correlation between body weight and the amount of phospholipid in BAL fluid and lavaged lung tissue. In both controls and *cftr*$^{m1HGU/m1HGU}$ mice there were significant positive correlations between the amounts of phospholipid in BAL fluid or lavaged lung tissue and the body weight of the animals (fig 2). We therefore corrected the amount of phospholipid in BAL fluid for the individual body weights of mice. After such correction the amount of phospholipid in total BAL fluid from *cftr*$^{m1HGU/m1HGU}$ mice was 43.4 (39.0 to 47.9) nmol/total BAL fluid/g body weight (table 1), while it was constantly lower in BAL fluid from MF1, BALB/c, C57BL/6, and C3H/He mice (26.5 (24.7 to 28.5), 27.1 (23.0 to 31.2), 25.2 (20.4 to 30.0), and 22.6 (16.5 to 29.1) nmol/total BAL fluid/g body weight, respectively). The relative increase in BAL fluid concentrations of phospholipid in *cftr*$^{m1HGU/m1HGU}$ mice compared with the in-

Table 1 Amounts of phospholipid (PL) and apparent surfactant protein A (SP-A) in BAL fluid and of phospholipid in lavaged lung tissue of cftr$^{m1HGU/m1HGU}$ mice versus individual healthy control strains

	cftr$^{m1HGU/m1HGU}$ (n=25)	*MF1 (n=25)*	*BALB/c (n=11)*	*C57BL/6 (n=13)*	*C3H/He (n=8)*
PL (µmol/BAL fluid)	1.02 (0.89 to 1.16)	0.69 (0.63 to 0.75)***	0.50 (0.42 to 0.57)***	0.52 (0.40 to 0.64)***	0.45 (0.27 to 0.63)***
PL (µmol/lavaged lung)	4.04 (3.86 to 4.22)	4.78 (4.54 to 5.02)***	3.32 (3.16 to 3.47)***	3.97 (3.78 to 4.16)	3.63 (3.54 to 3.73)
Body weight (g)	23.3 (22.0 to 24.7)	26.0 (24.8 to 27.2)*	18.5 (17.1 to 19.8)**	20.6 (18.3 to 22.9)	19.9 (18.1 to 21.7)
PL (nmol/total BAL fluid/g BW)	43.4 (39.0 to 47.9)	26.5 (24.7 to 28.5)***	27.1 (23.0 to 31.2)***	25.2 (20.4 to 30.0)***	22.6 (16.5 to 29.1)***
PL (nmol/lavaged lung/g BW)	175 (166 to 185)	185 (177 to 193)	182 (169 to 194)	197 (185 to 209)	185 (170 to 200)
BAL-PL (% of total lung PL)	19.9 (18.0 to 21.8)	12.6 (11.8 to 13.4)***	13.0 (11.2 to 14.7)***	11.3 (9.2 to 13.5)***	11.0 (7.5 to 14.5)***
Protein (mg/total BAL fluid)	0.63 (0.52 to 0.74)	0.68 (0.56 to 0.80)	0.67 (0.58 to 0.76)	0.74 (0.67 to 0.81)	0.51 (0.40 to 0.61)
SP-A (ng/total BAL fluid)	259 (239 to 279)[a]	249 (222 to 274)[b]	235 (208 to 263)	219 (197 to 241)	219 (159 to 280)
SP-A (ng/nmol PL)	0.27 (0.23 to 0.31)[a]	0.40 (0.34 to 0.46)[b]	0.47 (0.35 to 0.58)**	0.42 (0.36 to 0.53)*	0.49 (0.33 to 0.63)*

Data are presented as means and 95% confidence intervals (in brackets) of the indicated numbers of experiments.
[a]n=14; [b]n=13.
* p<0.05; ** p<0.01, *** p<0.001 versus *cftr*$^{m1HGU/m1HGU}$.

dividual controls was therefore 64 (52 to 76)%, 60 (39 to 89)%, 72 (45 to 113)%, and 92 (49 to 163)%, respectively.

Table 2 Phospholipid composition of bronchoalveolar lavage (BAL) fluid and lung tissue in cftr$^{m1HGU/m1HGU}$ mice versus MF1 and other control (BALB/c; C57BL/6; C3H/He) mice

Phospholipid	*cftr$^{m1HGU/m1HGU}$ (n=13)*	*MF1 (n=12)*	*Other controls (n=21)*[a]
BAL fluid			
PC	84.2 (81.2 to 87.1)	83.3 (81.8 to 84.8)	85.3 (83.8 to 86.8)
PG	11.1 (9.0 to 13.3)	12.0 (10.6 to 13.4)	10.4 (7.6 to 12.0)
SPH	1.1 (0.8 to 1.4)	0.8 (0.4 to 1.1)	1.1 (0.5 to 1.5)
PE	2.1 (1.2 to 2.9)	3.2 (2.1 to 4.5)	1.1 (0.7 to 1.4)
PI	1.6 (1.2 to 2.0)	0.7 (0.4 to 1.1)	2.1 (1.2 to 2.7)
PS	trace	trace	trace
	cftr$^{m1HGU/m1HGU}$ (n=13)	*MF1 (n=6)*	*Other controls (n=25)*[b]
Lung tissue			
PC	51.3 (49.8 to 52.9)	50.0 (48.7 to 51.3)	49.4 (48.3 to 50.3)
PG	4.2 (3.1 to 5.4)	4.2 (2.5 to 5.9)	3.4 (2.7 to 4.1)
SPH	8.2 (7.9 to 8.5)	8.4 (8.0 to 8.7)	8.3 (8.0 to 8.6)
PE	34.1 (31.9 to 36.2)	36.2 (34.1 to 38.5)	36.7 (35.5 to 37.9)
PI	2.2 (1.8 to 2.6)	1.2 (1.0 to 1.4)	2.2 (1.8 to 2.7)
PS	trace	trace	trace

Pc=phosphatidylcholine; PG=phosphatidylglycerol; SPH=sphingomyelin; PE=phosphatidylethanolamine; PI=phosphatidylinositol; PS=phosphatidylserine.
[a]BALB/c (n=7), C57BL/6 (n=8) and C3H/HE (n=6). [b]BALB/c (n=11), C57BL/6 (n=8) and C3H/He (n=6).

Table 3 Molecular species of phosphatidylcholine (PC) in bronchoalveolar lavage (BAL) fluid and lung tissue in cftr$^{m1HGU/m1HGU}$ versus MF1 and other control (BALB/c; C57BL/6; C3H/He) mice

PC species	*cftr$^{m1HGU/m1HGU}$ (n=9)*	*MF1 (n=14)*	*Other controls (n=17)*[a]
BAL fluid			
PC16:0/14:0	5.3 (4.9 to 5.8)	5.7 (5.3 to 6.1)	4.7 (3.9 to 5.4)
PC16:0/16:0	57.9 (56.4 to 59.4)	58.3 (57.3 to 59.3)	58.3 (56.0 to 60.7)
PC16:0/16:1	19.8 (18.8 to 20.9)	17.9 (16.9 to 18.9)	13.5 (10.3 to 16.8)*
PC16:0/18:1	4.5 (4.1 to 5.0)	5.2 (5.0 to 5.4)	7.2 (6.4 to 8.0)***
PC16:0/18:2	6.3 (5.6 to 6.9)	6.9 (6.5 to 7.2)	9.0 (8.0 to 9.9)**
PC18:0/18:2	0.4 (0.2 to 0.6)	0.2 (0.1 to 0.3)	1.2 (0.4 to 2.0)
PC16:0/20:4	1.1 (0.9 to 1.2)	1.2 (0.6 to 2.6)	1.4 (1.1 to 1.7)
PC18:0/20:4	0.4 (0.2 to 0.6)	0.2 (0.1 to 0.3)	0.8 (0.5 to 1.2)
PC18:1/18:2	0.4 (0.2 to 0.5)	0.5 (0.1 to 0.9)	0.5 (0.3 to 0.7)
Others	3.9 (2.9 to 4.9)	3.7 (3.3 to 4.1)	3.4 (2.7 to 4.3)
	cftr$^{m1HGU/m1HGU}$ (n=16)	*MF1 (n=23)*	*Other controls (n=17)*
Lung tissue			
PC16:0/14:0	3.6 (3.0 to 4.1)	4.0 (3.5 to 4.5)	2.9 (2.6 to 3.1)
PC16:0/16:0	34.8 (33.2 to 36.3)	35.6 (34.8 to 36.3)	33.2 (32.1 to 34.3)
PC16:0/16:1	10.8 (9.9 to 11.8)	8.4 (7.8 to 9.1)**	7.8 (6.5 to 9.0)***
PC16:0/18:1	11.1 (10.2 to 12.0)	14.2 (13.5 to 15.0)***	12.9 (12.6 to 13.3)*
PC16:0/18:2	10.8 (10.1 to 11.4)	10.2 (9.3 to 11.2)	11.6 (11.1 to 12.2)
PC18:0/18:2	3.6 (3.5 to 3.8)	3.3 (2.9 to 3.6)	4.0 (3.7 to 4.3)
PC16:0/20:4	4.3 (4.0 to 4.6)	4.0 (3.6 to 4.4)	4.9 (4.6 to 5.2)
PC18:0/20:4	4.0 (3.7 to 4.4)	3.8 (3.3 to 4.2)	4.8 (4.5 to 5.2)
PC16:0/22:6	3.4 (3.1 to 3.7)	4.9 (4.4 to 5.4)	3.2 (3.0 to 3.5)
PC18:1/18:2	1.4 (1.2 to 1.6)	2.7 (1.4 to 3.9)	1.6 (1.4 to 1.7)
Others	12.2 (11.5 to 12.9)	8.9 (8.1 to 9.7)	13.2 (12.5 to 13.9)

PC16:0/14:0=palmitoylmyristoyl-PC; PC16:0/16:0=dipalmitoyl-PC; PC16:0/16:1=palmitoylpalmitoleoyl-PC; PC16:0/18:1=palmitoyloleoyl-PC; PC16:0/18.2=palmitoyllinoleoyl-PC; PC18:0/18:2=stearoyllinoleoyl-PC; PC16:0/20:4=palmitoylarachidonoyl-PC; PC18:1/18:2=oleoyllinoleoyl-PC; others=minor identified (dioleoyl-PC, stearoyloleoyl-PC) or not identified PC species.
Data are means and 95% confidence intervals (in brackets) of the indicated numbers (n) of experiments. [a]BALB/c (n=10), C57BL/6 (n=4) and C3H/He (n=3).
* p<0.05, ** p<0.01, *** p<0.001 versus *cftr*$^{m1HGU/m1HGU}$.

In lavaged lung tissues from *cftr*$^{m1HGU/m1HGU}$ mice the amount of phospholipid was 4.04 (3.86 to 4.22) µmol/whole lung and 175 (166 to 185) nmol/whole lavaged lung/g body weight. While the absolute values per lavaged lung displayed some significant differences between *cftr*$^{m1HGU/m1HGU}$ mice and two of the control strains – namely, MF1 and BALB/c – there were no significant differences after correcting these values for body weight (table 1). Consequently, BAL fluid from *cftr*$^{m1HGU/m1HGU}$ mice contained 19.9 (18.0 to 21.8) mol% of total lung phospholipid (BAL fluid + lavaged lung), while in MF1, BALB/c, C57BL/6, and C3H/He mice the BAL fluid contained only 12.6 (11.8 to 13.4) mol%, 13.0 (11.2 to 14.7) mol%, 11.3 (9.2 to 13.5) mol%, and 11.0 (7.5 to 14.5) mol%, respectively (table 1). The apparent concentration of SP-A, a major component of

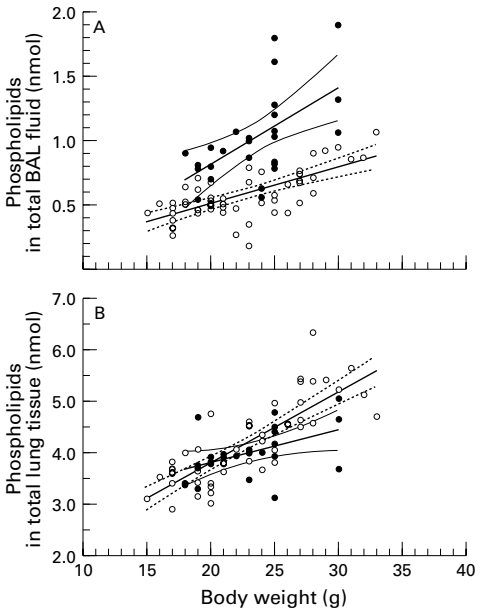

Figure 2 Correlation between the amounts of lung phospholipid and body weight. Linear regression curves were calculated between the total amount of phospholipids in (A) BAL fluid or (B) lavaged lung tissue from cftr$^{m1HGU/m1HGU}$ (●; n=25) and control mice (○; n=57) and the individual body weights of the mice. Thick bars represent regression curves while thin (cftr$^{m1HGU/m1HGU}$ mice) or dotted (control) lines represent 95% confidence intervals. In BAL fluid of cftr$^{m1HGU/m1HGU}$ mice r=0.591, p <0.01; controls r=0.671, p <0.00001. In lung tissue of cftr$^{m1HGU/m1HGU}$ mice r=0.458, p <0.05; controls r=0.826, p <0.00001.

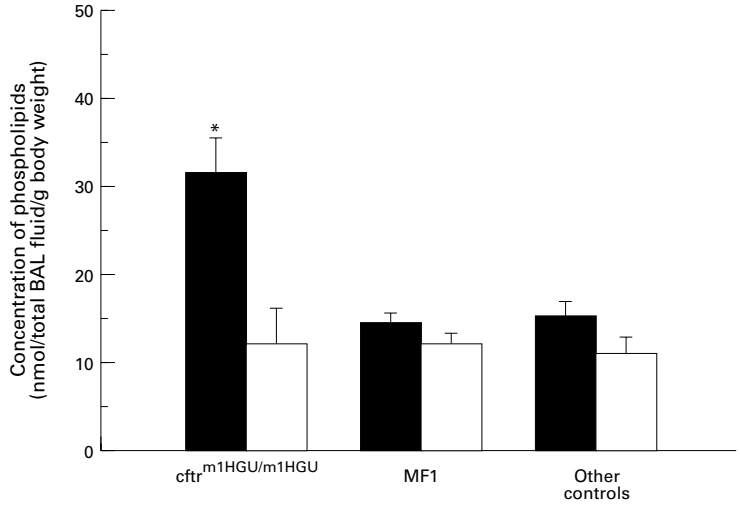

*Figure 3 Concentrations of phospholipids in P60000 (■) and S60000 (□) of BAL fluid of cftr$^{m1HGU/m1HGU}$, MF1, and other control mice. BAL fluid was centrifuged for one hour at 60 000 g and at 4°C. Phospholipid concentrations were determined in the pellet (P60000) and supernatant (S60000) as described in the Methods section. cftr$^{m1HGU/m1HGU}$ (n=6); MF1 (n=3); other control mice: BALB/c (n=4); C57BL/6 (n=3); C3H/He (n=5). Bar height represents 95 confidence interval. * p <0.05 versus MF1 and other controls.*

Table 4 Surface tension function (in mN/m) of 60000g pellet (P60000) from bronchoalveolar lavage (BAL) fluid in cftr$^{m1HGU/m1HGU}$ versus MF1 and other control (BALB/c; C57BL/6; C3H/He) mice

PC species	*cftr$^{m1HGU/m1HGU}$* (n=7)	*MF1* (n=7)	*Other controls* (n=13)[a]
γequil	24.7 (19.1 to 30.3)	25.0 (24.2 to 25.9)	28.6 (25.5 to 31.6)
γmin			
Pulsation 1	17.4 (14.4 to 20.5)	21.6 (20.0 to 23.1)	18.2 (13.4 to 22.9)
Pulsation 3	15.6 (11.3 to 19.8)	18.0 (13.1 to 22.9)	16.6 (10.8 to 22.4)
Pulsation 9	11.1 (5.3 to 16.8)	13.6 (7.0 to 20.2)	15.5 (10.1 to 20.9)
Pulsation 100	2.8 (0.1 to 5.5)	3.2 (0.1 to 6.7)	2.6 (1.7 to 3.5)

γequil = equilibrium surface tension; γmin = minimal surface tension.
Data are means and 95% confidence intervals (in brackets) of the indicated numbers (n) of experiments.
[a]13 experiments from BALB/c (n=4), C57BL/6 (n=5), and C3H/He (n=4) mice.

surfactant and important for its interactions with type II alveolar cells, phospholipids, bacteria and alveolar macrophages, was 259 (239 to 279) ng/total BAL fluid of *cftr*$^{m1HGU/m1HGU}$ mice which was identical to the concentrations in BAL fluid of control mice (table 1). Due to the increase in the concentration of phospholipids in the BAL fluid, the ratio of SP-A to phospholipid was decreased by 33–45% from 0.40 (0.34 to 0.46), 0.47 (0.35 to 0.58), 0.42 (0.36 to 0.53), and 0.49 (0.33 to 0.63), respectively, in the individual control strains to 0.27 (0.23 to 0.31) in *cftr*$^{m1HGU/m1HGU}$ mice (table 1).

COMPOSITION OF PHOSPHOLIPID CLASSES AND PHOSPHATIDYLCHOLINE (PC) MOLECULAR SPECIES

Investigation of phospholipid compositional changes in BAL fluid and lung tissue of *cftr*$^{m1HGU/m1HGU}$ mice was in the first instance addressed by HPLC analysis of phospholipid classes. As shown in table 2 there were no differences in the relative composition of phospholipid classes in BAL fluid and lung tissue of *cftr*$^{m1HGU/m1HGU}$ and control mice. Importantly, the contributions of PC and phosphatidylglycerol (PG) were not decreased, nor were those of sphingomyelin (SPH) or phosphatidylethanolamine (PE) increased in either BAL fluid or lung tissue of *cftr*$^{m1HGU/m1HGU}$ mice. Because there were no differences in the phospholipid compositions of BALB/c, C57BL/6 and C3H/He mice, these data were combined and are shown as "other controls". Since the key molecule of surface tension function of lung surfactant is PC16:0/16:0, and since alterations in the concentrations of individual molecular species of PC are a more sensitive indicator of pulmonary surfactant alterations than the determination of PC fatty acids by gas chromatography,[19] we subsequently investigated these PC molecular species in both BAL fluid and lavaged lung tissue. The BAL fluid and lavaged lung tissue of *cftr*$^{m1HGU/m1HGU}$ mice predominantly contained PC16:0/16:0 (table 3), comprising 57.9 (56.4 to 59.4) mol% and 34.8 (33.2 to 36.3) mol% of total PC, respectively. These values were identical to those from control mice. Other typical components of surfactant – for example, palmitoyl-myristoyl-PC (PC16:0/14:0) and palmitoyl-palmitoleoyl-PC (PC16:0/16:1) – were also without major changes in *cftr*$^{m1HGU/m1HGU}$ mice compared with controls. The highly unsaturated PC species palmitoylarachidonoyl-PC (PC16:0/20:4), stearoylarachidonoyl-PC (PC18:0/20:4), and palmitoyldocosahexaenoyl-PC (PC16:0/22:6) were not detected in significant amounts in BAL fluid but made up 12–14% in lung tissue. Again, there were no major differences between *cftr*$^{m1HGU/m1HGU}$ and control mice.

SURFACE TENSION FUNCTION OF SURFACTANT IN BAL FLUID

The relative concentration of active surfactant isolated by differential ultracentrifugation at 60 000*g* (P60000) compared with the phospholipids in the supernatant (S60000) of the BAL fluid is shown in fig 3. In BAL fluid from *cftr*$^{m1HGU/m1HGU}$ mice the amount of phospholipid in P60000 was significantly higher than in controls. To evaluate whether the surface tension function of the P60000 was different from that of controls we measured the surface tension function of this material. Equilibrium surface tension (γequil) after 10 seconds of static adsorption of P60000 from *cftr*$^{m1HGU/m1HGU}$, MF1, and other control mice was 24.7 (19.1 to 30.3) mN/m, 25.0 (24.2 to 25.9) mN/m, and 28.6 (25.5 to 31.6) mN/m, respectively (table 4). Dynamic measurement of minimal surface tension (γmin) showed that P60000 from *cftr*$^{m1HGU/m1HGU}$ mice reached values below 5 mN/m as quickly as surfactant from the control mice (table 4).

Discussion

Cystic fibrosis is an inherited disease with a defect in the *CFTR* gene resulting in the expression of a defective cAMP-dependent chloride channel.[1 2] Previous reports of impairment to the biophysical properties of airway secretions, as well as lipid extracts of such secretions in patients with cystic fibrosis as-

sociated with an altered phospholipid composition, have been attributed to the associated chronic inflammatory processes.[1–5] The possibility of a primary defect in surfactant processing in cystic fibrosis has not previously been considered.[1 2 25] However, recent RNA in situ hybridisation studies have demonstrated that the *CFTR* gene is expressed in the lung periphery in type II alveolar epithelial cells which produce both surfactant proteins and phospholipids.[6 7] Moreover, an increased phospholipid turnover was described in isolated human fibroblasts and blood platelets from patients with cystic fibrosis.[10] Hence, lung surfactant metabolism could be affected by the basic defect of cystic fibrosis prior to and independent from the chronic inflammatory processes in the lungs.[1 2 25] To address this issue we employed the *cftr*$^{m1HGU/m1HGU}$ mouse[12] as an animal model to study aspects of lung surfactant metabolism in cystic fibrosis prior to chronic pulmonary infection. The *cftr*$^{m1HGU/m1HGU}$ mouse is a useful model for human cystic fibrosis because, unlike the absolute "null" mutant mouse, this model shows normal growth rate and good long term survival. This is due to the "leaky" nature of the mutation which results in 5–10% residual wild type after expression. The *cftr*$^{m1HGU/m1HGU}$ mouse is thus analogous to patients with cystic fibrosis carrying CFTR mutations which retain partial CFTR function.

Our data show, for the first time, that BAL fluid concentrations of surfactant phospholipid as an index for the intra-alveolar surfactant pool are primarily increased by about 60% in a CFTR-deficient organism compared with all other tested control strains, whereas the amount of phospholipid in lung tissue is not altered. While we could not demonstrate biochemical alterations of alveolar phospholipid material, the quantitative changes in alveolar surfactant phospholipids were due to an increase in material which could be pelleted by ultracentrifugation at 60 000*g* (P60000) as previously described in the mouse by other investigators.[22] In that study it was demonstrated that the P60000 consisted of large surfactant aggregates, while so-called small surfactant aggregates remained in the 60000*g* supernatant (S60000). Moreover, it was demonstrated that P60000 consisted of surface active material while S60000 contained only inactive material. Our functional data are consistent with these observations since P60000 present in BAL fluid from both *cftr*$^{m1HGU/m1HGU}$ and control mice was highly surface active. It was suggested that, during the dynamic processing of surfactant from its secretion to its re-uptake into type II pneumocytes, P60000 represents freshly secreted surface active material while S60000 represents inactivated "older" surfactant which shows poor surface tension function.[22 26] It is therefore reasonable to attribute the increased alveolar surfactant pool size in *cftr*$^{m1HGU/m1HGU}$ mice to an increase in newly secreted active surfactant ("large aggregates") rather than an accumulation of inactivated older surfactant ("small aggregates")[22] in the alveolar space.[27]

Several mechanisms could lead to an accumulation of surfactant phospholipid in the alveolar space. Decreased clearance of surfactant along the conductive airways due to the impaired bronchopulmonary clearance in CFTR deficiency is unlikely to play a major role since this is typically a minor route for surfactant clearance.[27] Recent findings in isolated cells from patients with cystic fibrosis, however, support the concept that a primary alteration of phospholipid metabolism[10] may contribute to this increased surfactant phospholipid pool in the alveolus. In isolated fibroblasts and platelets from patients with cystic fibrosis phospholipid synthesis was increased, but such cells do not secrete major amounts of phospholipid. However, despite the tempting explanations outlined above, other explanations for an increase in surfactant phospholipids in the alveolar space should be mentioned. The *cftr* gene transcript regulates the cAMP-dependent Cl^- current and thereby the transport of other ions and of water through cells.[2] *Cftr* expression in type II alveolar epithelial cells[6 7] could indirectly alter the homeostasis of the watery alveolar hypophase and of cAMP, thereby influencing surfactant phospholipid homeostasis.

The alterations in *cftr*$^{m1HGU/m1HGU}$ mice described in this study are different from those previously described in chronically infected airways of human subjects. While in studies on airway secretions from patients with cystic fibrosis alterations in phospholipid classes, fatty acid composition of phospholipids, and biophysical properties (adsorption velocity) were described,[25] we did not detect such changes in BAL fluid from *cftr*$^{m1HGU/m1HGU}$ mice without infection. Similarly, no impairment of surface tension function of the surfactant isolated from the BAL fluid of *cftr*$^{m1HGU/m1HGU}$ mice was detected. We therefore conclude that such alterations are due to chronic inflammation and leucocyte infiltration of cystic fibrosis lungs, secondary to colonisation with opportunistic bacteria. Our data support the concept that any alteration in the phospholipid composition is due to chronic infection and inflammation rather than an effect of *CFTR* mutations.

While phospholipids were increased in the BAL fluid, the amount of SP-A remained unchanged. This unexpected finding is in agreement with a recent study by Doyle *et al* who showed that phospholipid and SP-A secretion into the alveolus are independently regulated.[26] While the mechanisms of such changes in *cftr*$^{m1HGU/m1HGU}$ mice are still uncertain and await further investigation, the potential consequences for pulmonary defence of an increased amount of phospholipid and a decreased ratio of SP-A to phospholipid are more obvious. Surfactant contributes to pulmonary integrity as a first line of defence.[11] Phospholipid inhibits the activity of SP-A to opsonise bacteria and depresses the respiratory burst of alveolar macrophages upon challenge.[11 28] Increased phospholipid concentrations together with a decreased SP-A to phospholipid ratio in *cftr*$^{m1HGU/m1HGU}$ mice, and possibly in patients with cystic fibrosis, may

impair the resistance of the lungs against bacteria.[28] Since human SP-A was used as a standard in the immunoassay, the values obtained for the mouse SP-A are not absolute and are only for use for the comparison of SP-A levels within the different strains of mice examined in this study. Nevertheless, our results are consistent with recent findings on BAL fluid from patients with cystic fibrosis which showed an increased concentration of PC, while the ratio between SP-A and phospholipid was even more decreased in the BAL fluid of patients with cystic fibrosis than in that of *cftr*$^{m1HGU/m1HGU}$ mice.[29] However, while the data from human BAL fluid originate from lungs that already show symptoms of chronic impairment and, possibly, proteolytic degradation of SP-A due to inflammation, the *cftr*$^{m1HGU/m1HGU}$ mice used in this study were kept under specific pathogen-free conditions. Histological examination of lungs confirmed the absence of leucocytic infiltrations or other visible alteration of lung structure and cellular distribution, and the absence of increases in membrane phospholipids such as PE or SPH in BAL fluid from *cftr*$^{m1HGU/m1HGU}$ mice provides further evidence of minimal damage to their lungs. It is therefore possible that the greater decrease in the SP-A to phospholipid ratio in BAL fluid from patients with cystic fibrosis[29] compared with *cftr*$^{m1HGU/m1HGU}$ mice may in part be due to secondary degradation of SP-A caused by chronic inflammation of the lung.

We conclude that, in *cftr*$^{m1HGU/m1HGU}$ mice without chronic inflammatory lung disease, the composition of phospholipid classes and phosphatidylcholine molecular species, the concentration of SP-A, and the surface tension function of surfactant are all unchanged compared with control mice. However, the total amount of phospholipid and the ratio of phospholipid to SP-A was increased in the BAL fluid, possibly as a result of altered surfactant phospholipid metabolism.

The authors acknowledge the expert advice of Professor K B M Reid (Oxford University, Oxford, UK) and A D Postle (Southampton University, Southampton, UK) and the excellent technical assistance of Mrs Ch Acevedo and Mrs K Westermann. This work was supported by the Deutsche Forschungsgemeinschaft (Ha 1959/1-2; 2-1) and the Medical Research Council.

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