Pulmonary surface-active materials in the Chediak–Higashi syndrome

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Abstract Beige mice express the Chediak–Higashi syndrome. Large inclusions, identified as abnormal lysosomes, are found in many cells. The inclusions in type II alveolar epithelial cells are enlarged lamellar bodies and they are associated with an increase in total lung surface-active material and phospholipid. Comparison of recovery of phospholipid in surface-active materials from beige and black (normal) mice indicates that in the beige mice there is an increase in total phospholipid and disaturated phosphatidylcholines in whole lung and in surface-active materials in residual lung after lavage. Phosphatidylcholine and phosphatidylglycerol are increased as percentages of total lung phospholipid. Calculated alveolar surface coverage of surface-active materials isolated from residual beige lungs is greater than three times that of normal lungs. Surface-active materials recovered from beige mice are qualitatively similar in phospholipid composition and in surface activity to materials recovered from normal mice. The quantity of surface-active material phospholipid recovered in the lavage of beige mouse lungs was normal. The basis for the abnormal accumulation of lamellar body lipids is not known.

Supplementary key words lamellar body · type II alveolar epithelial cell · disaturated phosphatidylcholine · lysosome

The beige mouse (C57BL/6J–bg^J/bg^J), a mutant strain of the black mouse (C57BL/6J–+/+), expresses the Chediak–Higashi syndrome. This syndrome is inherited in an autosomal recessive pattern and has been described in man, cow, and mink, as well as mouse (1, 2). Characteristic intracellular inclusions, identified as abnormal lysosomes, are found in a variety of cells including leukocytes (3, 4), kidney tubule cells (5, 6), and hepatocytes (7). We have found abnormally large lamellar bodies in type II alveolar epithelial cells in beige mice (8, 9) and an associated increase in total lung surface-active material (SAM) and SAM phospholipid (8). In the investigations reported here we sought to characterize the SAM in beige lung and to determine whether or not the enlarged lamellar bodies were associated with an abnormality of the distribution of SAM between the intracellular storage site and the alveolar space.

MATERIALS AND METHODS

Black (C57BL/6J–+/+) and beige (C57BL/6J–bg^J/bg^J) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine, or bred in the animal facility of the Department of Pathology, University of Washington, from stock obtained from the same source. Adult male mice, 25–30 g, were used in all experiments. Animals were killed by cervical dislocation and weighed. Animals were then handled in one of two ways: *1*) lungs were removed, freed from connective tissue and major airways, weighed, homogenized in 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, and extracted as described below to remove lipids; or *2*) lungs were lavaged in situ with 1 ml of homogenization medium via a tracheostomy cannula with the thorax open. The fluid was injected and withdrawn gently with a syringe five times in each animal. Symmetrical inflation and deflation of all lobes with lavage fluid was verified by inspection during the procedure. Lavage fluid from five animals was pooled in each experiment. The lungs after lavage were homogenized as described above. The initial weights of the lavaged lungs were estimated by multiplying the body weights of these animals by the lung weight:body weight ratios from animals of similar age and weight determined for unlavaged lungs.

SAM was isolated from lavage fluid and homogenate of residual lung (lung after lavage) by a modification of the method of Frosolono et al. (10), as described by Pawlowski et al. (11). The procedure was as follows. All centrifugation was done at 48,000 *g* in an SS-34 rotor in a high-speed refrigerated centrifuge (RC2-B, Sorvall, Inc., Newtown, CT) at 0°C. Sucrose solutions were made in homogenization medium. Lavage fluid or homogenate was layered on 0.75 M sucrose and

Abbreviations: DPC, disaturated phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; PL, phospholipid; SAM, surface-active material; Sph, sphingomeyelin; PAS, periodate–Schiff reagent.

centrifuged for 40 min. The interfacial material was aspirated with a pipet, suspended in distilled water, and centrifuged for 20 min. The pellet was suspended in 0.68 M sucrose and centrifuged for 40 min. The pellicle was aspirated and the concentration of sucrose was reduced to 0.25 M by adding homogenization medium. The suspension was centrifuged for 40 min. The pellet was resuspended in water and centrifuged for 20 min. This pellet, designated SAM, was resuspended in distilled water and stored at −20°C until it was analyzed for lipid composition and surface activity.

Lipids in SAM fractions and lung homogenates were extracted and purified according to the procedure of Folch, Lees, and Sloane Stanley (12). Lipid phosphorus was measured by the method of Bartlett (13). Total phospholipid (PL) was estimated by multiplying lipid phosphorus by 25. The phospholipids were separated into acidic and neutral fractions by diethylaminoethyl (DEAE) cellulose (Cellex D, Bio-Rad Laboratories, Richmond, CA) chromatography according to the procedures of Rouser, Kritchevsky, and Yamamoto (14). Lipid phosphorus distribution was determined by thin-layer chromatography on silica gel H (E. Merck AG, Darmstadt, Germany) in a solvent system of chloroform–methanol–water–15 M ammonium hydroxide 60:35:4:1 (v/v). Experimental losses of phospholipid during these analyses were determined by measuring recovery of phospholipid phosphorus. Recoveries from column and thin-layer separations were 98.8 ± 4.3% (mean ± 1 SD), n = 20, and 95.2 ± 5.2%, n = 20, respectively. Lipids were identified by comigration with known standards (Supelco, Inc., Bellefonte, PA) after visualization by brief exposure of the plates to iodine vapor; the phosphorus content of each spot was measured by the method of Parker and Peterson (15). Phosphatidylglycerol was also identified on chromatograms by characteristic reaction with PAS and the absence of a reaction with ninhydrin. Disaturated phosphatidylcholines (DPC) in lipid extracts of lung and SAM were measured according to the method of Mason, Nellenbogen, and Clements (16). Recovery of DPC in these experiments, determined using an internal standard of 1,2-di[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (Applied Science Laboratories, Inc., State College, PA), was 93.2 ± 4.7% (mean ± 1 SD), n = 30.

Variability in recovery of surface-active material PL from lavage of airways was high. To determine if this was attributable to the methods used to isolate SAM and if the methodology may have obscured a difference between the groups, we also measured PL in cell-free lavage fluid, not further fractionated, that was obtained from individual animals in additional experiments. Lungs were lavaged as described above, and the fluid obtained from each animal was centrifuged at 150 *g* for 5 min. The supernatant was extracted for lipids and analyzed for lipid phosphorus as described above.

Surface activity of SAM was measured at room temperature (21–22°C) and 37°C on modified Wilhelmy balances. The balance troughs and barriers were constructed of Teflon. The barriers were driven linearly at constant velocity, and surface area and surface tension changes during each compression–expansion cycle were recorded on an *x*–*y* plotter. Surface tension was measured as the force exerted on a platinum paddle suspended in the hypophase from a force transducer (Statham, G 10B, Hato Rey, PR) calibrated by measuring the responses to known weights. The balance used in the studies at room temperature had a maximum surface area of 52 cm² and a minimum surface area of 7 cm². The balance used for measurements at 37°C was enclosed in a double-walled Plexiglass chamber through which heated water was circulated to maintain the solution in the trough at 37 ± 0.5°C. This balance had a maximum surface area of 522 cm² and a minimum surface area of 49 cm². Suspensions of SAM were spread in isopropyl alcohol 1:1 (v/v) from a microsyringe. Five minutes were allowed for spreading of materials and evaporation of the solvent. The smallest aliquot of the aqueous SAM suspension that would decrease surface tension of clean Ringer's solution, pH 7.4, to less than 10 dynes/cm during area reduction was determined. The weight of lung that was required to yield the SAM in that aliquot was calculated and divided by the surface area at which the surface tension had been lowered to 12 dynes/cm. Estimates of potential alveolar surface coverage, calculated as cm² per gram of lung, were determined from these data as described by Clements, Nellenbogen, and Trahan (17). Surface concentrations for lipid phosphorus were also calculated by dividing the weight of phosphorus in the aliquot of SAM by the surface area at 12 dynes/cm (18).

Differences between means were tested for significance by Student's *t* test.

RESULTS

No differences were observed in lung weight:body weight ratios between beige (6.33 ± 0.69 mg/g [mean ± 1 SD, n = 9]) and normal (6.81 ± 0.97 mg/g [mean ± 1 SD, n = 7]) mice. Total lung and residual SAM PL (SAM PL recovered from lung after lavage) were

TABLE 1. Phospholipid in lung and surface-active materials

Animal	Lung		Surface-Active Materials	
	Homogenate	Lavage	Residual Lung	Lavage
Black	29.37 ± 2.28[a] (4)[b]	2.59 ± 0.52 (5)	2.51 ± 1.47 (4)	0.60 ± 0.16 (5)
Beige	40.59 ± 3.54 (4)	2.40 ± 0.67 (4)	6.58 ± 2.34 (7)	0.48 ± 0.31 (5)
P	<0.005	>0.50	<0.025	>0.40

[a] Values are mg PL/g lung, mean ± 1 SD.
[b] The number of experiments is indicated in parentheses.

significantly increased in beige mice compared to black mice. There was no difference between the two groups in the alveolar PL measured either in the total lavage or in the lavage SAM fraction (**Table 1**). However, lavage SAM PL expressed as a percentage of total (lavage plus residual) SAM PL was significantly decreased in beige mice (**Fig. 1**).

Whole lung DPC was increased in beige compared to black mice (**Table 2**). The proportion of whole lung PL represented by DPC was also increased in the beige mice. In residual SAM the amount of DPC per gram of lung was increased in the beige mice, but there was no difference between the two strains in the percentage of residual SAM PL represented by DPC. In lavage SAM, DPC as a percentage of lavage SAM PL was also similar in the two groups.

Qualitative analysis of the phospholipids in whole lungs of beige mice indicated that both phosphatidylcholine and phosphatidylglycerol were increased. Phosphatidylethanolamine and combined phosphatidylserine and phosphatidylinositol fractions were proportionately decreased in lungs of beige mice (**Table 3**). An unidentified phospholipid that did not react with ninhydrin, PAS, or Draggendorf reagents migrated between phosphatidylglycerol and the solvent front in the acidic phospholipid fractions; it accounted for 1–2% of the lipid phosphorus in lungs of both black and beige mice (Table 3).

Analysis of phospholipid distribution in residual SAM suggests that these materials in beige and black mice are qualitatively similar (Table 3). In both groups phosphatidylcholine accounted for about 75–80% of phospholipid phosphorus. Phosphatidylglycerol was the second most abundant phospholipid in residual SAM in both groups and accounted for about 10% of phospholipid phosphorus.

Surface tension measurements of SAM isolated at room temperature indicated similar surface concentrations of lipid phosphorus at 12 dynes/cm in black and beige groups (**Table 4**). There was, however, a threefold increase in surface area coverage of SAM recovered from the residual lungs of beige mice. The relationship of surface tension to specific surface area ($cm^2/\mu g$ lipid phosphorus) of a beige residual SAM sample is shown in **Fig. 2**. These materials lowered surface tension to less than 10 dynes/cm at 37°C.

DISCUSSION

Our results show that there was an increase in total PL and DPC in whole lung and residual SAM in beige mice. The percentage of total lung PL contributed by DPC, the major component of SAM, was also increased in beige whole lung. The decrease in lavage SAM PL as a percentage of total SAM PL in beige mice indicates an abnormality in compartmental distribution of SAM, although the amount of SAM PL recovered from the air spaces was not different between the two strains. This indicates an increase in the intracellular compartment of SAM in beige mice.

The increase in whole lung PL and DPC is consistent with the observed increase in intracellular SAM in beige mice. Since the PL in residual SAM is composed of about 50% DPC, an increase in whole lung PL of about 10 mg/g lung in beige mice (Table 1) should be associated with an increase in whole lung DPC of about 5 mg/g lung if the increase in PL was due to SAM. Whole lung DPC is somewhat less than 25% of total lung PL in normal mice, so an increase in PL due to components other than SAM would be expected to yield an increase in lung DPC of 2.5 mg/g or less. The observed increase in DPC of about 5 mg/g

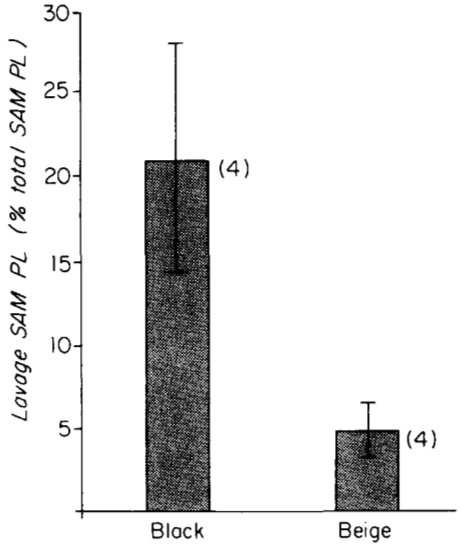

Fig. 1. Comparison of lavage SAM PL as a percentage of total SAM PL between black and beige mice. The number of experiments is indicated in parentheses. Values are mean ± 1 SD, $P < 0.005$.

TABLE 2. Disaturated phosphatidylcholines in lung and surface active materials

Animal	Lung		Surface-Active Materials			
	Homogenate		Residual Lung		Lavage	
	% PL	*mg/g lung*	*% PL*	*mg/g lung*	*% PL*	*mg/g lung*
Black	23.3 ± 1.3[a] (4)[b]	6.80 ± 0.18 (4)	48.5 ± 0.4 (4)	1.22 ± 0.72 (4)	52.9	0.30
Beige	29.8 ± 1.7 (4)	12.11 ± 1.33 (4)	49.4 ± 2.2 (6)	3.28 ± 1.26 (6)	52.1	0.24
P	<0.001	<0.001	>0.20	<0.025		

[a] Values are mean ± 1 SD.
[b] The number of experiments is indicated in parentheses. Materials from four experiments were pooled for analysis of lavage SAM.

lung (Table 2) thus is consistent with a marked increase in total lung SAM and with our previous morphologic evidence for abnormally enlarged lamellar bodies in type II alveolar epithelial cells in beige mice (8, 9).

There is an apparent discrepancy between the increase of DPC in whole lung and the recovery of DPC in SAM. The increase of DPC in SAM in beige mice was about 2 mg/g lung compared to about 5 mg/g lung in whole lung homogenates. This is attributable at least in part to experimental losses of SAM during the several steps of the isolation procedure. When SAM that had been labeled in vivo with [1-^{14}C]palmitic acid was added to homogenates of lungs and recovery was measured after the isolation of SAM, it ranged from 57 to 74%. Even though the isolation method does not permit a reliable quantitative estimate of total SAM, it does allow the recovery of material that can be analyzed qualitatively for phospholipid composition and surface activity.

Further support for the contention that the abnormality in beige mouse lung lipids is largely, if not exclusively, related to SAM storage is provided by the data indicating an increase in the proportion of total lung phospholipid provided by phosphatidylcholine and phosphatidylglycerol, the two major SAM phospholipid constituents (19–22) (Table 3).

The relative increase in surface area coverage by residual SAM from beige lungs was somewhat greater than threefold. On the basis of an estimated alveolar surface area in the mouse of about 3,000 cm² per gram of lung (23), we calculate, using the data in Table 4, that about 1.5 times as much SAM as is necessary to cover the alveolar surface with a monolayer at a surface tension of 12 dynes/cm was recovered from residual lung of black mice and about five times as much SAM as is necessary for alveolar coverage was recovered from residual lung of beige mice. These calculations provide minimal estimates of surface coverage in view of the experimental losses of SAM described above.

There does not appear to be any qualitative abnormality of SAM phospholipid in beige mouse lungs. The percentage of SAM PL that was DPC was similar, comprising 52% in the lavage fractions and 49% in the residual fractions from both groups of mice (Table 2). In residual SAM in both groups of mice, phosphatidylcholine accounted for about 75–80% of the phospholipid phosphorus. Phosphatidylglycerol was the next most abundant phospholipid, comprising about 10% of phospholipid phosphorus. That phos-

TABLE 3. Percentage distribution of phospholipid phosphorus in lung and surface-active materials

Phospholipid	Beige Lung		Black Lung	
	Homogenate	Residual SAM	Homogenate	Residual SAM
	(4)[a]	(6)	(4)	(3)
Sph	6.4 ± 0.5[b]	2.3 ± 1.3	7.2 ± 1.3	3.0 ± 1.2
PC	60.8 ± 2.0[c]	77.8 ± 2.4	55.1 ± 2.6	75.2 ± 2.3
PS + PI	8.4 ± 1.4[c]	4.1 ± 0.5	11.4 ± 1.0	5.4 ± 1.4
PE	16.1 ± 1.0[c]	3.0 ± 1.3	18.9 ± 1.4	2.4 ± 0.3
PG	6.2 ± 1.1[c]	9.1 ± 2.1	4.6 ± 0.5	10.6 ± 1.0
Unk[d]	2.0 ± 0.3	1.7 ± 0.7	2.5 ± 0.3	1.1 ± 0.1

[a] The number of experiments is indicated in parentheses. Each SAM was isolated from pooled lungs of five animals.
[b] Values are mean ± 1 SD.
[c] $P < 0.025$ vs. black.
[d] Unk, unknown phospholipid.

TABLE 4. Comparison of surface concentration and surface area coverage of residual SAM isolates

Animal	Surface Conc. Phosphorus	Surface Area Coverage
	μg/cm²	*cm²/gm lung*
Black	0.035 ± 0.006[a] (3)[b]	3,753 ± 593 (4)
Beige	0.039 ± 0.006 (3)	15,261 ± 3,106[c] (4)

[a] Values are mean ± 1 SD.
[b] The number of experiments is indicated in parentheses.
[c] $P < 0.001$ vs. black.

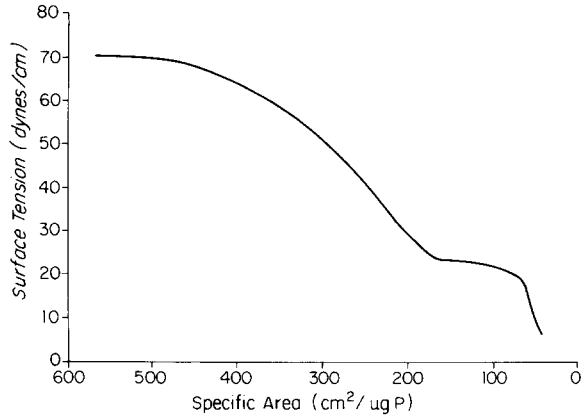

Fig. 2. Relationship of surface tension and specific surface area of residual SAM from beige mice. Measurements were made at 37°C during surface area reduction using a modified Wilhelmy balance.

phatidylglycerol is the second most common phospholipid in SAM has also been shown in lungs of dogs (21), rats, rabbits, monkeys, and humans (22). Surface concentrations of PL phosphorus at 12 dynes/cm were also essentially identical (Table 4). In addition, SAM from beige mice lowered surface tension to less than 10 dynes/cm at 37°C (Fig. 2). Thus, the materials meet at least two major criteria of a pulmonary surfactant. These are *1*) enrichment in DPC compared to whole lung, and *2*) ability to lower surface tension of an air–liquid interface to less than 10 dynes/cm at physiological temperature (18).

If the increased DPC and PL in beige mouse lung were simply the result of a defect in secretion of lamellar bodies by type II cells, then we would expect to find diminished intraalveolar PL and SAM. Our failure to observe such a deficit indicates a more complex situation; for instance, the increase in lamellar body mass could represent an increase in synthesis of SAM as effective compensation for a decrease in secretory efficiency. Measurements of synthesis and secretion of DPC are under way. Until results from these experiments are available, it seems unwarranted to speculate on the relationship of the large lamellar bodies to the defect in microtubule assembly in Chediak-Higashi syndrome described by Oliver and Zurier (24).

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