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Changes in Pulmonary Surfactant and Phosphatidylcholine Metabolism in Rats Exposed to Chrysotile Asbestos Dust

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1. Pulmonary surfactant was isolated from rats that had been exposed to chrysotile asbestos dust for from 3 days to 15 weeks. 2. Asbestos-treated rats showed a progressive increase in amounts of surfactant. After 15 weeks, treated animals contained 4 times as much as non-treated. 3. No significant change was seen in the total protein or total fatty acid composition of surfactant with exposure. 4. The increase in surfactant phosphatidylcholine normally seen on maturation of rat lung was accelerated by exposure of animals to asbestos. 5. An increase in the activity of phosphorylcholine glyceride transferase in lung homogenates and free cell populations was found. 6. Lysosomal phospholipase A was relatively unaffected by dust exposure. 7. It is suggested that the increase in surfactant amounts could be due to an increase in its synthesis without a corresponding alteration in its degradation.

The presence of a surface-active agent in pulmonary-oedema fluid was noted by Pattle & Thomas (1961). A similar material had also been isolated from whole-lung extracts (Clements *et al.*, 1958), and was later found to be a lipoprotein complex which lines the alveoli and, by decreasing surface tension, prevents lung collapse during respiration (Morgan, 1971; Pattle, 1965). Surface-active material which has been obtained by endobronchial lavage and subsequently purified consists of 80–90% lipid and 10–20% protein, together with a small amount of carbohydrate (Goerke, 1974; King, 1974; Harwood *et al.*, 1975). Although some of the protein is derived from plasma, evidence has been presented for the presence of at least one unique protein (King *et al.*, 1973; Sawada & Kashiwamata, 1977). Of the lipid components, dipalmitoyl phosphatidylcholine, which usually represents about half of the total surfactant, is generally considered to be the most important for developing low surface tensions (King & Clements, 1972*a*,*b*). Other lipid constituents and the unsaturated molecular species of phosphatidylcholine may help to speed the spreading of the surfactant layer within the alveolus (King, 1974). The newly identified component phosphatidylglycerol (Rooney *et al.*, 1974; Hallman & Gluck, 1975; Sanders & Longmore, 1975) has also been suggested to act as a modifier of surfactant function (Hallman & Gluck, 1976).

The exceptionally high concentration of saturated phosphatidylcholine in surfactant, together with convincing demonstrations of its importance in lowering surface tension (Morgan *et al.*, 1965; Watkins, 1968), have led to considerable research on its metabolism in lung. Although the methylation pathway for synthesis from phosphatidylethanolamine has been found (Morgan, 1969; Motoyama & Rooney, 1974), it is generally agreed that the route involving CDP-choline is by far the more important (Bjornstad & Bremer, 1966; Goerke, 1974; Spitzer *et al.*, 1968, 1969; Tierney, 1974). The 'turnover' of lipids in surfactant has been studied by using radiolabelled palmitic acid, and values for the half-life of 12–14h have been published (Tierney *et al.*, 1967; Thomas & Rhoades, 1970). It is perhaps unfortunate that this fatty acid was chosen, since experiments on labelling of molecular species of phosphatidylcholine have revealed a very rapid acyl exchange of palmitate (Akino *et al.*, 1971; Vereykin *et al.*, 1972), possibly catalysed by a microsomal acyltransferase (Frosolono *et al.*, 1971). However, labelling of phosphatidylcholine from [^{3}H]glucose (Tierney *et al.*, 1967) also indicates a short (14–20h) half-life and, in addition, that the glycerol and fatty acid moieties turn over at similar rates. Although the mechanism of surfactant removal from the alveoli has not been elucidated, one obvious possibility is hydrolysis of the phospholipid components by phospholipase A, which is present in lung. The alveolar macrophages, which have a very efficient lysosomal apparatus and move in an environment rich in surfactant (Tetley *et al.*, 1976), may also be involved in degradation of the material.

Pulmonary surfactant is probably synthesized by alveolar type-II cells (Chevalier & Collett, 1972; Massaro & Massaro, 1972; Meyrick & Reid, 1973; Goerke, 1974; Snyder & Malone, 1975; Douglas & Teel, 1976), which contain lamellar bodies (Page-Roberts, 1972). Extensive studies (e.g. by Frosolono *et al.*, 1970; Gil & Reiss, 1973; Rooney

et al., 1975) have shown that the composition and properties of lamellar-body material resembles, but is not identical with, surfactant isolated by endobronchial lavage. Phospholipid synthesis has been studied by using lamellar bodies (Spitzer *et al.*, 1975), and it is likely that material from this source forms most of the pulmonary surfactant secreted into the alveoli.

Only recently has the vital role of pulmonary surfactant in normal and abnormal lung function been recognized. Notably, its deficiency is the cause of acute-respiratory-distress syndrome (Gluck *et al.*, 1972), and the huge increase in lung lipids after exposure of animals to silica dust (Heppleston *et al.*, 1974; Marks & Marasas, 1960) or in human alveolar proteinosis (Dobson & Karlish, 1975; Ramirez-R & Harlan, 1968; Sahu *et al.*, 1976) is probably due to surfactant accumulation. The herbicide paraquat also apparently causes changes in alveolar surfactant, in this case a decrease, which results in a loss of alveolar stability (Fletcher & Wyatt, 1972; Fisher *et al.*, 1974). Tetley *et al.* (1976) showed that asbestos dust, like silica, would also give rise to a considerable increase in amounts of pulmonary surfactant. This effect has been further investigated and the results are now reported.

Materials and Methods

Sphingomyelin, phosphatidylcholine and fatty acid standards were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Lipid standards were homogeneous by t.l.c., and fatty acids were homogeneous by g.l.c. Phosphatidylinositol, purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K., was purified by t.l.c. Bovine serum albumin (fraction V) and Tween 20 were obtained from Sigma.

CDP-[*Me*-^{14}C]choline (60mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [^{14}C]Phosphatidylcholine was isolated from soya beans which had been germinated in [1-^{14}C]acetate (58mCi/mmol) by the method of Harwood (1975). All other reagents were of highest available grades and were purchased from BDH Chemicals, Poole, Dorset, U.K., from Boehringer, London W.5, U.K., or from Sigma.

Animals

Male specific-pathogen-free rats (Wistar strain; 7–9 weeks old) were divided into two groups which were matched for age and weight. Animals in the control group were given water and food *ad lib.*, and animals in the treated group, in addition, inhaled Rhodesian chrysotile asbestos [International Union against Cancer (Timbrell *et al.*, 1968) standard reference sample]. The asbestos was given at a dose of 12.5mg/m^3 for 7h per day, 5 days per week, as previously described (Timbrell *et al.*, 1970; Tetley *et al.*, 1976). The rats and inhalation-chamber facilities were kindly provided by the M.R.C. Pneumoconiosis Unit, Llandough Hospital, Penarth, Wales, U.K. After periods of 3 days to 15 weeks the animals were killed by CO_2 asphyxiation and their lungs immediately removed as previously detailed (Tetley *et al.*, 1976).

Isolation of lung free cell population and surfactant

A lung lavage technique using iso-osmotic NaCl as developed by Myrvik *et al.* (1961) and modified by Tetley *et al.* (1976) was performed on the isolated rat lungs. The washings (ten) from each animal were pooled and the free cell number was determined by using a Neubaur haemocytometer. Pooled washings from individual animals within each group were then combined and the lung free cells and surfactant purified as detailed previously (Harwood *et al.*, 1975; Tetley *et al.*, 1976).

Enzyme analyses

Whole lung material was homogenized in iso-osmotic KCl (100mg wet wt./ml) for 2min in an Omni-Mix apparatus, and then sonicated at 20kHz for a total period of 2min. The lung free cells were suspended at a concentration of approx. 5×10^6cells/ml in iso-osmotic KCl and similarly sonicated. All operations were performed at 4°C.

Phosphorylcholine glyceride transferase (EC 2.7.8.2) was assayed as described by Skurdal & Cornatzer (1975). The reaction was terminated by the addition of chloroform/methanol (1:2, v/v) and the lipids were extracted by the method of Garbus *et al.* (1963). A sample of the lower phase was counted for radioactivity in PCS scintillant (The Radiochemical Centre). Control experiments showed that phosphatidylcholine was quantitatively extracted and was the only radiolabelled compound in the lower phase.

Lysosomal phospholipase A activity was measured by the method of Mellors & Tappel (1967). Soya-bean [^{14}C]phosphatidylcholine was used as substrate and dispersed by sonication (20kHz for a total of 2min at 4°C) in 0.25M-sucrose/0.1M-Tris/HCl (pH4.6)/5% foetal bovine serum. After incubation, lipids were extracted as described above and separated by t.l.c. on silica-gel G (E. Merck, Darmstadt, Germany) plates in chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Lipid bands were revealed by exposure to I_2 vapour, and bands corresponding to unesterified fatty acids and phosphatidylcholine were removed and radioactivity was determined as previously described (Harwood, 1975).

Chemical determinations

Total and individual fatty acids, total phosphorus, phospholipid phosphorus and protein were deter-

Table 1. *Pulmonary-surfactant content and free cell population of rats exposed to chrysotile asbestos*
Results are expressed as average free cell population ($\times 10^{-6}$)/animal and as mg dry wt. of surfactant/animal. The numbers of animals from which material was pooled are given in parentheses. For methods of isolation and estimation see the Materials and Methods section. Control experiments indicate an average error of about 10% for surfactant weight estimations. Means ± S.D. for free cell population are shown.

	Free cell population		Surfactant	
Experimental period	Untreated rats	Treated rats	Untreated rats	Treated rats
3 days	10.6±3.4	9.2±2.5	1.1 (4)	1.7 (3)
3 weeks	14.9±5.6	9.4±3.0	2.0 (4)	4.2 (3)
6 weeks	16.6±6.5	20.7±3.9	2.1 (4)	4.2 (3)
9 weeks	11.0±1.5	19.8±5.0	1.3 (3)	7.7 (3)
15 weeks	18.6±3.2	31.4±7.7	1.5 (4)	6.5 (3)

Table 2. *Phosphorus content of pulmonary surfactant*
For details see the text. Means ± S.D. are given (three experiments). Results are expressed as % (w/w). Significance was estimated by Student's *t* test: n.s., not significant ($P > 0.1$).

Experimental period	Untreated rats	Treated rats	Significance (P)
3 days	1.93±0.20	2.99±0.06	<0.01
3 weeks	1.59±0.01	3.53±0.27	<0.005
6 weeks	4.07±0.20	4.58±0.30	n.s.
9 weeks	3.73±0.25	4.12±0.25	n.s.
12 weeks	4.10±0.16	4.92±0.68	n.s.
15 weeks	3.26±0.10	3.19±0.07	n.s.

mined as described previously (Harwood *et al.*, 1975). For each time-interval, determinations were performed on the pooled material from at least three animals.

Results

The problems that are associated with the isolation and characterization of surface-active material from lungs have been discussed at some length by previous workers (Goerke, 1974; King, 1974; Harwood *et al.*, 1975). During the present study every precaution was taken to prepare material conforming to criteria outlined by Harwood *et al.* (1975). Care was also taken to ensure that control and treated samples were isolated in an identical manner.

Previous work (Tetley *et al.*, 1976) had indicated that rats that had inhaled asbestos dust showed a considerable increase in amounts of pulmonary surfactant. In Table 1 the changes in pulmonary surfactant during exposure of animals to chrysotile asbestos for different periods are shown. After only 3 weeks of exposure the treated rats contained twice as much surfactant as untreated controls. This difference became more marked with time, so that a 4-fold increase was seen after 9–15 weeks exposure. The amounts of pulmonary surfactant in control rats throughout the experimental period were in the normal range previously found (Harwood *et al.*, 1975). After 9 weeks of exposure to dust, the animals also showed a significant increase in the free cell population obtained by lavage (Table 1).

After finding such a marked increase in total surfactant content after exposure to chrysotile asbestos, we decided to test whether any qualitative changes had occurred in the material derived from asbestos-treated animals. Accordingly, the surfactant was analysed with respect to its protein and lipid components. No significant differences were found in total protein content either between treated and control animals or with age. Protein was in the range 11–12%, which agrees with previously published values (cf. Harwood *et al.*, 1975; King, 1974; King & Clements, 1972*a*). Phosphorus analysis was carried out as a routine on whole surfactant, since control experiments had indicated that the contribution of non-lipid material was negligible. As Table 2 shows, an increase in phosphorus occurred with age in the untreated group of rats. Moreover, the treated group had phosphorus values that were significantly different from the control groups after 3 days and 3 weeks of exposure. The phosphorus contents of asbestos-exposed rats at these times was similar to that of the older control animals.

Surfactant samples from both treated and control groups at 3 weeks were analysed further. The higher content of phosphorus in the treated animals could be entirely accounted for by the increase in phosphatidylcholine content. This represented 59% of the total surfactant, a value comparable with that in normal adult rats (Harwood *et al.*, 1975).

In contrast with the changes in phosphorus content, there were no extensive alterations in fatty acid composition of the total surfactant (Table 3) or in the fatty acid pattern of individual phospholipids (T. D. Tetley, R. J. Richards & J. L. Harwood, unpublished work). The very high content of hexadecanoic acid, characteristic of pulmonary surfactant,

Table 3. *Fatty acid composition of isolated pulmonary surfactant*

Results are expressed as means are corrected to one decimal place. Additional experiments showed that $C_{16:1}$ was palmitoleic acid, $C_{18:1}$ was oleic acid and $C_{18:2}$ was linoleic acid.

Experimental period	Animal group	No. of experiments	Fatty acid (% of total)					
			$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
3 days	Untreated	2	2.2	71.7	8.0	4.4	7.7	6.5
	Treated	2	2.2	69.4	8.8	5.1	7.7	7.0
3 weeks	Untreated	3	2.1	71.6	9.0	4.6	7.8	5.0
	Treated	3	1.7	71.8	10.5	2.8	8.9	4.4
6 weeks	Untreated	2	3.2	68.1	11.5	4.3	8.2	4.8
	Treated	2	2.9	70.3	11.1	2.8	8.1	5.4
9 weeks	Untreated	2	1.7	68.4	10.6	7.1	8.7	3.6
	Treated	2	1.4	67.7	10.0	3.7	11.9	5.2
12 weeks	Untreated	3	1.0	69.5	10.0	5.2	9.8	4.4
	Treated	3	1.6	70.5	8.2	5.2	10.7	3.7
15 weeks	Untreated	3	1.2	73.2	9.0	4.9	8.4	3.3
	Treated	2	2.1	73.7	6.9	4.2	10.7	2.3

is shown in Table 3. Surface-tension studies that were carried out on the same pulmonary-surfactant samples (M. McDermott & M. H. Clay, unpublished work) showed that all produced minimum surface tensions of less than 0.01 $N \cdot m^{-1}$ at 20°C.

It has been pointed out by several workers that the high anenoic phosphatidylcholine content of pulmonary surfactant is vital for lung compliance and normal function (cf. Goerke, 1974). Accordingly, attention was focused on changes in enzyme activities in dust-treated animals that could account for the increases in surfactant reported above. The final enzyme of the CDP-base pathway, phosphorylcholine glyceride transferase, was measured, since this is normally thought to be rate-limiting for the pathway. Degradation of phosphatidylcholine takes place principally by phospholipase A action, and the lysosomal enzyme was also measured. The two enzymes were measured in whole lung homogenates as well as in the free cell population. Since the protein content in the free cell population can vary with age or treatment of rats, enzyme activity in this case is expressed in terms of cell number rather than protein.

Large increases in phosphorylcholine glyceride transferase occurred on exposure of animals to chrysotile asbestos both within the free cell population and for the whole-lung homogenate (Figs. 1 and 2). The greatest differences during the experimental period were seen after 15 weeks of treatment, when the free cell phosphorylcholine glyceride transferase showed a 7-fold increase over the control values. This could be due to a change in the nature of the treated free cell population, such as an increase in the proportion of type-II cells or, alternatively, to a general increase in membrane turnover. The increase in phosphorylcholine glyceride transferase activity in lung homogenates may be also partly due to proliferation of alveolar type-II cells, which are responsible for surfactant synthesis. Indeed, electron-microscopic examination of lungs from asbestos-treated rats suggests an increase in the number of type-II cells (McDermott *et al.*, 1977).

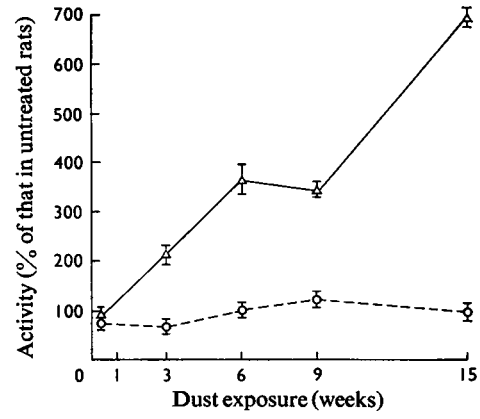

Fig. 1. *Phosphorylcholine glyceride transferase* (△) *and lysosomal phospholipase A* (○) *activities in pulmonary free cell populations after asbestos exposure*

The values for treated animals are given as a percentage of the values for untreated rats in each case. Means ± S.E.M. ($n = 2$) are shown. Untreated rats gave values of 3.1–5.4 nmol of phosphorylcholine incorporated into phosphatidylcholine/h per 10^6 cells for the transferase and 5.7–9.2 nmol of fatty acids released/h per 10^6 cells for phospholipase A.

In contrast with the increase in potential synthesis of phosphatidylcholine as represented by phosphorylcholine glyceride transferase, phospholipase A activity showed no consistent increase (Figs. 1 and 2). Furthermore its values were frequently lower in treated than in untreated rats.

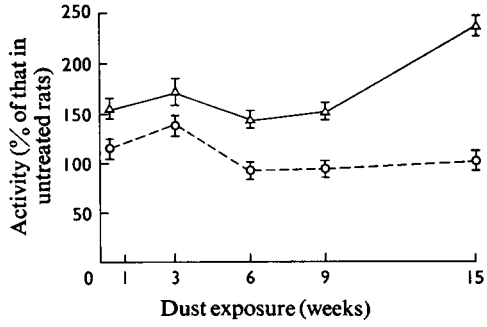

Fig. 2. *Phosphorylcholine glyceride transferase* (△) *and lysosomal phospholipase A* (○) *activity in lung homogenates after asbestos exposure*

Untreated rats gave values of 5.1–13.3nmol of phosphorylcholine incorporated into phosphatidylcholine/h per mg of protein for the transferase and 7.9–16.5nmol of fatty acids released/h per mg of protein for phospholipase A. For explanation see the legend to Fig. 1.

Discussion

The considerable increase in amounts of pulmonary surfactant in rats exposed to chrysotile asbestos, which was first reported by Tetley *et al.* (1976), has now been confirmed. It is noteworthy that these changes are significant after only 3 weeks' exposure. An increase in lung lipid has also been observed after silica-dust treatment (Marks & Marasas, 1960; Heppleston *et al.*, 1974), and their data indicate that such changes are due to alterations in amounts of surfactant. Indeed, it has been suggested that proliferation of alveolar type-II cells, which are presumed to be responsible for surfactant synthesis (Goerke, 1974; Snyder & Malone, 1975; Douglas & Teel, 1976) and which increase after asbestos exposure (McDermott *et al.*, 1977), may be a common response to toxic lung injury (Witschi, 1976).

Examination of a number of relevant criteria of the isolated surfactant showed that the material from asbestos-treated animals was basically similar to that from control rats with respect to total protein, phosphorus and fatty acid pattern (Tables 2 and 3). The only difference observed was that the phospholipid composition of treated rat surfactant at 3 days and 3 weeks resembled that of the more-mature control animals. Since the animals were relatively young at the start of the experiment (7–9 weeks), the effect of dust exposure appears to increase the maturation of lung tissue. Data from several groups of workers on lipid composition of lung or lung exudates from a number of species have emphasized the changes in phospholipid composition, and particularly increases in phosphatidylcholine content with age (Brumley, 1971; Hallman *et al.*, 1976; Jiminez *et al.*, 1975; Motoyama *et al.*, 1976; Tordet & Marin, 1976). Moreover, these results agree with the observations by Richards *et al.* (1974) that chrysotile asbestos promotes an aging effect or early maturation of lung cells in culture.

Our data therefore indicate a large quantitative rather than qualitative alteration in lung surfactant. However, an exhaustive analysis of individual proteins or fatty acid composition of all the acyl-lipids at each stage was not undertaken; therefore some very minor changes in the surfactant cannot be excluded. However, since the surfactant isolated from the lavage of treated animals also lowered surface tension in a similar manner to that from control animals (M. McDermott & M. H. Clay, unpublished work), any alterations in lipid compositions are probably of minor importance in comparison with the huge increase in material within the alveolar space. The condition thus induced in the rats by asbestos inhalation is reminiscent of the human complaint alveolar (lipo-)proteinosis (Dobson & Karlish, 1975; Sahu *et al.*, 1976).

The metabolism of pulmonary surfactant is normally under strict metabolic control (cf. Goerke, 1974; King, 1974; Tierney, 1974). It has been suggested that exposure to silica dust alters the rates of dipalmitoyl phosphatidylcholine synthesis and degradation (Heppleston *et al.*, 1974). In lung, the CDP-base pathway is the most important source of phosphatidylcholine (see the introduction), and phosphorylcholine glyceride transferase is believed to be the rate-limiting stage for this pathway. Measurement of its activity (Figs. 1 and 2) showed significant increases in whole-lung homogenates and free cell preparations. In the absence of a corresponding increase in catabolism of phosphatidylcholine, and assuming an adequate supply of precursors for synthesis, this would lead to an increase in phosphatidylcholine. Since the latter is the major component of surfactant, its synthesis may well be the rate-limiting stage under normal conditions. Although the route for disposal of pulmonary surfactant is not understood, lysosomal phospholipase A is known to be active in degrading phosphatidylcholine (Waite *et al.*, 1969). There is little evidence to indicate that macrophage cells (which form part of the free cell population) can degrade pulmonary surfactant, although this has been suggested by Naimark (1973). If these cells do degrade surfactant, and lysosomal phospholipase A is important in this process, then it is perhaps surprising that the activity of this enzyme does not increase after the increase in amount of surfactant in asbestos-treated rats. It may also be argued that a delay in the catabolism of surfactant results from an impairment in the rate of ingestion of the material by alveolar cells (including macrophages) that have been damaged by asbestos. Thus from the results

presented it seems highly likely that surfactant synthesis is promoted in asbestos-treated rats and that the material accumulates owing to a failure to stimulate correspondingly degradation or clearance from the alveolar space.

One intriguing question that this work raises is why such a large response in amount of surfactant to dust exposure takes place at all? Asbestos and silica fibres have been shown to be highly cytotoxic to cells *in vitro* (Allison *et al.*, 1966; Harington *et al.*, 1973; Richards & Jacoby, 1976), and the action of these mineral particles *in vivo* is also well documented (Wagner *et al.*, 1973, 1974). It can be suggested that the increase in pulmonary surfactant represents an attempt by animals to neutralize the effect of inhaled dust. For example, experiments *in vitro* have shown that pulmonary surfactant is strongly bound on to the surface of mineral particles (Richards *et al.*, 1977), and it is the most effective material yet examined in preventing chrysotile-induced lysis of cells (Desai *et al.*, 1975). Alternatively, the increase in surfactant may help in physical movement of the dust particles out of the alveoli, a role that has been previously suggested (Scarpelli, 1968) for this material.

Future studies should attempt to answer these questions and also the way in which foreign particles, such as asbestos, can alter the activities of pulmonary enzymes and lipids.

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