Studies on the Fate of Pulmonary Surfactant in the Lung

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1. Radioactively labelled pulmonary surfactant was prepared in an isolated perfused lung system provided with [^{14}C]hexadecanoate. 2. After intratracheal administration of pulmonary surfactant radioactively labelled components were rapidly distributed into different lung fractions, including macrophages (free cells), but most of the radioactive label was accumulated by the lung tissue. 3. Alveolar macrophages, maintained in a variety of culture media in the presence and absence of mineral particles, incorporated a low percentage (11%) of radioactively labelled components when incubated with the surfactant, although evolution of labelled CO_2 (6% of the original total activity) suggested that some breakdown of the components had taken place. 4. In similar cultures little intracellular accumulation or extracellular release of non-esterified fatty acids was demonstrated, indicating minimal catabolism of the high-molecular-weight lipid components of surfactant (particularly phosphatidylcholine). 5. However, experiments *in vitro* designed to simulate the lysosomal degradation of endocytosed surfactant indicated that the macrophage had enzymes capable of releasing non-esterified fatty acids, particularly hexadecanoate, from the lipoprotein complex. 6. It is argued that lung cells, other than alveolar macrophages, may also have a role in surfactant turnover.

Pulmonary surfactant, a complex lipoprotein material (King, 1974; Harwood *et al.*, 1975), is adsorbed at the alveolar air/liquid interface and assists in maintaining alveolar stability. The surface-active properties of surfactant are attributed to its high phospholipid content, particularly dipalmitoyl phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), and some specific protein components are considered to have an important role in the organization and rate of secretion of the complex (Goerke, 1974; King, 1974). The importance of surfactant in maintaining alveolar stability is demonstrated in the infant disease respiratory distress syndrome (Gluck *et al.*, 1972), although the material is also considered vital in many other aspects of pulmonary function (Scarpelli, 1968).

Recent research has shown that the amount of surfactant may be influenced by a variety of environmental factors. Inhalation of asbestos dust by rats induces up to 11-fold increases in the amounts of pulmonary surfactant (Tetley *et al.*, 1976), indicating a similar lung response to toxic particles to that observed by Heppleston *et al.* (1974), who found a 53-fold increase in lung dipalmitoyl phosphatidylcholine in rats inhaling quartz. These effects appear analogous to the pathological condition of human alveolar (lipo)proteinosis (Ramirez-R & Harlan, 1968). Similarly, a variety of pharmacological agents produce an elevation in the amount of lung lipids and pulmonary surfactant (Lullman *et al.*, 1975;

Brody *et al.*, 1975; R. J. Richards, J. L. Harwood & M. McDermott, unpublished work).

Attempts have been made to rationalize these results on the basis of altered rates of surfactant synthesis or degradation. Heppleston *et al.* (1974) have suggested that in rats inhaling quartz the synthesis is increased but catabolism is retarded, and Tetley *et al.* (1977a) have suggested that asbestos inhalation leads to an elevated synthesis of surfactant, owing to a numerical increase in the number of Type II epithelial cells (McDermott *et al.*, 1977).

It is generally accepted that the Type II epithelial cell represents the site of surfactant synthesis (Mecklin, 1954; Buckingham *et al.*, 1964; Adamson & Bowden, 1973; Goerke, 1974). Naimark (1973) has suggested that alveolar macrophages are responsible for degradation of the lipoprotein complex.

As part of the long-term aim in understanding the metabolism of pulmonary surfactant, the current study investigates the uptake and metabolism of surfactant components by alveolar macrophages or free lung cells (see Myrvik *et al.*, 1961; Brain, 1970) both *in vivo* and *in vitro*. For this study radioactively labelled pulmonary surfactant was prepared by means of a lung-perfusion technique. The alveolar macrophage has an important role in the clearance of mineral particles from the lung. In addition, Desai (1977) has shown that particulate matter rapidly absorbs components of pulmonary surfactant and that this reaction probably occurs in advance of

cellular phagocytosis in the lung, thus providing a protective mechanism by decreasing dust toxicity (Desai *et al.*, 1975). Thus the reaction of cultured alveolar macrophages incubated with asbestos (chrysotile) and quartz that had been pretreated with radioactively labelled surfactant was also assessed. Finally, the ability of a preparation of hydrolytic enzymes, derived from isolated alveolar macrophages, to degrade pulmonary surfactant in the presence and absence of asbestos was investigated.

Materials and Methods

Chemicals

Fatty acid and phosphatidylcholine standards were purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Silica gel G was purchased from E. Merck, Darmstadt, Germany. All other chemicals were of the highest available grade. L-[4,5-³H]Leucine and [1-¹⁴C]hexadecanoic acid were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. PCS (Phase-Combining System) scintillator and PPO (2,5-diphenyloxazole) were purchased from Hopkin and Williams, Romford, Essex, U.K., and dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] was obtained from Fisons, Loughborough, Leics., U.K.

Media and animals

Foetal bovine serum was purchased from Flow Laboratories, Irvine, Scotland, U.K., and minimum essential medium was from Gibco-Biocult, Paisley, Scotland, U.K. Mature male Dutch rabbits were used in most experiments. Sheep lungs were obtained directly from the slaughterhouse with the co-operation of Mr. W. Lewis and used with minimum delay after death.

Mineral dusts

U.I.C.C. standard reference Rhodesian Chrysotile A asbestos was obtained from the M.R.C. Pneumoconiosis Unit, Llandough, S. Wales, U.K., and Brazilian silica was kindly supplied by Dr. F. Pooley, University College, Cardiff.

Preparation of radioactively labelled pulmonary surfactant by using the isolated perfused lung

A rabbit liver was perfused for 90 min with homologous heparinized (0.02%, w/v) blood, as described by Curtis *et al.* (1970); 250 μ Ci of [1-¹⁴C]hexadecanoic acid (specific radioactivity 56 mCi/mmol) in 2 ml of 5% (v/v) ethanol was added to the perfusate (150 ml) after removal of the isolated liver and 8 min before the attachment of the isolated lung to the perfusion apparatus. A rabbit was anaesthetized with ether and

the trachea cannulated. The jugular vein was then cannulated for the administration of Nembutal (60 mg/kg body wt.). The thoracic cavity was opened and the tracheal cannula connected to a respiratory pump. Two ligatures were placed loosely around the pulmonary artery, which was cannulated with a glass cannula, and through this homologous heparinized blood at 37°C was slowly passed. The blood applied through the cannula was allowed to drain out through the heart, by opening the wall of the left auricle. The isolated lung was then removed, attached to the perfusion apparatus and perfused with homologous heparinized blood at 37°C. The rate of flow of perfusate was recorded and blood samples (0.2 ml) were taken at regular intervals (up to 3 h) to determine the radioactivity remaining in the perfusate (see Table 1). After perfusion the lung was infused with heparinized 0.15 M-NaCl via the pulmonary artery to remove blood from the vascular bed. Lung lavage (10 washes) was carried out with 50 ml volumes of iso-osmotic saline, and pulmonary surfactant was isolated as described previously (Desai *et al.*, 1975).

Determination of radioactivity in perfusate and lung fractions

Samples (maximum volume 500 μ l) or portions of tissue (maximum weight 500 mg) were burnt in a model 1N 4101 tissue combustion apparatus (Inter-technique, Portslade, Sussex, U.K.) and the resulting CO₂ was trapped in an ethanolamine-based scintillator (1600 ml of toluene, 1800 ml of methanol, 600 ml of ethanolamine, 37.5 g of PPO and 2.7 g of dimethyl-POPOP) and counted for radioactivity in a Packard Tri-Carb model 3375 liquid-scintillation spectrometer.

Table 1. *Distribution of radioactivity after a 3 h lung perfusion with [1-¹⁴C]hexadecanoate*

Originally 1.18×10^6 c.p.m./ml of blood perfusate (total volume 150 ml) was present, which fell to 0.93×10^6 c.p.m./ml after 10 min. At the start of the experiment the perfusate flow rate through the lung was 50 ml/min, and fell to 19 ml/min after 3 h. For experimental details see the Materials and Methods section.

Lung fraction	$10^{-6} \times$ Radioactivity (c.p.m.)	Percentage of total radioactivity incorporated into lung
Lung tissue (after lavage)	46.90	94.2
Total lung lavage	2.80	5.8
Free lung cells (macrophages)	0.47	0.9
Pulmonary surfactant	1.12	2.3

Lipid extraction, separation and determination of radioactivity

Lipids were extracted from the different lung fractions, cultured macrophage cells and pulmonary surfactant by the method of Garbus *et al.* (1963) and separated by t.l.c. on silica gel G plates in chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). The lipid bands were revealed by I₂ vapour and the phospholipids were identified by co-chromatography with standards. Individual phospholipid and non-esterified fatty acid bands were scraped from dry plates into glass scintillation vials, 10ml of PCS/xylene (2:1, v/v) scintillator was added, and the vials were left for 12h to allow the silica gel to settle before counting.

Distribution of radioactivity following incubation of [¹⁴C]hexadecanoate and ¹⁴C-labelled rabbit pulmonary surfactant with normal and mineral-treated alveolar macrophages in vitro

Rabbit alveolar macrophages were isolated by lung lavage with 0.15M-NaCl (Richards & Wusteman, 1974) and cultured at 37°C in Leighton tubes (1 × 10⁶ cells/tube) in 1ml of 0.15M-NaCl or minimal essential medium or minimal essential medium + 10% foetal bovine serum. Each incubation mixture contained 0.11 mg of ¹⁴C-labelled pulmonary surfactant (specific radioactivity 38 500 c.p.m./mg)/ml. The pulmonary surfactant was sterilized by passage through a 0.45 µm membrane filter and the distribution of radioactivity in the lipid fractions and the stability of this material in 0.15M-NaCl at 37°C over 6h is shown (Table 2). Brazilian silica and asbestos (Chrysotile A, U.I.C.C. sample) were first incubated for 60min at 37°C with the culture media containing radioactive surfactant at concentrations of 100 µg of mineral/ml before addition to the macrophage cells. Normal and mineral-treated cultures were

incubated for periods of up to 12h, and during this time an assessment of cell viability was recorded by the Eosin dye-exclusion test. At intervals, duplicate samples of normal and mineral-treated cultures were removed and the cells attached to the glass gently removed by a rubber 'policeman'. The macrophages were then separated from the culture medium by centrifugation (300g for 20min) and the lipid content of both fractions was extracted, separated and the radioactivity determined as described above. Further experiments were carried out with untreated alveolar macrophages to determine the distribution of label after incubation of the cells with [¹⁴C]hexadecanoate. In addition, after incubation of normal cells with 0.57mg of labelled pulmonary surfactant (specific radioactivity 10150 c.p.m./mg), the distribution of total radioactivity into CO₂ produced by the cultures was determined and the radioactivity associated with the cell surface and that accumulated intracellularly were monitored. Label associated with CO₂ was determined after removal of a small piece of filter paper, soaked in KOH, which was suspended above the culture medium on a long needle insert via the stopper of the sealed Leighton tube. After the removal of culture medium and the re-addition of 'floater macrophages' [pelleted by centrifugation (300g for 20min) of the medium] to the Leighton flask the cells were exposed to trypsin/EDTA [50 µg of trypsin/ml of 0.01% EDTA/phosphate-buffered saline (8g of NaCl, 0.2ml of KCl, 0.2g of KH₂PO₄, 0.15g of Na₂HPO₄ dissolved in 1 litre of water and adjusted to pH 7.4)] for a period of 20min, during which time cell viability (as assessed by Eosin dye-exclusion on complementary cultures) was unaffected. The material removed by trypsin treatment was separated by centrifugation (300g at 20min) and the amount of label in this fraction taken as that in the material associated with the cell surface. The amount of label in the remaining cell pellet from this procedure was assumed to be located intracellularly (Table 5).

Table 2. *Distribution of ¹⁴C label in the major lipid fractions of rabbit surfactant prepared by one single lung perfusion for use in some of the cellular studies in vitro*

Zero time is the point at which the material was added to the culture: 'time 6h' is a stability control after incubation for 6h alone at 37°C. All values are the means for three determinations.

Lipid component	Zero time radioactivity		Time 6h radioactivity	
	(c.p.m./mg of surfactant)	(% of total)	(c.p.m./mg of surfactant)	(% of total)
Sphingomyelin	240	0.6	430	1.1
Phosphatidylinositol	560	1.5	790	2.0
Phosphatidylcholine	34120	91.8	35440	90.3
Phosphatidylethanolamine	480	1.3	490	1.2
Non-esterified fatty acids	1780	4.8	2110	5.4

Table 3. *Distribution of label in rabbit lung tissue, free cells and pulmonary surfactant after intratracheal injection of ^{14}C -labelled pulmonary surfactant in vivo*

Results are expressed as percentages of detectable radioactivity. All values represent the means for three determinations.

Isolated fraction	Distribution of label at different time intervals after introduction into lungs (%)		
	1h	2h	8h
Pulmonary surfactant	28.7	28.0	28.0
Free cells (macrophages)	15.6	8.2	8.0
Lung tissue (after lavage)	32.1	40.2	51.7
Other lung lavage fractions	23.7	23.6	12.1
Recovery (% of original injected label)	57.5	55.2	49.5

Accumulation of radioactivity from ^3H leucine-labelled serum proteins by alveolar macrophages

Radioactively labelled rabbit serum proteins were prepared by sterile intravenous injection of 1.0mCi of L-[4,5- ^3H]leucine (specific radioactivity 1.0Ci/mmol) in 0.15M-NaCl. After 12h the rabbit was killed by air embolism, the blood drained, allowed to clot and the serum separated at 4°C. After centrifugation (1300g for 20min) the serum was dialysed for 24h at 4°C against several volumes and changes of 0.15M-NaCl. Rabbit alveolar macrophages (0.5×10^6 cells) were cultured at 37°C in 1 ml of minimum essential medium + 10% ^3H -labelled rabbit serum of specific radioactivity 2915c.p.m./mg of protein. Duplicate cultures were taken at intervals, attached cells removed from the glass surface as described above, and after centrifugation (300g for 20min) the cell-associated radioactivity and that in the culture medium were determined by using PCS scintillator.

Degradation of sheep surfactant in vitro by enzymes from a lysosome-rich fraction isolated from sheep alveolar macrophages

Sheep lungs were lavaged (four or five times) with 0.15M-NaCl and the free cell population (mostly macrophages) was pelleted by centrifugation at 300g for 20min. A lysosome-rich fraction was obtained from these cells by a procedure based on that described by de Duve *et al.* (1955). The lysosome fraction was suspended in 0.2M-sodium acetate buffer (pH 5.0), sonicated at 20kHz for 2min at 4°C and then sterilized by passage through a 0.45 μm membrane filter. Sheep pulmonary surfactant was prepared as described previously (Desai *et al.*, 1975) from the same animal and dialysed against 0.15M-NaCl. A sterile sample of chrysotile asbestos (20mg) was incubated with 4ml of sheep surfactant (1 mg/ml). Then 6ml of the lysosomal enzyme preparation was added to the

incubation mixture of surfactant plus chrysotile and also to a tube containing 4ml of surfactant (1mg/ml). Control incubations containing lysosomal enzyme preparation, surfactant alone or chrysotile (20mg) plus surfactant were treated identically. The reaction vessels were maintained for 22h at 35°C, undergoing continuous gentle shaking, and duplicate 1ml samples were removed at selected intervals. Lipids were extracted from the samples by the method of Garbus *et al.* (1963) and were separated by t.l.c. on silica gel G plates in light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (90:10:1, by vol.). The fatty acid components were analysed by g.l.c. after transesterification with BF_3 . An internal standard of pentadecanoate was added for quantification of results. Separation was achieved in 15% (w/w) diethylene glycol succinate on Chromosorb WAW (80–100 mesh; Supelco Inc., Bellefont, PA 16823, U.S.A.) by using isothermal programming at 180°C on a Perkins–Elmer F33 gas chromatogram.

Accumulation and distribution of radioactivity in lung fractions after intratracheal administration of ^{14}C -labelled pulmonary surfactant

Labelled pulmonary surfactant was prepared by lung perfusion by using [^{14}C]hexadecanoate as described above. The material had a specific radioactivity of 12406d.p.m./mg, and 73% of the incorporated label was found in phosphatidylcholine, 7.0% in phosphatidylinositol, 7.8% in sphingomyelin and 6.9% as non-esterified fatty acids.

Rabbits were anaesthetized by parenteral injections of sodium pentobarbitol (Nembutal), the jugular vein was cannulated for introduction of further anaesthetic, and a tracheotomy carried out. Approx. 5.6mg of labelled surfactant (see above) in 3ml of 0.15M-NaCl was introduced by cannula into the lungs (half in each bronchus) of each experimental animal, which was maintained under anaesthesia for periods of up to 8h. At intervals of 1, 2 and 8h animals were killed by exsanguination from the dorsal aorta and the lung tissue was perfused with 0.15M-NaCl to remove blood from the vascular bed. Pulmonary surfactant and other lung fractions (see Tetley *et al.*, 1977b) were isolated and the distribution of total radioactivity in the lipid components of the fractions was determined as described above.

Results

The distribution of radioactivity in major alveolar fractions after a 3h lung perfusion with [^{14}C]hexadecanoate is shown in Table 1. Most of the incorporated label (94%) was found in the lung tissue after lavage, and only a small percentage (2.3%) was associated with the pulmonary-surfactant fraction. Nevertheless, 9.3mg of surfactant was isolated

and contained 1.12×10^6 c.p.m. Further details on the biosynthesis of labelled surfactant are detailed elsewhere (Tetley *et al.*, 1977b). After dialysis, a sample of labelled surfactant thus prepared was analysed further to determine the distribution of the [^{14}C]-hexadecanoate in the major lipid fractions before use in experimental studies (Table 2). Most of the label (92%) was found in the phosphatidylcholine moiety and less than 5% was associated with non-esterified fatty acids. Incubation of this labelled surfactant alone at 37°C for 6 h did not induce any change in the distribution of radioactivity (Table 2).

The distribution of radioactivity in rabbit lung tissue, the free cells and pulmonary surfactant after the intratracheal injection of ^{14}C -labelled pulmonary surfactant is shown in Table 3. After 1 h only 30% of the total radioactivity was associated with the pulmonary surfactant, and this value was maintained throughout the experiment (Table 3). The lung tissue (after lavage) contained 32% of the label 1 h after injection, but this value increased to 52% within 8 h. The free cell population contained 16% of the label after 1 h, but this was lower after 8 h (8%). Similarly, other fractions obtained by lavage (see Tetley *et al.*, 1977b) showed a decrease in associated label with increase in time. Of the original label 57% was detectable in lung fractions 1 h after injection of the material (Table 3). This recovery decreased with time and may be accounted for in part by the distribution of label into CO_2 (see below, Table 5), blood and extrapulmonary tissues. In the same experiment the distribution of label in the major lipid fractions of pulmonary surfactant and the free cell population was determined (Table 4). At 2 h after injection only 48% of the original label was found in the phos-

phatidylcholine fraction of the pulmonary surfactant, whereas 73% of the label was associated with this phospholipid in the original lipoprotein material. There was a considerable increase in the proportion of label associated with the non-esterified fatty acids of the pulmonary surfactant, but no radioactivity was detectable in phosphatidylinositol, sphingomyelin or other lipid fractions of the pulmonary surfactant after its injection into the animals (Table 4). The distribution of label in the major lipid classes of the free cell population was somewhat different from that found in pulmonary surfactant. In the former, the label in the non-esterified fatty acid fraction remained relatively constant with time, whereas that associated with sphingomyelin was high after 1 h, but undetectable after 8 h, and there was a proportional increase with time in the radioactivity associated with the phosphatidylcholine fraction (Table 4).

When normal alveolar macrophages were exposed to ^{14}C -labelled pulmonary surfactant most of the label remained in the culture medium, but decreased over a 4 h period (Table 5). Only 13–16% of the label was associated with the cells, and of this 7–11% was located intracellularly, the remainder (trypsin-treated material) being associated with the cell surface. A small percentage of the label was found in CO_2 , and this increased over a period of 4 h. By contrast, when similar cultures were incubated with [^{14}C]hexadecanoate alone approx. 50% of the label was associated with the cells, 40% of which was intracellular, and a corresponding decrease occurred in the amount of label in the culture medium.

Subsequent experiments indicated that varying the composition of the culture medium (0.15M-NaCl alone; minimal essential medium; minimal essential

Table 4. Distribution of label in the lipid fractions of (a) pulmonary surfactant and (b) free lung cells after intratracheal injection of ^{14}C -labelled pulmonary surfactant *in vivo*

Percentage recovery is shown in Table 3. Abbreviation: n.d., no detectable activity.

Lipid fraction of	Distribution of label at different time intervals (%)			
	0 (original material)	1 h	2 h	8 h
(a) Pulmonary surfactant				
Phosphatidylcholine	73.0	64.9	48.1	55.2
Phosphatidylinositol	7.0	n.d.	n.d.	n.d.
Sphingomyelin	7.8	n.d.	n.d.	n.d.
Non-esterified fatty acids	6.9	35.1	51.9	44.8
Other	5.3	n.d.	n.d.	n.d.
(b) Free cells				
Phosphatidylcholine		48.2	64.0	74.5
Phosphatidylinositol		n.d.	n.d.	n.d.
Sphingomyelin		24.3	4.2	n.d.
Non-esterified fatty acids		27.6	31.7	25.5
Other		n.d.	n.d.	n.d.

Table 5. *Distribution of radioactivity from ^{14}C -labelled pulmonary surfactant and $[^{14}\text{C}]$ hexadecanoate after addition to normal rabbit alveolar macrophages maintained in vitro*

Compound administered	Time interval (h)	Percentage of total administered radioactivity associated with:				
		CO_2	Extracellular medium	Cells (no treatment)	Intracellular treatment*	Cell surface*
Labelled surfactant	1	1.0	85.5	13.5	7.1	6.4
	2	2.3	81.5	16.2	11.3	4.9
	3	3.2	82.2	14.5	9.7	4.8
	4	6.3	77.3	16.4	11.6	4.8
Free hexadecanoate	4	5.9	44.3	49.8	41.1	8.7

* For details see the text.

medium+10% foetal bovine serum) makes very little difference to the amount of label from pulmonary surfactant that was associated with the cells (maximum 10–12% of total label administered). In normal cultures treated with labelled pulmonary surfactant, the label associated with the cells reached a maximum after 4 h (Fig. 1*a*), an effect that was not so clearly marked when similar cultures were treated with ^3H -labelled serum proteins (Table 6). In the former cultures the association of labelled phospholipids

other than phosphatidylcholine with the macrophage cells was not measurable, and the amount of label of these phospholipids in the culture medium did not alter. The amount of cell-associated non-esterified fatty acids was also negligible, and there was little or no increase in the amount of ^{14}C -labelled non-esterified fatty acids in the culture medium with time (Fig. 1*a*). The presence of silica dust or chrysotile asbestos in the cultures had a negligible effect on the distribution of label derived from the pulmonary surfactant (Figs. 1*b* and 1*c*).

The ability of a lysosomal enzyme preparation derived from sheep alveolar macrophages to degrade sheep pulmonary surfactant was demonstrated by measuring the release of total non-esterified fatty acids, and specifically hexadecanoate, from the lipoprotein complex (Table 7). After incubation of surfactant or lysosomal enzyme preparation alone there was little detectable increase in the amounts of non-esterified fatty acid or hexadecanoate. When the surfactant was incubated with the lysosomal enzyme preparation there was a time-dependent increase in the amounts of non-esterified fatty acid and hexadecanoate detectable in the medium (Table 7). The presence of chrysotile asbestos did not interfere with this process.

Table 6. *Distribution of radioactivity from $[^3\text{H}]$ leucine-labelled serum proteins after addition to normal rabbit alveolar macrophages maintained in vitro*

Time interval (h)	Recovery of label (%)	Percentage of total recovered radioactivity associated with	
		Culture medium	Cells
0	98	97.2	2.8
1	98	90.6	9.4
3	97	89.4	10.6
5	93	86.8	13.2
7	96	86.3	13.7

Table 7. *Release of free hexadecanoate ($\text{C}_{16:0}$) and total non-esterified fatty acids (NEFA) from sheep surfactant incubated at pH5.0 and 35°C for 22 h with a lysosomal enzyme preparation from sheep free cells (alveolar macrophages)*
Asbestos sample is U.I.C.C. Chrysotile A. Values are means for 3 determinations.

Preparation mixture	Free fatty acid or hexadecanoate detected at different time intervals ($\mu\text{g/ml}$)							
	5 min		30 min		4 h		22 h	
	NEFA	$\text{C}_{16:0}$	NEFA	$\text{C}_{16:0}$	NEFA	$\text{C}_{16:0}$	NEFA	$\text{C}_{16:0}$
Lysosomal enzyme preparation+ surfactant	20.5	8.1	28.0	14.6	37.0	17.8	41.5	21.1
Lysosomal enzyme+asbestos +surfactant	22.3	9.0	31.2	14.2	36.2	19.0	40.0	21.4
Surfactant alone (or+asbestos)	8.0	2.5	8.3	2.3	8.5	2.6	8.5	3.0
Lysosomal enzyme preparation alone (or +asbestos)	9.6	4.5	9.7	4.6	12.0	5.0	11.7	5.2

Fig. 1. Accumulation of radioactively labelled components from ^{14}C -labelled rabbit pulmonary surfactant in minimum essential medium + 10% foetal bovine serum by (a) normal rabbit alveolar macrophages (10^6 cells) and cells treated with (b) silica (Brazilian, $100\mu\text{g}$) and (c) chrysotile asbestos (U.I.C.C. A, $100\mu\text{g}$)

Symbols: ○, cell-associated ^{14}C -labelled phosphatidylcholine; ●, extracellular ^{14}C -labelled phosphatidylcholine; ■, extracellular ^{14}C -labelled non-esterified fatty acids.

Discussion

The alveolar macrophage or free lung cell could be considered to be well equipped to degrade surfactant, as this cell exhibits active endocytosis, is found in a surfactant-rich environment and has a well-developed lysosomal enzyme complex for the catabolism of pinocytosed organic materials. Following the suggestion of Naimark (1973) that pulmonary surfactant is continually endocytosed and subse-

quently degraded by alveolar macrophages, a series of experiments was designed utilizing radioactively labelled and unlabelled surfactant to test this hypothesis.

The lung-perfusion technique proved to be suitable for obtaining radioactively labelled pulmonary surfactant for use in subsequent studies *in vitro* and *in vivo*. The distribution of the ^{14}C hexadecanoate into the major lipid fractions of the surfactant prepared by this procedure was not always identical, although in most instances most of the incorporated hexadecanoate label was associated with phosphatidylcholine (70–90%).

When radioactively labelled surfactant was injected intratracheally into rabbits there was a rapid distribution of the labelled components into a variety of alveolar and lung fractions. This suggests a rapid half-life for some of the components of surfactant. In addition, the amount of label associated with the phosphatidylcholine fraction of surfactant decreased, whereas the amount of label in the non-esterified fatty acid fraction increased with time, suggesting that the surfactant is degraded at the alveolar surface.

Macrophage cells incubated *in vitro* with labelled pulmonary surfactant accumulated 16% of the total radioactivity available within 4h, and of this only 11% (maximum) was 'intracellular', whereas 5% was associated with the cell surface. In contrast, when the macrophages were incubated with ^{14}C hexadecanoate the results suggested that the low-molecular-weight material was more readily accumulated by the cells than were the components of the complex lipoprotein material. Thus cultured alveolar macrophages have a limited capacity to accumulate components derived from pulmonary surfactant. This capacity was neither enhanced or suppressed if they were exposed to silica dust or chrysotile asbestos, although it might be expected that as a result of phagocytosis of the particles (which are known to be coated with pulmonary surfactant; Desai, 1977) a greater amount of label would be found in the cells. Also, it was surprising that after the uptake of components from the labelled surfactant there was only a slight increase in the concentration of non-esterified fatty acids in the cells or in the culture medium. Despite this apparent lack of evidence for surfactant degradation, other experiments *in vitro* demonstrated that a lysosomal enzyme preparation derived from isolated macrophages was capable of breaking down components of the lipoprotein complex. In this system, however, only 10% of the available surfactant was degraded to non-esterified fatty acids after 22h incubation, which may be accounted for by enzyme inactivation or alternatively by the accumulation of breakdown products. The apparent inconsistencies between the two studies *in vitro* are open to a number of interpretations. Arguably, macrophages may utilize the phosphatidylcholine component of sur-

factant directly or, after degradation of lipid components of surfactant, the resulting non-esterified fatty acids are immediately reutilized to synthesize phosphatidylcholine, which is an important component of macrophage membranes. Equally, it may be stated that both of the systems used *in vitro* are artificial, as the lysosomal study was designed to ensure maximum contact between surfactant substrate and hydrolytic enzymes and the cell-culture study could not reproduce the precise physiological conditions found *in vivo*.

From the present experiments and those described by Naimark (1973) it is difficult to assess whether or not the alveolar macrophage is the major cell responsible for surfactant degradation. Although lipid inclusions, possibly derived from surfactant, have been identified in normal alveolar macrophages (Nichols, 1976), the results presented here suggest that these cells do not contribute significantly to the digestion of labelled surfactant. Some 15% of the injected label from surfactant was associated with the free cell fraction after 1 h, and this closely agreed with the cell-culture study, which suggested that components of the lipoprotein complex are utilized by the macrophages. However, as previously suggested by Geiger *et al.* (1975), it is equally possible that alveolar cells (Type I and Type II) other than macrophages may also have a role in removing components of pulmonary surfactant and that any degradation products, particularly fatty acids, are then reutilized by both macrophages and other lung cells for energy purposes (finally releasing CO₂) and/or further phosphatidylcholine synthesis. Because of this rapid reutilization of [¹⁴C]hexadecanoate-labelled products or lipid components of pulmonary surfactant, further studies are necessary to determine the turnover rate of the lipoprotein complex.

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