A method for preparing radiolabelled rat pulmonary surfactant

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A method is described for the preparation of rat pulmonary surfactant, radiolabelled specifically in the phosphatidylcholine species, which may be used for degradative studies of the lipoprotein complex. Intravenously administered [*methyl*-¹⁴C]choline chloride is maximally incorporated into alveolar surface surfactant 8 h after injection, and more than 97% of this radiolabel is present in the phosphatidylcholine fraction of the surfactant and, of this, 75% is associated with the dipalmitoyl phosphatidylcholine species. Electron microscopy indicates that the isolated surfactant has a similar physical form to that found at the alveolar surface. The mineral α -quartz can be used to increase the yield of surfactant lavaged from the lung surface, but the complex isolated from rats treated in this manner has a low specific radioactivity (< 1000 d.p.m./mg) compared with that prepared from control animals (22860 d.p.m./mg).

INTRODUCTION

The lipoprotein complex known as pulmonary surfactant is primarily responsible for maintenance of low surface tension at the alveolar surface, but is suggested to have numerous other functions in the lung (Jarstrand, 1984; Richards & Curtis, 1984; Harwood & Richards, 1985). The material has been isolated from lung tissue by a variety of different methods (Harwood & Richards, 1985), and most preparations satisfy the basic criteria necessary for the complex to be designated as 'pulmonary surfactant'. Thus the isolated sample should be capable of producing stable low surface tensions, have a high lipid-to-protein ratio, and the lipid component should be predominantly phosphatidylcholine, existing mainly in the dipalmitoyl form.

The biosynthesis of pulmonary surfactant *in vivo* and by cultures of isolated type II cells has been well studied, but, to date, comparatively little information has been presented concerning the removal and/or degradation of the complex (Desai *et al.*, 1978; Hallman *et al.*, 1981; Jobe & Jacobs, 1984; Harwood & Richards, 1985; Richards & Lewis, 1985). In order to determine the exact route of surfactant degradation in both normal and disease states and to obtain quantitative data on its rate of turnover, a radiolabelled preparation of the lipoprotein complex would prove useful. A natural preparation of radiolabelled surfactant could have certain advantages in degradation studies over 'artificial' surfactant preparations consisting of radiolabelled phospholipid mixtures. Artificial mixtures may be difficult to prepare complexed with the proteins found in natural surfactant. These proteins may affect the turnover/reutilization of the lipoprotein complex (Benson *et al.*, 1984), and thus both the distribution and rates of removal of artificial preparations could provide erroneous results regarding surfactant degradation. In addition, artificial preparations may not represent the correct morphological form of surfactant as found at the alveolar surface.

Studies on the turnover of pulmonary surfactant have involved monitoring the removal of either radiolabelled synthetic phospholipid mixtures (Geiger *et al.*, 1975; Scarpelli *et al.*, 1975; Obladen *et al.*, 1979; Oyarzun *et al.*, 1980) or naturally derived surfactant from the alveolar

surface. However, examination of the studies involving naturally prepared surfactants shows that the natural product was altered in each case before intratracheal instillation: by freeze-drying (Hallman *et al.*, 1981), by mixing with amniotic fluid (Glatz *et al.*, 1982) or by combination with radiolabelled synthetic phospholipids (Jacobs *et al.*, 1983). To date, only one study (Desai *et al.*, 1978), has been carried out on the degradation of pulmonary surfactant using a naturally prepared unmodified radiolabelled lipoprotein complex. In this study, surfactant was isolated by a modification of the method of Abrams (1966) and chemically characterized as described by Harwood *et al.* (1975). The surfactant was prepared in the isolated perfused rabbit lung, utilizing [¹⁴C]hexadecanoate as the precursor molecule. This method had the advantage of yielding an isolated surfactant with relatively high specific radioactivity. However, lung perfusions are time-consuming and difficult to perform correctly and consistently, particularly for the rat. Furthermore, the use of [¹⁴C]palmitate as the precursor molecule ensures that all the acyl lipids will be radiolabelled. Consequently, in subsequent degradation studies of a palmitate-radiolabelled complex, some difficulties may arise in the interpretation of results if there is differential turnover of the phospholipid fractions or selective reutilization of the surfactant fatty acids.

The objective of the present study was to devise a simple procedure to prepare radiolabelled surfactant of high enough specific radioactivity such that it would be of use in subsequent degradative studies both *in vivo* and *in vitro*. The precursor molecule chosen was [*methyl*-¹⁴C]choline chloride, since this compound should label significantly only the major lipid component of surfactant, namely phosphatidylcholine. The animal model chosen was the rat, as this species has previously been employed in numerous surfactant studies and is used by several laboratories for the isolation of type II cells. The rat has one disadvantage in preparing bulk samples of radiolabelled surfactant in that only approx. 1.0 mg (dried weight) of the complex can be lavaged from the alveolar surface (Richards & Curtis, 1984). Attempts were therefore made to produce radiolabelled surfactant from rats which had previously been instilled with the mineral α -quartz. This mineral produces a rapid lipoproteinosis,

resulting in the accumulation of large amounts of surfactant at the lung surface (Richards & Curtis, 1984). Therefore both control and quartz-treated rats were given intravenous injections of [*methyl*- ^{14}C]choline chloride and alveolar surface surfactant isolated at various time intervals after precursor injection in an attempt to determine the time of maximal incorporation of radiolabel into the lipoprotein complex. The isolated material was chemically characterized, the distribution of radiolabel determined and the morphological form of the complex examined by electron microscopy.

MATERIALS AND METHODS

Phospholipid and fatty acid standards were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. [*methyl*- ^{14}C]Choline chloride (2.15 GBq/mmol) was obtained from Amersham International, Amersham, Bucks., U.K. Fluothane was supplied by ICI Ltd., Macclesfield, Cheshire, U.K. All other chemicals were of the highest grade available and purchased from BDH Chemicals, Poole, Dorset, U.K., or from Sigma. The DO_{12} quartz sample was kindly given by Dr. C. J. Hardy, Huntingdon Research Centre, Huntingdon, Cambs., U.K. Male specific-pathogen-free (COBS-CD) rats were purchased from Charles River Ltd., Manston, Kent, U.K. All the animals were housed on wire-bottomed cages and allowed food (Pilsburys Small Animals Diet; Pilsburys Ltd., Birmingham, U.K.) and water *ad libitum*.

Instillation procedure and administration of radiolabel

Quartz samples (5 mg), sterilized by heating to 140 °C for 2 h, were suspended in 0.5 ml of sterile 0.15 M-NaCl and sonicated before instillation. Animals were anaesthetized with fluothane and the dust instilled via the trachea as previously described (Richards *et al.*, 1981). To prepare radiolabelled surfactant, 0.5 ml of sterile 0.15 M-NaCl containing the [*methyl*- ^{14}C]choline chloride (usually 5–10 μCi) was injected into anaesthetized animals via the penal vein. Pulmonary surfactant was then isolated at various time intervals (up to 24 h) after injection of radiolabel.

Lavage technique and pulmonary-surfactant isolation

Animals were given a lethal dose of nembutal anaesthetic, the thorax opened and the trachea clamped to prevent entry of blood into the lungs. Residual blood was cleared from the pulmonary circulation by cardiac perfusion with 0.15 M-NaCl. The lungs were removed, the trachea cannulated and lavaged with 6 $\times$ 10 ml aliquots of 0.15 M-NaCl. Pulmonary surfactant was then isolated as described previously (Abrams, 1966; Harwood *et al.*, 1975). Briefly, lung lavage fluid was centrifuged (300 g, 20 min) to remove the free cell population, which was resuspended and washed in 10 ml of 0.15 M-NaCl and re-centrifuged under the same conditions. The cell-wash supernatant was pooled with the primary supernatant and centrifuged (1000 g, 60 min). The resulting pellet was suspended in 10 ml of 4 M-NaCl and centrifuged at 1500 g for 25 min. This resulted in a three-phase separation, the upper floating pellicle being designated 'pulmonary surfactant'. All fractionation procedures were carried out at 4 °C, and the isolated surfactant exhaustively dialysed against distilled water for 48 h.

Chemical determinations

Lipids were extracted from surfactant samples by the method of Garbus *et al.* (1963) and the phospholipids separated by t.l.c. on silica-gel G plates in chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Lipids were detected by spraying the plates with 8-anilino-4-naphthalene-2-sulphonic acid in methanol (0.2%, w/v) and the spots revealed under u.v. light. The dipalmitoyl species was separated from other phosphatidylcholines on silica-gel G plates impregnated with AgNO_3 (20%, w/w) in a solvent system of chloroform/methanol/water (65:25:4, by vol.) by the method of Arvidson (1968). Phospholipids were quantified, after transesterification of the fatty acid components with H_2SO_4 in dry methanol (2.5%, v/v), by g.l.c. as described previously (Harwood *et al.*, 1975). Surfactant protein was also determined as described previously (Harwood *et al.*, 1975). Aqueous samples and those in organic solvents (evaporated to dryness under nitrogen) were counted for radioactivity in 9 ml of PCS scintillant by mean of an Intertechnique LS-4000 liquid-scintillation counter. Quench correction was obtained by the addition of an internal standard of [^{14}C]hexadecane.

Electron microscopy of pulmonary-surfactant samples

Surfactant samples were mixed (1:1, v/v) with freshly prepared fixative [2.5% (w/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4, containing 3 mM- CaCl_2] and centrifuged at 3000 g for 10 min. The supernatant was discarded and the surfactant pellet fixed for 1 h at 4 °C by the addition of 5 ml of fresh fixative. Samples were washed four times with sodium cacodylate buffer and post-fixed with 1% (w/v) OsO_4 for 1 h at 4 °C. These preparations were dehydrated successively with 50, 70, 80 and 95% ethanol (15 min each at 4 °C) and then washed at room temperature four times with 100% ethanol and twice with 100% propylene oxide. The samples were infiltrated with propylene oxide and Araldite resin (1:1, v/v) for 12 h and embedded in freshly mixed resin for 3 days at 60 °C before sectioning. Sections were viewed by transmission electron microscopy.

RESULTS

Preparation of radiolabelled surfactant from quartz-treated rats

Pulmonary surfactant accumulates rapidly in the lungs of rats instilled with quartz such that between 10 and 20 times as much lipoprotein complex may be obtained from rats exposed to the mineral compared with untreated animals (Richards & Curtis, 1984). Two preliminary experiments were carried out on rats exposed to quartz to determine whether instillation of this mineral would provide a suitable method for preparing a bulk sample of radiolabelled surfactant. In the first experiment, rats were instilled with 5 mg of quartz and injected with radiolabelled choline when surfactant accumulation was approaching maximal values (up to 40 mg/animal; results not shown) 10 weeks later. Surfactant was then isolated at time periods of up to 24 h after the injection of 10 μCi of radiolabel. Maximum incorporation of radiolabel into pulmonary surfactant (750 d.p.m./mg of surfactant) was found at 8 h after administration of the radiolabel (Fig. 1). At 24 h after injection of the choline

Fig. 1. Incorporation of [methyl- ^{14}C]choline chloride into pulmonary surfactant over 24 h in quartz-treated rats

Results are the means of two determinations, and the bars represent the range of values.

Fig. 2. Incorporation of [methyl- ^{14}C]choline chloride into pulmonary surfactant over 8 h in quartz-treated rats

Results are given as the means of two determinations and bars represent the range of values.

the radiolabelled surfactant recovered by lavage had a specific radioactivity of 100 d.p.m./mg of surfactant, which is only 13% of that noted at 8 h. A second experiment was carried out on animals instilled with 5 mg of quartz and then injected with 5 μCi of radiolabelled choline 9 weeks later. Incorporation of the radiolabel into pulmonary surfactant was time-dependent up to 8 h, whereupon the lipoprotein complex had a maximum specific radioactivity of 1000 d.p.m./mg of surfactant (Fig. 2). A similar pattern of incorporated radiolabel was observed in the 300 g supernatant (see the Materials and methods section), which contains all the acellular material (including surfactant) at the lung surface, and in the 1000 g supernatant, which contains lipids but is also rich in soluble surface protein (Richards & Curtis, 1984) (results not shown). The amount of radioactivity associated with phosphatidylcholine and its dipalmitoyl molecular species was determined. Previous studies (R. W. Lewis, unpublished work) had shown that the

Fig. 3. Incorporation of [methyl- ^{14}C]choline chloride into pulmonary surfactant over 8 h in untreated rats

Results are given as the means of duplicate determinations on pooled samples from four rats at each time period.

surfactant isolated from quartz-treated rats was indistinguishable from that derived from untreated rats. In this preliminary study using quartz-treated animals, phosphatidylcholine was the only lipid band separated by t.l.c. which was appreciably radiolabelled. About half of this radiolabel was found in the dipalmitoyl molecular species. Maximum incorporation of radiolabel was again observed at 8 h for both total phosphatidylcholine and for its dipalmitoyl form, although the low specific radioactivity of the starting material (1000 d.p.m./mg) made accurate quantitative assessment of the radiolabel associated with the separated phospholipid moieties very difficult (results not shown).

Preparation of radiolabelled surfactant from untreated rats

In a preliminary experiment, untreated rats were injected with 5 μCi of [methyl- ^{14}C]choline chloride and the surfactant isolated after 2, 4, 6 and 8 h. As found with the quartz-treated animals, there was a gradual increase in the specific radioactivity found in the surfactant with time after injection of radiolabel (Fig. 3). The surfactant isolated from untreated rats at 8 h had a much higher specific radioactivity (16000 d.p.m./mg of surfactant) than that found in quartz-treated rats (1000 d.p.m./mg of surfactant). The incorporation of radiolabel with time presented a similar pattern for the 300 g and 1000 g supernatant samples from control animals (Fig. 4), as found for quartz-treated animals (results not shown).

Finally, a bulk preparation of surfactant was synthesized by using 40 control rats injected intravenously with 5 μCi of radiolabelled choline. A pooled surfactant preparation (33.5 mg) was isolated at 8 h after radiolabel injection and found to have a specific radioactivity of 22860 d.p.m./mg of surfactant. Chemical analysis of this material indicated that 98% of the incorporated radiolabel was associated with the purified surfactant lipid fraction and that practically all of the radiolabel was detected in the phosphatidylcholine species (Table 1). Further separation of the molecular species of phosphatidylcholine showed that, in this bulk sample, 75% of the radioactivity was found in the dipalmitoyl phosphatidylcholine moiety.

Fig. 4. Incorporation of [methyl- ^{14}C]choline chloride into alveolar-lavage fractions over 8 h in untreated rats

Results are given as the means of duplicate determination on pooled samples; 300 g and 1000 g refer to supernatant fractions (see the text).

Table 1. Distribution of [methyl- ^{14}C]choline chloride in a bulk preparation of rat pulmonary surfactant

Normal male rats were injected with 5 μCi of [methyl- ^{14}C]choline chloride and killed after 8 h (see the Materials and methods section). [Of the incorporated radiolabel, 98% was detected in the lipid fraction as isolated by Garbus *et al.* (1963); 75% of this radioactivity was found in dipalmitoyl phosphatidylcholine.]

Phospholipid component	Percentage of detected radioactivity (total approx. 4000 d.p.m.)
Phosphatidylinositol	1.0
Sphingomyelin	0.6
Phosphatidylcholine	97.0
Phosphatidylglycerol	0.8
Phosphatidylethanolamine	< 0.1
Neutral lipids	0.5

The bulk aqueous preparation of surfactant, which had been stored at -20°C under nitrogen (to prevent lipid oxidation), was examined by electron microscopy and found to contain both tubular myelin-like configurations and common myelin (results not shown). These two morphological forms are thought to be representative of the structure of pulmonary surfactant as it exists in the alveoli, and as such are unlikely to arise as artefacts of the extraction and/or fixation procedures. When the material was freeze-dried and stored at -20°C under nitrogen before electron microscopy, it then appeared more amorphous in nature, with less tubular myelin figures (results not shown).

DISCUSSION

A method is described for obtaining a purified preparation of specifically radiolabelled pulmonary surfactant which may be used for turnover/degradative studies of the lipoprotein complex.

Pretreatment of rats with quartz increases the yield of surfactant. In one experiment 170 mg of material was derived from eight rats: a potential increase of 20 times that found in untreated animals. Unfortunately this material had a low specific radioactivity, presumably because the newly synthesized and released surfactant is considerably diluted by the amount of unlabelled lipoprotein complex already present at the lung surface. Surfactant isolated by the methods described from quartz-treated rats had the same phospholipid and protein composition as that found in control animals (R. W. Lewis, unpublished work). However, surfactant derived from the mineral-treated animals could have certain disadvantages in subsequent experiments. Firstly, free quartz particles would almost certainly bind strongly to the preparations of isolated surfactant (Richards & Curtis, 1984) and this may affect subsequent metabolic studies by damaging lung cell cultures or the alveolar surface on to which the isolated surfactant complex was reintroduced. Secondly, although the surfactant of low specific radioactivity could possibly be employed in degradative studies with isolated lung cells, the preparation is of little use in whole-animal investigations on the turnover of the complex. This is because a large, total unphysiological, amount of prepared surfactant would have to be instilled on to the lung surface in order to detect redistribution of radiolabel. Thus, despite the potential saving in animal numbers and the cost of precursor, radiolabelled surfactant derived from quartz-treated rats has only limited use for turnover studies.

Untreated rats may be used to isolate a bulk preparation of radiolabelled surfactant of high specific radioactivity (22860 d.p.m./mg) with maximum incorporation of the choline precursor at 8 h after injection of radiolabel. Chemical analysis of the phospholipid components indicated that the surfactant preparation fulfills all necessary criteria (Harwood *et al.*, 1975) and that practically all of the radiolabel is associated with phosphatidylcholine moieties, most being found in the major surfactant component, namely dipalmitoyl phosphatidylcholine. In addition, results from electron microscopy indicated that the isolated material has a very similar morphology to that found under natural conditions at the lung surface (Harwood & Richards, 1985), although subsequent freeze-drying, to permit longer storage, may well change the physical form of the preparation.

The cost of preparing the isolated radiolabelled surfactant is relatively high and the process is time-consuming. Nevertheless, the isolated complex is ideal for examining the postulated role of type II cells in surfactant turnover (Geiger *et al.*, 1975; Richards & Lewis, 1985). Furthermore, the present interest in the use of surfactant-replacement therapy for the treatment of acute-respiratory-distress syndrome may benefit from a rational animal study with respect to dose levels and turnover phenomena of instilled surfactant.

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