Phosphatidylcholine composition of endotracheal tube aspirates of neonates and subsequent respiratory disease

M R Ashton, A D Postle, M A Hall, S L Smith, F J Kelly, I C S Normand

Abstract
The phosphatidylcholine (PC) content of the initial endotracheal tube aspirate was measured in 105 infants intubated for resuscitation or for ventilation for respiratory distress syndrome, using high performance liquid chromatography and postcolumn fluorescence derivatization with diphenyl-1,3,5-hexatriene. Sixty eight had measurable PC. Of the infants who developed respiratory distress syndrome, with or without subsequent chronic lung disease, neither the percentage of dipalmitylophosphatidylcholine (DPPC) nor the ratio of DPPC to palmitoyloleophosphatidylcholine (POPC), showed any correlation with gestational age. However, both parameters were significantly lower overall in this group than in the group of infants who did not develop respiratory distress syndrome. Infants with a ratio of DPPC:POPC <3:0 developed respiratory distress syndrome irrespective of gestational age, but there was considerable overlap between groups for values greater than this.

The infants with respiratory distress syndrome who went on to develop chronic lung disease had the same initial PC profile as those with respiratory distress syndrome who did not develop chronic lung disease, but differed as a group by being lighter and more premature. The development of chronic lung disease was not associated with a particular initial PC composition. Other factors related to increasing prematurity must therefore be involved in rendering infants vulnerable to developing chronic lung disease.

Despite the introduction of surfactant replacement treatment, respiratory distress syndrome remains one of the major causes of morbidity and mortality in premature infants, and one of its sequelae, chronic lung disease, continues to cause respiratory problems through into infancy. A biochemical parameter that accurately predicts the development of respiratory distress syndrome would enable targeting of 'prophylactic' surfactant replacement treatment, with consequent financial implications. Further, early identification of infants who are at risk for developing chronic lung disease could enable timely intervention, such as diuretic or dexa-methasone treatment, in an attempt to alter outcome.

The major cause of respiratory distress syndrome is a deficiency in both quantity and quality of pulmonary surfactant. This substance is composed of phosphatidylcholine (PC) species, other phospholipids, and surfactant proteins. It has been shown that changes in the PC composition occur with increasing maturity. In human postmortem studies an increase in disaturated PC has been demonstrated with increasing gestation. Attempts to establish a reliable assessment of surfactant status have included quantitation of the disaturated PC species dipalmitylophosphatidylcholine (DPPC), as this particular species is the most surface active component of pulmonary surfactant. However, such attempts have been restricted largely to the analysis of total disaturated PC. It has been demonstrated recently that lung fluid at term contains significant quantities of another disaturated PC species myristoylpalmitoylphosphatidylcholine, which is not surface active, emphasising the limitation of total disaturated PC as an index of surfactant maturity.

A sensitive method for the analysis of individual PC species has been described, and employed in the study of a guinea pig model. In this model DPPC, specifically, has been shown to increase with gestation, and palmitoyloleophosphatidylcholine (POPC), an unsaturated species, to decrease. Amniotic fluid and nasopharyngeal aspirates of term infants were shown to have a higher percentage of DPPC and a lower percentage of POPC when compared with amniotic fluid taken at 20 weeks' gestation. The ratio of DPPC to POPC may be an index of surfactant maturity, and has been proposed as possibly predictive for the development of respiratory distress syndrome.

Currently available methods for the analysis of surfactant maturity, including the one described here, take too long to perform to be of use in predicting postnatally which babies might benefit from the use of 'prophylactic' surfactant treatment—the diagnosis of respiratory distress syndrome would be obvious clinically before the results were available. However, initial endotracheal tube aspirates are easily obtained and can form the basis for feasibility studies into any biochemical parameter that might be predictive for the development of respiratory distress syndrome, with more rapid techniques being established later.

The techniques used previously have been employed to study the initial endotracheal tube aspirates of 105 babies admitted to the neonatal unit. Differences in PC composition between groups of infants who developed respiratory distress syndrome and chronic lung disease have been examined. The aims of the study were to determine whether the PC composition of initial endotracheal tube aspirates was predictive of
Demographic data

(2a) Respiratory distress syndrome or chronic lung disease. In addition we report the first description of PC species composition of endotracheal tube aspirates from infants with respiratory distress syndrome.

Subjects and methods

Subjects

A total of 105 infants of ≤37 weeks’ gestation who were admitted onto the neonatal unit, and who required endotracheal intubation for either resuscitation or ventilation for respiratory distress syndrome, were studied. The infants were divided into the following groups, according to their developing respiratory distress syndrome (as defined clinically and radiologically) and chronic lung disease (defined as requiring supplemental oxygen at 28 days, with an abnormal chest radiograph): (1) no respiratory disease and (2) respiratory distress syndrome (a) without progression to chronic lung disease and (b) with progression to chronic lung disease. Classification was done by MAH without knowledge of the analysis results.

Specimen collection

Endotracheal tube aspirates were collected by deep suction either at resuscitation, in which case no saline was used for irrigation of the endotracheal tube, or within four hours of birth at a time of normal endotracheal tube toilet. In the latter cases 0.25-0.5 ml normal saline were introduced into the endotracheal tube and allowed to dwell for approximately 10 seconds before deep suction. Traps and catheters were washed out with 1-6 ml normal saline and the washings centrifuged at 1000 g for 10 minutes to remove cellular debris. The supernatants were stored at −20°C until analysis.

Extraction and fractionation

Total lipids were extracted with chloroform:methanol, after PC 14:0/14:0 (dimyristoyl-PC) had been added as an internal standard, and were dried under nitrogen before being dissolved in chloroform. The PC fraction was obtained by selective elution from Bondelut NH2 columns (Jones Chromatography) with chloroform:methanol (3:2, v:v). Individual PC species were resolved by isocratic reverse phase high performance liquid chromatography (HPLC). A 25 cm Apex II ODS column was used, with a mobile phase of 40 mM choline chloride in 92.5% methanol, 7.5% water at 1 ml/minute.

Demographic data for infants with measurable PC. Results are mean (SD)

<table>
<thead>
<tr>
<th>No of infants</th>
<th>Gestational age (weeks)</th>
<th>Birth weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) No respiratory disease</td>
<td>22</td>
<td>32.8 (2.1)</td>
</tr>
<tr>
<td>(2) All babies with respiratory distress syndrome</td>
<td>46</td>
<td>31.0 (3.2)*</td>
</tr>
<tr>
<td>(2a) Respiratory distress syndrome, no chronic lung disease</td>
<td>36</td>
<td>31.9 (2.8)</td>
</tr>
<tr>
<td>(2b) Respiratory distress syndrome and chronic lung disease</td>
<td>10</td>
<td>27.9 (2.3)**</td>
</tr>
</tbody>
</table>

*p < 0.05 compared with (1); **p < 0.0001 compared with (1) and with (2a).

Derivatisation by diphenyl-1,3,5-hexatriene fluorescence

Eluted peaks were quantified by postcolumn fluorescence. The methanol HPLC stream was mixed with an aqueous stream of 1,6-diphenyl-1,3,5-hexatriene at 3 ml/minute, and the resultant fluorescent peaks detected by excitation at 340 nm and emission at 460 nm. The lower limit of detection for each molecular species was 200 pmol.

Figure 1 Infants with no respiratory disease. (A) %DPPC v gestational age and (B) DPPC:POPC ratio v gestational age. Open squares represent infants who did not develop chronic lung disease.

Figure 2 Infants developing respiratory distress syndrome. (A) %DPPC v gestational age and (B) DPPC:POPC ratio v gestational age. Open circles represent infants who did not develop chronic lung disease and filled circles represent infants who developed chronic lung disease. Correlation coefficients represent all infants with respiratory distress syndrome.
Analysis of results was by the pooled Student's t test, and when appropriate, the construction of contingency tables, the use of the χ² test, and of regression analysis, using the Statgraphics software package.

Ethical approval was given by the Southampton and South West Hampshire joint ethical committee for the collection of the endotracheal tube aspirates.

### Results

Gestational age and birthweight data for infants with detectable PC are shown in the table. Of the 105 infants studied detectable PC was present in 68. Twenty two of the 37 infants in group 1 had detectable PC, as did 36 of the 50 infants in group 2(a), and 10 out of the 18 in group 2(b). The differences between the groups in the proportion of aspirates with detectable PC were not significant (χ² 5.41 in 2 df).

As would be expected, infants developing respiratory distress syndrome were less mature than infants who did not. This difference was due mainly to those infants who went on to develop chronic lung disease, this subgroup being significantly less mature than both the 'no respiratory disease' group and the group of infants who developed respiratory distress syndrome, but did not go on to develop chronic lung disease. Indeed, the differences in gestational age between these latter two groups do not achieve significance. Similar differences between the groups are seen for birth weight, though the difference between the no respiratory disease group and the group of all infants developing respiratory distress syndrome was not significant.

The area under each derived peak of the chromatographs obtained in this study is proportional to the concentration of that particular PC species in the sample. Determination of absolute concentration was not possible as the exact volume of each aspirate sample, and the degree of dilution of the sample by the lavage saline, was not known. Results have therefore been expressed in terms of the percentage of DPPC of the total PC recovered (%DPPC), and in terms of the ratio of DPPC to POPC (DPPC:POPC ratio).

Figure 1 shows clearly that, for the infants in group 1 (no respiratory disease), there is no correlation with gestational age for either %DPPC or DPPC:POPC ratio. Similarly, fig 2 shows there is no correlation between either parameter and gestational age for infants in group 2 (respiratory distress syndrome). However, the mean values for both parameters are significantly lower for the respiratory distress syndrome group when compared with the no respiratory disease group (figs 3 and 4). These differences are not explained by the differences in gestational age between the two main groups (table) as we failed to demonstrate a correlation for either parameter with gestational age. In addition the differences persist for the 'respiratory distress syndrome, no chronic lung disease' subgroup, when it is compared with the no respiratory disease group, despite there being no significant differences in gestational age between these two groups. The mean values for both %DPPC and the DPPC:POPC ratio for the 'respiratory distress syndrome and chronic lung disease' subgroup are also significantly lower than those for the no respiratory disease group. Importantly, however, the mean values for both parameters are identical for both respiratory distress syndrome subgroups—there is no difference between the results for infants who do or do not progress to chronic lung disease.

![Graph](attachment:image.png)

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (SE)</th>
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</thead>
<tbody>
<tr>
<td>(1)</td>
<td>53.7 (3.4)</td>
</tr>
<tr>
<td>(2)</td>
<td>43.6 (1.9)</td>
</tr>
</tbody>
</table>

![Graph](attachment:image2.png)

**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (SE)</th>
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</thead>
<tbody>
<tr>
<td>(1)</td>
<td>5.6 (0.5)</td>
</tr>
<tr>
<td>(2)</td>
<td>3.1 (0.2)</td>
</tr>
</tbody>
</table>

![Graph](attachment:image3.png)

**Table 3**

<table>
<thead>
<tr>
<th>Group</th>
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</thead>
<tbody>
<tr>
<td>(1)</td>
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<td>(2)</td>
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</tr>
</tbody>
</table>

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Phosphatidylcholine of infants and neonates and subsequent respiratory disease

One final important result can be seen in fig 4. All infants with an initial DPPC:POPC ratio of less than 3:0 developed respiratory distress syndrome irrespective of their gestational age. However, above this value, there is considerable overlap between groups.

Discussion

It is recognised that soon after birth premature infants, especially those ventilated for respiratory distress syndrome, may have few endotracheal tube secretions and that the detection of surfactant phospholipids in them may not be possible. This was proved to be the case in this study, with one third of infants having no detectable PC in their aspirates, but no bias between groups of infants was found. The technique of deep endotracheal tube suction should ensure that smaller airway secretions are being sampled, but endotracheal tube secretion analysis will always be open to the criticism that the composition of tracheal secretions may not accurately reflect that of the alveolar surfactant pool.

With increasing gestation, the quality of pulmonary surfactant improves in terms of biochemical composition and, in animals at least, in terms of function. The percentage of saturated PC species, especially of DPPC, increases at the expense of unsaturated species. The amount of surfactant proteins increases and the appearance of another phospholipid, phosphatidylglycerol, heralds the maturation of surfactant. The data presented here show a lack of correlation between two parameters that reflect surfactant maturity (the %DPPC and the DPPC:POPC ratio) and gestational age. The lack of ‘improvement’ in surfactant with gestational age is at first sight surprising. However, the only human data available for PC species composition refers to just two time points during gestation — 20 weeks and term — serial data is not available, so the time course of ‘maturation’ of the PC species profile of surfactant in humans is unknown. Surfactant production pathways may mature at different rates in individual fetuses. It is possible that ‘mature’ surfactant production had been induced early in gestation in the premature infants that did not develop respiratory distress syndrome as part of natural variation, or perhaps as the result of obstetrical intervention with antenatal dexamethasone, and that mature surfactant production had not been induced in those infants who developed respiratory distress syndrome. If this was the case then no correlation between PC composition and gestational age would be expected, but differences between groups of infants developing respiratory distress syndrome and infants not developing respiratory distress syndrome should be found.

The means of both the %DPPC and the DPPC:POPC ratio are significantly lower for the infants who developed respiratory distress syndrome compared with the infants who did not. Because of the lack of correlation between either parameter and gestational age, this suggests that a certain composition of surfactant will predispose an infant to the development of respiratory distress syndrome, irrespective of gestational age. Of the group of infants with measurable PC in their initial endotracheal tube aspirate, all those with a DPPC:POPC ratio less than 3:0 developed respiratory distress syndrome. Though values below this could reasonably be taken as predictive for the development of respiratory distress syndrome, there is considerable overlap between groups for values above 3:0. The %DPPC shows even greater overlap between groups and would be of even less value in predicting respiratory distress syndrome.

Of the infants that developed respiratory distress syndrome, it was the lighter, less mature ones who tended to go on and develop chronic lung disease. In this study, the respiratory distress syndrome and chronic lung disease group had a mean gestational age of 27·9 weeks and a mean birth weight of 1049 g, compared with 31·9 weeks and 1885 g for the respiratory distress syndrome, no chronic lung disease group. Despite this difference in gestational age there were no differences in the %DPPC and the DPPC:POPC ratio — neither parameter was predictive for the development of chronic lung disease.

This finding does not support the suggestion that infants who later develop chronic lung disease have a poorer ‘surfactant phospholipid profile’ early in the course of their disease. It is probable that it is not the composition of pulmonary surfactant at birth that predisposes infants to developing chronic lung disease but some other factor, or factors, associated with increasing prematurity that are responsible. Possible factors include low numbers of alveoli and, if surfactant has a role in the pathogenesis of chronic lung disease, poor surfactant function or poor continued production. Alternatively, more premature infants might tend to have immature antioxidant systems or be vitamin A deficient and thus be more prone to oxidative damage, and less able to instigate epithelial repair.

We were unable to define a parameter predictive of respiratory distress syndrome but have shown a lack of correlation between the initial PC composition of endotracheal tube aspirates and gestational age for infants who develop respiratory distress syndrome. This suggests that a particular surfactant composition will lead to respiratory distress syndrome, irrespective of gestational age. We have also demonstrated that the initial PC composition of tracheal aspirates from infants who go on to develop chronic lung disease does not differ from those who do not develop chronic lung disease. Factors other than initial surfactant composition predispose infants to developing chronic lung disease; these factors are related to increasing prematurity.

The methodology used here proved to be sensitive in detecting individual PC species in endotracheal tube aspirates, and has great potential in the study of other aspects of neonatal respiratory disease, especially of the turnover of exogenous surfactant, and of the effects of dexamethasone in the treatment of chronic lung disease.
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