Early increase of TNF\(\alpha\) and IL-6 in tracheobronchial aspirate fluid indicator of subsequent chronic lung disease in preterm infants

Baldvin Jónsson, Kjell Tullus, Annelie Brauner, Ying Lu, Gerd Noack

Abstract

Aim—To investigate if early changes in concentrations of proinflammatory cytokines in tracheobronchial aspirate fluid (TAF) from preterm infants could be used to detect infants at risk of chronic lung disease (CLD) and help in the selection of patients for early steroid treatment.

Methods—Twenty-eight preterm infants less than 34 weeks of gestation (median 26 weeks) were intubated and daily measurements of TAF concentrations of tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) and the interleukins IL-1\(\beta\), IL-6, and IL-8 were made, using enzyme immunoassay techniques.

Results—Seventeen of the infants developed CLD. The infants who developed CLD had significantly increased concentrations of TNF\(\alpha\), IL-1\(\beta\), IL-6 on days 2 and 3. TNF\(\alpha\), IL-6, and IL-8 concentrations were significantly related to gestational age and duration of supplemental oxygen; TNF\(\alpha\), IL-6, and IL-8 concentrations also correlated with length of time on the ventilator.

Conclusion—These data indicate that tracheobronchial aspirate fluid cytokine concentrations may be used as a predictor of subsequent CLD and may help select a group of preterm infants at high risk of developing CLD for early treatment.

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Keywords: cytokines; chronic lung disease; tracheobronchial aspirate fluid; mechanical ventilation

The major advances in neonatal intensive care in recent years have produced an increase in survival of very low birthweight (VLBW) infants. In these immature babies the incidence of chronic lung disease (CLD) is high. CLD increases the length of stay in neonatal intensive care and is also the most common chronic lung disorder in infants.\(^1\) Pathophysiological changes in CLD include epithelial and endothelial cell damage, increased permeability, surfactant inactivation, and recruitment/activation of neutrophils and alveolar macrophages.\(^2\) Proinflammatory cytokines are thought to have an important role in modulating and promoting the initial lung tissue response to injury.\(^3\) Expression of individual inflammatory mediators in tracheobronchial aspirate fluid (TAF) from preterm infants has been described.\(^4-8\) In a cross-sectional study we found increased concentrations of proinflammatory cytokines in infants with CLD, and a decrease after treatment with corticosteroids.\(^9\)

Our hypothesis is that the early appearance of cytokines in TAF can help to identify patients at high risk of developing CLD, and to select infants for early treatment with intravenous or inhaled corticosteroids. We therefore compared the antigen titres of the proinflammatory cytokines tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) and the interleukins IL-1\(\beta\), IL-6, and IL-8 in TAF from intubated infants during the first weeks of life, who subsequently developed CLD, with those in a control group of infants with respiratory distress syndrome (RDS) that resolved without sequelae. We also studied the effect of gestational age, fraction of inspired oxygen (FIO\(_2\)) and insufflation pressure during mechanical ventilation on the proinflammatory cytokine values.

Methods

This study was conducted from January through December 1994 on mechanically ventilated infants, of 34 weeks gestation or less, admitted to the neonatal intensive care unit at the Karolinska Hospital in Stockholm, Sweden. Tracheobronchial fluid (TAF) was collected daily. A total of 32 patients were sampled. The median (range) gestational age for all infants was 26 (24–33) weeks and the birthweight was 871 (530–2356) g. Infants with perinatal infections (two infants, one with \textit{Ureaplasma urealyticum} and the other with group B Streptococcus), congenital malformations and/or syndromes (\(n = 2\)) were excluded from further analysis. Of the remaining 28 infants, 17 developed CLD and these were compared with the remaining 11 patients with uncomplicated RDS. Data on perinatal factors (mode of delivery, rupture of membranes, prenatal steroid treatment) were documented.

Respiratory distress syndrome was diagnosed in infants on the basis of characteristic radiographic findings, respiratory distress, and an increasing FIO\(_2\) requirement.\(^10\) Infants diagnosed with RDS and an FIO\(_2\) > 0.6 received rescue treatment with natural porcine surfactant 200 mg/kg (Curosurf, Serono, Italy), after intubation.

The infants were mechanically ventilated using a Sechrist 100B infant ventilator...
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Table 1 Perinatal characteristics of RDS and CLD infants

<table>
<thead>
<tr>
<th></th>
<th>RDS group (n=11)</th>
<th>CLD group (n=17)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birthweight (g)</td>
<td>1039 (959-2365)</td>
<td>709 (530-1766)</td>
<td>0.0044</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>27 (26-33)</td>
<td>25 (24-30)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Prenatal steroid</td>
<td>7 (64)</td>
<td>11 (65)</td>
<td>NS</td>
</tr>
<tr>
<td>PROM</td>
<td>5 (45)</td>
<td>4 (23)</td>
<td>NS</td>
</tr>
<tr>
<td>Delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cesarean</td>
<td>6</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Vaginal</td>
<td>5</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

Values are shown as number (%); PROM denotes prolonged rupture of membranes.

Table 2 Ventilatory and clinical characteristics of study population

<table>
<thead>
<tr>
<th></th>
<th>RDS group (n=11)</th>
<th>CLD group (n=17)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max PIP (cm)</td>
<td>23 (20-26)</td>
<td>24 (22-28)</td>
<td>NS</td>
</tr>
<tr>
<td>Inspiratory &gt; 21% (hours)</td>
<td>80 (48-62)</td>
<td>1848 (1008-5760)</td>
<td>0.001</td>
</tr>
<tr>
<td>Ventilatory rate (hours)</td>
<td>72 (48-120)</td>
<td>624 (120-1680)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Surfactant</td>
<td>8 (73)</td>
<td>15 (88)</td>
<td>NS</td>
</tr>
<tr>
<td>PDA</td>
<td>3 (27)</td>
<td>15 (88)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Air leaks</td>
<td>2 (18)</td>
<td>7 (41)</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Values are shown as median (range) or number (%); PIP denotes peaked inspiratory pressure; PDA denotes patent ductus arteriosus.

The study was approved by the local ethics committee of the Karolinska Hospital.

SAMPLING PROCEDURE
The collection of TAF samples was begun on day 2 of life and was repeated daily, in the same 8 hour period. TAF samples were only collected when endotracheal suctioning was clinically indicated. No additional procedures were needed. Parental consent was obtained for this modified nursing procedure. Before initiating the procedure FIO2 was adjusted to maintain saturation between 92–94% by pulse oximetry. In infants who received surfactant the first sample was taken at least 6 hours after administration.

With the infant supine 0.5 ml of 0.9% sterile saline was instilled endotracheally via a 6.0 French catheter (Vygon, Ecouen, France) and a deep gentle endotracheal suctioning of lung and airway secretions was done by rotating the catheter and applying suction while withdrawing the catheter. To rinse aspirate from the catheter wall, the catheter was flushed with 0.5 ml of sterile saline. All remaining aspirated material was thus flushed into a sterile specimen trap. Tree to five manual breaths were given and the lavage procedure repeated.

The total duration of each instillation was less than 40 seconds. The recovery of aspirated fluid ranged between 70–80% of the amount instilled. Samples from the two instillations were pooled. Samples were excluded from further analysis if blood was visible in the aspirate.

Samples were centrifuged at 1200 rpm for 10 minutes. The supernatant fluid was frozen at −70°C until subsequent assay.

CYTOKINE ANALYSIS
The supernatant fluids were used for cytokine determination by enzyme immunoassay (EIA). TNF-α, IL-1β, IL-6 and IL-8 kits were obtained from R&D systems (Abingdon, Oxon, UK).

The limit of detection was defined as 0.5 pg/ml for TNF-α, 3.9 pg/ml for IL-1β, 3.13 pg/ml for IL-6 and 31.3 pg/ml for IL-8.

Cytokine concentrations are expressed as volume concentrations (pg/ml) as no other satisfactory correction factor is currently available. Albumin content, urea, or IgA secretory component have been used as correction units, but their use has been questioned because all are subject to individual variations depending on the disease state, sampling site, and fluid recovered. 6-11-13

STATISTICAL ANALYSIS
Intra- and intergroup differences were first tested using analysis of variance. For comparison of clinical variables and differences in cytokine values between groups, the Mann-Whitney U test was used. Differences in frequencies were tested using the χ² test. Correlations of cytokine concentrations with clinical variables were tested using the Spearman rank correlation coefficient test. For the correlation analysis only the first sample from each patient was used (n=28). A p value of less than 0.05 was considered significant.

RESULTS
There were no differences in regard to sex, method of delivery, prolonged rupture of membranes (PROM) or prenatal steroid use between infants who subsequently developed CLD and those with uncomplicated RDS (table 1). The infants who developed CLD had significantly lower gestational ages and birth-weights than infants with RDS (table 1). There were also significant differences between the groups in time spent on a ventilator and duration of supplemental oxygen (table 2). The infants who developed CLD had significantly more air leaks and PDA (table 2). We found no significant correlation between the time of diagnosis or closure of the PDA with concentrations of individual cytokines. No early deaths occurred in either group of infants. Two infants in the CLD group died at 6 months of age from CLD related complications.

By days 2 (p = 0.0133) and 3 (p = 0.026) neonates who developed CLD had significantly higher TNF-α values than the RDS group of infants (fig 1A). In CLD infants and RDS control infants median (range) TNF-α values on day 2 were 6.5 (3–9) pg/ml and 2.5 (1–4)

(Sechrist Corp., Anaheim, California, USA) with initial ventilator settings of FIO2 > 0.6, a rate of 60 breaths per minute, a peak inspiratory pressure (PIP) of < 30 cm H2O, a positive end expiratory pressure (PEEP) of 3–4 cm H2O and an inspiration:expiration ratio (I:E) of 1:2. If, despite conventional ventilation and surfactant administration, the infant had increasing pCO2, hypoxia and acidosis, high frequency oscillatory ventilation was started using the Sensor Medics 3100A oscillator (Sensor Medics Corp., CA).

Chronic lung disease (CLD) was diagnosed in infants who had been ventilated during the first week of life, had respiratory symptoms, an oxygen requirement and radiological findings at 28 days of age. Air leak was defined as the radiological presence of pulmonary interstitial emphysema and/or pneumothorax. Patent ductus arteriosus (PDA) was diagnosed from clinical signs and confirmed by echocardiography.

The study was approved by the local ethics committee of the Karolinska Hospital.

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Infants; p > 0.05 on days 6 between CLD and RDS; p < 0.05 on days 2 and 3.
Concentrations:*denotes separatedays. (A) TNFα concentrations: *denotes p < 0.05 on days 2 and 3 between CLD and RDS infants; †p > 0.05 on days 6 and 7 compared with days 2 and 3 for CLD infants. (B) IL-1β antigen titres: *p < 0.05 between RDS and CLD infants on day 2. (C) IL-6 concentrations: †p < 0.05 on days 2 and 3 between the two groups of infants. (D) IL-8 concentrations pg/ml, respectively. On day 2 TNFα concentrations of ≥ 5 pg/ml were found in six of seven samples in infants who subsequently developed CLD compared with none of the five control infants. In CLD infants TNFα values were significantly higher on days 6 and day 7 compared with day 3 (p = 0.044 and 0.041, respectively). TNFα concentrations were significantly correlated with lower gestational age, birthweight, time spent on a ventilator and duration of supplemental oxygen (table 3).
IL-1β concentrations were significantly higher only in CLD infants on day 2 (p = 0.046; fig 1B). IL-6 concentrations were significantly higher in CLD infants on days 2 (p = 0.014) and day 3 (p = 0.05) (fig 1C) compared with the RDS group. On day 2 median IL-6 concentrations were 1130 (390–3800) pg/ml in CLD infants and 106 (38–350) pg/ml in RDS infants. On day 2 IL-6 concentrations over 350 pg/ml were found in all five samples in CLD infants but in none of the six control infant samples. IL-6 concentrations correlated with lower gestational age, birthweight, time spent on a ventilator and duration of supplemental oxygen (table 3).
Overall, using an analysis of variance, IL-8 concentrations were significantly increased from day 2 to day 7 (p = 0.012) in CLD infants. There was no significant difference in values on separate days between the CLD and the RDS infants (fig 1D). IL-8 concentrations were significantly related to lower gestational age, duration of supplemental oxygen, time spent on a ventilator and maximal PIP (table 3).

**Table 3** Correlation between cytokine concentrations and clinical variables (Spearman rank correlation showing r, P value)

<table>
<thead>
<tr>
<th></th>
<th>TNFα</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age</td>
<td>-0.42 (0.0002)</td>
<td>-0.29 (0.027)</td>
<td>-0.41 (0.003)</td>
<td>-0.32 (0.026)</td>
</tr>
<tr>
<td>Birthweight</td>
<td>-0.15 (0.093)</td>
<td>-0.05 (0.70)</td>
<td>-0.18 (0.18)</td>
<td>0.04 (0.78)</td>
</tr>
<tr>
<td>Time with supplemental oxygen</td>
<td>0.29 (0.018)</td>
<td>0.07 (0.61)</td>
<td>0.36 (0.01)</td>
<td>0.18 (0.12)</td>
</tr>
<tr>
<td>Duration of mechanical ventilation</td>
<td>0.36 (0.024)</td>
<td>0.12 (0.37)</td>
<td>0.42 (0.003)</td>
<td>0.21 (0.10)</td>
</tr>
<tr>
<td>Maximal peak inspiratory pressure</td>
<td>-0.01 (0.92)</td>
<td>-0.36 (0.96)</td>
<td>0.14 (0.31)</td>
<td>0.25 (0.04)</td>
</tr>
<tr>
<td>Time of appearance of air leak</td>
<td>0.27 (0.082)</td>
<td>0.25 (0.09)</td>
<td>0.20 (0.05)</td>
<td>0.18 (0.098)</td>
</tr>
</tbody>
</table>

**Discussion**
We have shown that proinflammatory cytokines are increased in TAF from as early as day 2 of life in mechanically ventilated preterm infants who subsequently developed CLD. Compared with the control RDS infants their concentrations of TNFα, IL-1β and IL-6 were significantly higher on days 2 or 3 of life. Our finding of increased cytokines early in the course of CLD supports the hypothesis that they are important mediators in the early inflammatory response in the preterm lung. On day 2 a cut-off limit of 350 pg/ml of IL-6 and 5 pg/ml of TNFα in all cases selected only those infants who subsequently developed CLD. However, our study is to small to define such cutoff limits, but we suggest that further multicentre studies should aim at defining these break points, which could then indicate the potential for early treatment of evolving CLD.

The presence of a major ductal shunt is common in infants weighing less than 1000 g and is related to an increased incidence of CLD. PDA can increase pulmonary capillary pressure and promote the formation of oedema, and thus affect the concentrations of cytokines. We found that cytokine concentrations were higher in infants of lower gestational age and correlated with the duration of mechanical ventilation and oxygen exposure. This can reflect an exaggerated tissue response in the more immature lung, and the common presence of PDA and air leak are certainly important in this respect. Recent
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