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Cysteinyl leukotriene involvement in chronic lung disease in premature infants

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Cysteinyl leukotriene involvement in chronic lung disease in premature infants. A.J. Cook, B. Yuksel, A.P. Sampson, A. Greenough, J.F. Price. ©ERS Journals Ltd 1996.

ABSTRACT: The pathophysiology of chronic lung disease (CLD) in premature infants who require mechanical ventilation and prolonged oxygen supplementation has been well-described but the underlying mechanisms are not understood. Our aim was to test the hypothesis that excess cysteinyl leukotriene (LT) production was a contributing factor in CLD.

We compared LT production and lung function, at 7 months of age, in nine premature infants with CLD and in eight control infants without CLD. None of the control infants developed any neonatal respiratory problems, but two subsequently required bronchodilator therapy. Respiratory function was assessed by the measurement of thoracic gas volume (TGV), airways resistance (R_{aw}) and functional residual capacity (FRC). Total cysteinyl LT production was quantified by measurement of leukotriene E_4 (LTE_4) in a spot urine sample.

Although all patients were asymptomatic at follow-up, there was evidence of significant lung function abnormalities in infants with CLD. The CLD infants had significantly elevated TGV, R_{aw} and FRC values reflecting airway obstruction when compared to the controls. Urinary LTE_4 levels were significantly higher in the CLD infants when compared to the controls (geometric mean: 741 and 337 pmol·mmol^{-1} creatinine, respectively). There was no direct correlation between urinary LTE_4 levels in the CLD group and TGV, R_{aw} or FRC values.

Although this study is small and a direct correlation between lung function and urinary leukotriene E_4 was not demonstrated, pathological lung function and an enhanced urinary leukotriene E_4 production in infants with chronic lung disease would tend to suggest that the cysteinyl leukotrienes were involved in the sequelae of this disease.

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Many of the pathological features of neonatal chronic lung disease (CLD) have been attributed to the pulmonary effects of mechanical ventilation and prolonged supplementary oxygen [1]. Infants developing CLD subsequently have repeated respiratory infections and wheezing attacks, and an increased rate of hospital admission [2]. Impairment of lung function, as evidenced by reduced dynamic compliance, airway hyperreactivity, increased airway resistance and hyperinflation, has also been described [1, 3–7]. These abnormalities are evident in the first few years of life [3–5], and may persist throughout childhood [6, 7].

Several mechanisms have been implicated in the pathogenesis of CLD. These include an imbalance of protease/antiprotease production [8], increased lipid mediator (platelet-activating factor (PAF) and leukotrienes (LTs)) [9–11] and cytokine [11, 12] production, and immature development of the antioxidant system [13]. Increased neutrophil numbers and levels of neutrophil-derived products, elastase, LTB_4 and interleukin (IL)-8, have been demonstrated in bronchoalveolar lavage fluid of infants with CLD [8, 11], suggesting an involvement of neutro-phils in the pathology of CLD.

LTs are derived from the membrane lipid arachidonic acid. The potent proinflammatory properties of the cysteinyl LTs: bronchoconstriction [14], increased vascular permeability [15] and mucus secretion [16], and airway hyperreactivity [17], are analogous to many of the pathophysiological features seen in CLD. Elevated levels of cysteinyl LTs have been detected in the tracheal lavage fluid [9, 10] and urine [18] of infants with bronchopulmonary dysplasia (BPD) when compared to infants with hyaline membrane disease or control term infants with no respiratory disease. These studies suggest an involvement of the LTs in the early phase of BPD. However, the role of LTs in the persisting lung abnormalities resulting from acute lung injury in neonatal chronic lung disorders, such as CLD and BPD, has not been investigated. The aim of this study was to determine the relationship between cysteinyl LT production, lung function and CLD in premature infants.

Materials and methods

Subjects

Nine CLD infants and eight control infants were selected from a cohort of babies who were part of a prospective study examining respiratory problems after

premature birth. CLD infants were consecutively recruited into the study and the control infants were then matched with them for gestational and postnatal age. Control infants had to have had no neonatal respiratory problems. The CLD and control infants were reviewed at a median age of 7 months (range 5–9 months). Infants were diagnosed as having CLD if they were chronically oxygen-dependent at 28 days old, had received mechanical ventilation during the first week of life, and had an abnormal chest radiograph appearance at 28 days, with no evidence of other congenital abnormalities. The control infants had received minimal ventilation (<0.1 days) and supplementary oxygen therapy (<0.2 days) due to prematurity. All of the infants were asymptomatic at the time of the study and had not received any sort of medication on the day of testing prior to lung function measurements. The clinical characteristics of the CLD and control infants are shown in table 1.

In the CLD group, six infants had received bronchodilator therapy and three were receiving anti-inflammatory prophylaxis treatment because of repeated wheezy episodes. Two of the control infants had received bronchodilator therapy but none were receiving prophylactic anti-inflammatory treatment. Three of the CLD infants had recurrent wheezing attacks (as defined by two or more wheezing episodes since discharge) and six CLD infants had been readmitted to hospital with respiratory infections. None of the control infants had had repeated wheezing attacks or had been readmitted to hospital with respiratory infections. During the neonatal period, all nine of the CLD infants had received antibiotics, two had ribavirin treatment and four had surfactant replacement therapy. Six infants in the control group had received antibiotics and none had received either ribavirin or surfactant therapy during the neonatal period. There was no significant difference in the gender of each group. There was no evidence of liver or kidney disease in either the CLD or control infants.

Lung function measurements

Thoracic gas volume (TGV) and airways resistance (R_{aw}) were measured in eight infants with CLD and five control infants by whole body plethysmography as described previously by our group [2]. The infants were sedated with oral chloral hydrate (80–100 $mg \cdot kg^{-1}$) and lung function measurements were obtained during quiet sleep. TGV was measured at the end of normal inspiration and R_{aw} at two thirds of maximum inspiratory flow, using the techniques of DUBOIS and co-workers [19, 20] suitably modified for infants. R_{aw} was calculated from a minimum of 10 breaths and TGV was calculated from five breaths during five separate occlusions. TGV and R_{aw} measurements were collected for the apparatus dead space and for its resistance (8 $cmH_2O \cdot L^{-1} \cdot s$), and were measured at flows of 5–15 $mL \cdot min^{-1}$. All lung function traces were analysed blind. In all 17 patients functional residual capacity (FRC) was determined by helium gas dilution technique with a water-sealed spirometer (Gould Pulmonet III) [3]. FRC was recorded at 15 s intervals. When no change in FRC readout occurred over a 30 s period, equilibration was assumed to have occurred. FRC values were corrected for body temperature, pressure and saturation (BTPS) conditions. The coefficients of variation for TGV, R_{aw} and FRC have previously been reported by our group and were 6, 9 and 7.3%, respectively [3]. Measurement of FRC on two occasions in a group of 20 infants matched for gestational and postnatal age showed that the mean differences between paired values was 2.0 $mL \cdot kg^{-1}$. All lung function results are expressed as the median (range) values.

Urinary LTE_4 analysis

A single spot urine sample was collected from each patient and frozen at -20°C until purification. An aliquot (1 mL) was removed from each urine sample for creatinine analysis (Dept Clinical Biochemistry, King's College Hospital). The purification process was adapted from a technique previously described by our group [21]. Briefly, each sample was spiked with ^3H-LTE_4 (5 nCi) and partially purified using a Mega Bond-Elut C18 cartridge. Nonlipid contaminants were removed by washing with distilled water (20 mL), and the lipid containing fraction was eluted with methanol (20 mL), evaporated under vacuum (Rotavapor, UK), and stored at -20°C until high performance liquid chromatography (HPLC).

Each sample was resuspended in HPLC solvent (methanol:distilled water:glacial acetic acid, 80:20:0.01 v/v/v, pH 5.4), eluted from a column (Techsphere C18, 250×4.6 mm), and fractions (1 mL) were collected for 20 min. An aliquot (800 µL) was removed from each fraction, added to Optiphase HiSafe 3 scintillant (4 mL) and counted for 2 min on a scintillation counter (Beckman LS6000). The remaining aliquots were evaporated under vacuum and placed under nitrogen at -20°C prior to radioimmunoassay (RIA). The LTE_4 peak was identified by comparison with a known unlabelled synthetic LTE_4 standard, and only the fractions containing the ^3H-LTE_4 peak of interest were assayed.

Urinary LTE_4 levels were determined by a competitive RIA using a specific $LTC_4/D_4/E_4$ antiserum (kind gift of B. Peskar, University of Graz, Austria). Fractions were resuspended in duplicate in Tris buffer containing 0.1% gelatine (w/v) (pH 7.6), and incubated overnight at 4°C with ^3H-LTE_4 (3 nCi) and antiserum. The unbound ^3H-LTE_4 was removed by dextran-coated charcoal and centrifugation, and bound ^3H-LTE_4 was quantified by scintillation counting of the supernatant. The LTE_4 content of each sample was interpolated from a standard curve (range 8–4,096 pg), with 50% binding occurring at 100–140 pg/LTE_4. The LTE_4 levels were corrected for volume assayed, extraction losses and creatinine content, and expressed as the $pmol \cdot mmol^{-1}$ creatinine value. The theoretical lowest detection limit for urinary LTE_4 concentration was 20 $pmol \cdot mmol^{-1}$ creatinine.

Materials

Materials were obtained from the following sources: $LTC_4/LTD_4/LTE_4$ antiserum (kind gift of B. Peskar, University of Graz, Austria); unlabelled LTE_4 (Cascade Biochem Ltd, Reading, UK); ^3H-LTE_4 (specific activity 127 $Ci \cdot mmol^{-1}$) (Amersham International plc, Little Chalfont, UK); Optiphase HiSafe 3 scintillant and methanol (HPLC-grade) (Fisons Laboratories, Loughborough, UK); Mega

Table 1. – Clinical characteristics of the children with chronic lung disease (CLD) and the control group

	CLD group		Control group	
Admission				
Gestational age weeks#	29	(24–31)	29	(28–33)
Birth weight g#	1210	(740–1724)	1340	(770–1840)
Follow-up				
Age# months	7	(5–8)	7	(5–9)
Weight kg#	5.9	(5.2–6.6)	6.3	(5.6–7.6)
Height cm#	60	(56–65)	62	(59–65)
History of atopy†	2/9	(22)	1/8	(13)

#: Values are presented as the median, and range in parenthesis; †: absolute values, and percentage in parenthesis. There were no significant differences in any of the parameters listed above between the CLD and control group.

Bond-Elut C18 cartridges (Jones Chromatography, Mid-Glamorgan, UK); Tris hydrochloride, gelatine, dextran and activated charcoal (Sigma Chemical Co., Poole, UK); acetic acid (BDH Chemical Co., Poole, UK).

Statistical analysis

Previous studies in asthmatic and normal adults (TAYLOR *et al*. [22], 1989), and cystic fibrosis and normal children (SAMPSON *et al*. [21], 1990) have shown urinary LTE_4 levels to be $\log_{10}$ normally distributed by normal probability score (Minitab). Therefore, an unpaired Student's t-test was used on $\log_{10}$ normalized urinary LTE_4 values for each group, with the results expressed as the geometric mean (95% confidence intervals (95% CI)). A Mann-Whitney U-test was employed for comparisons between gestational and postnatal age, birth weight, height and weight at follow-up, and lung function in each group. The relationship between urinary LTE_4 levels and lung function measurements was examined using Pearson's correlation. Statistical significance was assumed if the p-value was less than 0.05.

Results

Patient history

Clinical characteristics of the CLD and control groups are shown in table 1. There was no significant difference in the gestational or postnatal age, birth weight, and weight and height at follow-up, between the CLD and control groups. The incidence of familial atopy was similar in both groups.

Lung function studies

The CLD group had evidence of more airway obstruction than the controls. Median (range) TGV values for the CLD group (38.5 (28–44) $mL \cdot kg^{-1}$; n=8) were significantly higher than the control group (31 (24–34) $mL \cdot kg^{-1}$; n=5) (p=0.02) (fig. 1a), and the R_{aw} values were significantly higher in the CLD infants when compared to the controls (54 (34–70) $cmH_2O \cdot L^{-1} \cdot s$; n=8 *versus* 32 (22–39) $cmH_2O \cdot L^{-1} \cdot s$; n=5, respectively) (p=0.01) (fig. 1b). The FRC values were also significantly higher in the CLD group (30 (26–33) $mL \cdot kg^{-1}$; n=9) than in the controls (27 (20–30) $mL \cdot kg^{-1}$; n=8) (p<0.005) (fig. 1c). The time to equilibration (median (range)) was significantly longer in the CLD infants (60 (45–60) s) than the controls (45 (45–60) s) (p<0.01). The median (range) FRC to TGV ratio did not differ significantly between the CLD (0.78 (0.69–0.93)) and control groups (0.85 (0.73–0.90)).

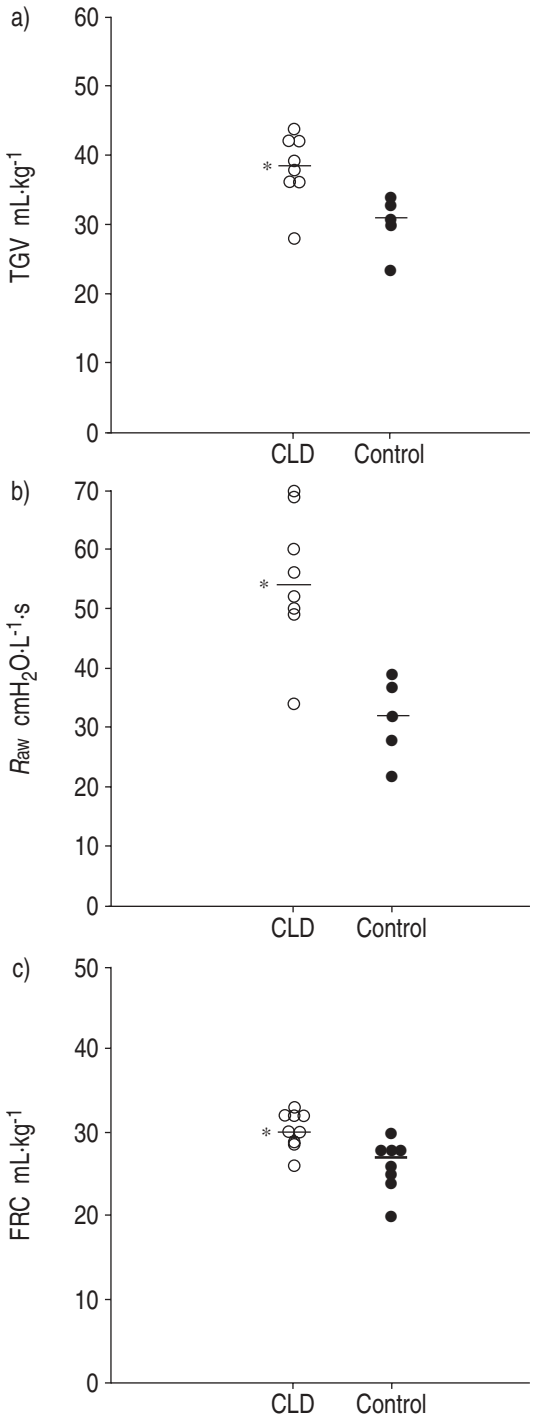

Fig. 1. – Individual: a) thoracic gas volume (TGV); b) airways resistance (R_{aw}); and c) functional residual capacity (FRC) values for the chronic lung disease (CLD) and control group. The median values are represented by the horizontal lines. *: p<0.05, as compared to the control infants (Mann-Whitney U-test).

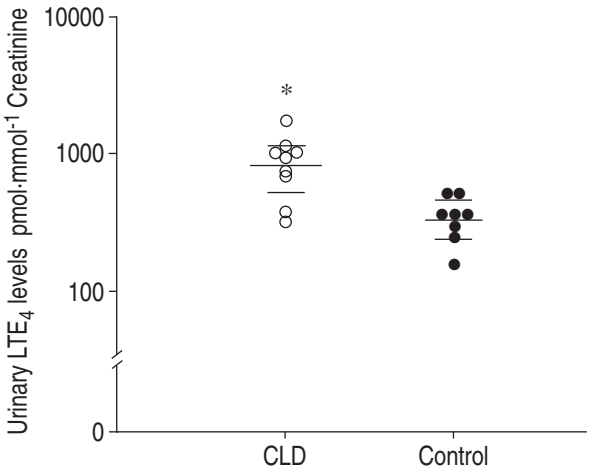

Fig. 2. – Individual urinary leukotriene E_4 (LTE_4) values for the chronic lung disease (CLD) and control group. The geometric mean and 95% confidence limits are represented by the three horizontal lines. Note the log scale for urinary LTE_4. *: $p<0.01$, as compared to the control infants (Student's unpaired t-test).

Urinary LTE_4 levels

The mean (±SEM) recovery of the internal ^{3}H-LTE_4 standard in the urine samples after purification was similar in both groups of premature infants, 52.7±5.0% in the CLD group (n=9) compared to 51.5±2.6% in the control group (n=8) (NS). Log_{10} normal distribution of urinary LTE_4 values was confirmed in both groups. Urinary LTE_4 levels are, therefore, expressed as the geometric mean (95% CI). The CLD group excreted significantly more urinary LTE_4 (741 (474–1,159) pmol·mmol^{-1} creatinine) than the control group (337 (240–473) pmol·mmol^{-1} creatinine) ($p<0.01$) (fig. 2). Creatinine excretion was similar in both groups (CLD 3.8 (2.0–6.5) mmol·L^{-1}; controls 4.4 (1.3–10.5) mmol·L^{-1} (NS)). Using the observed urinary LTE_4 values for the CLD and control group, the power to detect this difference was approximately 93%.

There were no correlations between log_{10} urinary LTE_4 levels and the duration of oxygen dependence (r=0.159; p=0.693; n=8), TGV (r=0.309; p=0.46; n=8), *R*aw (r=-0.063; p=0.88; n=8) and FRC (r=0.178; p=0.674; n=8) in the CLD group. However, there was a strong tendency for the duration of mechanical ventilation to correlate with log_{10} urinary LTE_4 levels (r=0.622; p=0.074; n=9), but this did not reach statistical significance.

Discussion

The CLD infants had significantly elevated TGV, *R*aw and FRC values, and excreted significantly more urinary LTE_4 when compared to the controls. However, no correlation was demonstrated between the lung function measurements and urinary LTE_4 in infants with CLD. There were no differences in the gestational and postnatal age, birth weight, and height and weight at follow-up, between the CLD and control infants. Family history of atopy was equally common in the CLD and control infants, a finding similar to CHAN *et al*. [23], (1988), but one which differs from NICKERSON *et al*. [24], (1980).

The apparent lack of correlation between urinary LTE_4 levels and lung function may be due to the small number of patients studied. Possible confounding factors may be the duration of mechanical ventilation or supplementary oxygen, involvement of other inflammatory mediators; or, alternatively, cysteinyl LTs may be playing a more indirect role in CLD, possibly in the development of airway hyperreactivity, which was not examined in the present study. There was no significant relationship between urinary LTE_4 levels and duration of ventilation or oxygen supplementation, suggesting these two factors were unlikely to affect cysteinyl LT production directly. Another possible complicating factor could be the heterogeneity of the CLD group with respect to treatment.

The TGV values of the CLD infants were significantly higher than the controls. In the presence of airway obstruction, it has been variously claimed that TGV may be over estimated [25] or underestimated [26]. In the present studies, to avoid errors, all measurements were made during quiet sleep [27], and occlusions were performed at end-inspiration [28] rather than at end-expiration [29]. We also found the FRC of the CLD infants to be higher than the controls. The former group, because of their higher airway resistance, would be predicted to have areas of lung with long time constants. The method of assessment of equilibration [2] used in this study, however, is likely to have afforded time for helium mixing in such units. As a consequence, the FRCs of the CLD infants were probably not underestimated and, although their FRC:TGV ratio was lower than that of the controls, the difference did not reach statistical significance.

Urinary LTE_4 production is widely used as a noninvasive indicator of total cysteinyl LT turnover *in vivo*. In the present study, LTE_4 levels were determined in spot urine samples obtained at the same time as lung function measurements. Until recently, the validity of LTE_4 measurement in spot urine samples, as opposed to 24 h samples, was unknown. However, in normal and stable asthmatic subjects there is no diurnal variation in basal endogenous LTE_4 excretion [30, 31], supporting the use of spot urine collection for cysteinyl LT analysis. Whether LT excretion differs in infants has not yet been investigated.

The levels of LTE_4 excreted in these premature infants are much higher than levels reported in normal children aged 1–13 yrs (88.4 (71.3–111) pmol·mmol^{-1} creatinine) [21]. This may be explained, in part, by the tendency for creatinine levels to be lower in the urine of infants (4.0 (1.3–10.5) mmol·L^{-1}) than older children (10.5 (1.7–34) mmol·L^{-1}). This would artificially raise the ratio of LT to creatinine. In the present study, enhanced urinary LTE_4 production in the CLD group could not be attributed to differences in creatinine excretion.

Two CLD infants had received ribavirin as part of a randomized, double-blind, placebo-controlled study investigating the effect of this agent on episodes of respiratory deterioration during the neonatal period. Neither of these patients had proven viral or bacterial infection. The exclusion of these patients, however, did not alter the significant differences in lung function or urinary LTE_4 between the CLD and control groups.

A recent study by SCHAUER *et al*. [32] (1994) comparing LTC_4 production in premature and term infants may help explain the observed increase in urinary LTE_4 production in infants with CLD. Eosinophils isolated

from premature children who subsequently developed bronchial hyperreactivity generated significantly more LTC_4 on stimulation with ionophore than premature infants who did not develop bronchial hyperreactivity.

Many studies support an involvement of inflammation in the early stages of CLD. Increased levels of soluble intercellular adhesion molecule-1, elastase and complement component C5a have been demonstrated in infants, at 10 days of age, who subsequently develop CLD [11, 12]. Similarly, PAF and LTs are present in tracheal lavage fluid, and LTE_4 in urine, of infants with CLD [9, 18]. The present results showing an enhanced urinary LTE_4 production at 7 months of age would tend to suggest that cysteinyl LTs were also involved in the sequelae of the disease.

Longitudinal studies examining pulmonary function and urinary leukotriene E_4 excretion in premature infants developing chronic lung disease and in premature infants with no lung disease are required to further clarify the association of enhanced cysteinyl leukotriene production with persisting airway abnormalities. If such studies reinforce the concept that leukotrienes are implicated in the pathogenesis of chronic lung disease, treatment with selective cysteinyl leukotriene antagonists and 5-lipoxygenase inhibitors may provide a novel approach in preventing or reducing chronic lung damage in ventilated premature infants.

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