Sputum tumour necrosis factor- α and leukotriene concentrations in cystic fibrosis

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Abstract

It is postulated that a vigorous host inflammatory response in the cystic fibrosis lung contributes to lung injury. Tumour necrosis factor- α (TNF- α) may play a part in that process and in the generation of leukotrienes. Therefore, the relationships between sputum TNF- α , leukotriene concentration, and lung function abnormalities in 16 children with cystic fibrosis were investigated. Each subject provided sputum samples and performed spirometry. TNF- α was measured by enzyme linked immunosorbent assay; individual leukotrienes were separated using high performance liquid chromatography and quantified by radioimmunoassay. The geometric mean concentration of TNF- α was 129.7 pg/ml and 95% confidence interval 48.2 to 348.3. Mean (SEM) leukotriene B₄ (LTB₄) was 97.8 (22.9) pmol/g and total cysteinyl leukotrienes were 60.9 (14.8) pmol/g. Mean (SD) forced expiratory volume in one second (FEV₁) of the group was 53 (15)% of predicted and forced vital capacity (FVC) was 65 (14)% of predicted. There was a significant positive correlation between TNF- α and both LTB₄ and the total cysteinyl leukotriene sputum content. An inverse relationship existed between TNF- α and FEV₁ and FVC. Moreover, a negative correlation was observed between sputum LTB₄ and FEV₁ and FVC. These results suggest that TNF- α and the leukotrienes may participate in the airways inflammation and airflow obstruction observed in cystic fibrosis subjects and support the hypothesis that TNF- α upregulates the 5-lipoxygenase pathway *in vivo*.

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the lung of cystic fibrosis patients and that it contributes to the enhanced generation of leukotrienes. We investigated whether TNF- α could be detected in sputa from those with cystic fibrosis, and if present, whether any relationship existed between it, leukotriene concentrations and the severity of airflow obstruction.

Patients and methods

Sixteen children (seven boys) who were attending the cystic fibrosis clinic at King's College Hospital or one of six outlying clinics were evaluated. Their mean age was 11.2 years, range 5.4–16.5 years. All the children had confirmed cystic fibrosis (sweat sodium concentration >70 mmol/l), were capable of expectorating sputum, and clinically stable. Sputum samples were sent for routine bacteriology. Forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC) were measured by spirometry and results were expressed as a percentage of that predicted for an individual's age, sex, and height.⁶ We excluded those patients taking regular oral or inhaled corticosteroids or theophylline because these compounds have potential immunomodulatory properties. For similar reasons, patients refrained from taking inhaled β_2 agonists for at least four hours before samples were collected. Two specimens of sputum, each approximately 1 ml in volume, were expectorated into separate sterile containers, then placed immediately in ice and stored at -70°C .

Informed written consent was obtained in all cases.

LEUKOTRIENE ANALYSIS

Leukotriene assay is a three stage procedure consisting of purification, separation, and quantification. These methods have been previously described and validated.⁵ Sputa were thawed on ice, weighed, and tritiated leukotrienes LTB₄, LTC₄, LTD₄, and LTE₄ were added before their homogenisation with 4 ml of ethanol for two minutes. All samples were left at 4°C for 30 minutes before debris and precipitated proteins were removed by centrifugation. The supernatants were evaporated to dryness in a vacuum, reconstituted in distilled water, and then purified on C₁₈ Sep-pak cartridges. Leukotriene containing material was eluted in methanol, evaporated to dryness, and stored under nitrogen at -70°C before reverse phase high performance liquid chromatography (HPLC). HPLC was performed using 5 μm C₁₈ analytical and guard columns with a mobile phase of methanol:water:acetic acid, 75/25/0.01 v/v/v, pH 5.6) at a flow rate of 1 ml/min.

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In cystic fibrosis, bacterial adherence occurs despite the presence of a vigorous host inflammatory response. It is widely believed that this process, in which polymorphonuclear leucocytes play a prominent part, contributes to lung injury. Tumour necrosis factor- α (TNF- α) is a monocyte/macrophage derived cytokine that is secreted in response to a variety of infectious and inflammatory stimuli. It is strongly chemotactic for neutrophils and can induce their degranulation.^{1,2} TNF- α upregulates the expression of vascular adhesion molecules *in vitro* and primes human 5-lipoxygenase *ex vivo*.^{3,4} Leukotrienes are arachidonic acid products of the 5-lipoxygenase pathway. They are present in sputum from patients with cystic fibrosis at concentrations capable of causing mucosal inflammation and bronchial lability.⁵

Therefore we postulated that TNF- α participates in the inflammatory response observed in

Figure 1 Association between sputum TNF- α and leukotriene concentrations.

Radioimmunoassay was performed for LTB₄, LTC₄, LTD₄, and LTE₄ using specific antibodies. Leukotriene immunoreactivity was corrected for the percentage tritium labelled internal standard recovered. Results are expressed in pmol/g weight of sputum.

TNF- α ESTIMATION

Before TNF- α estimation, mucolysis of specimens was achieved by adding an equal volume of *N*-acetylcysteine. Samples were then liquified using an Ultra-Turrax homogeniser and proteinaceous debris was removed by centrifugation. Immune reactive concentrations of TNF- α were assayed using a modified two site enzyme linked immunosorbent assay (ELISA) as previously described.⁷ The assay sensitivity was approximately 10 pg/ml. Aliquots of 100 μ l of monoclonal anti-TNF- α diluted in 100 mmol/l bicarbonate buffer (pH 9.5) to a concentration of 5 μ g/ml were coated onto ELISA plates. Plates were washed three times with phosphate buffered saline supplemented with 0.1% bovine serum albumin (BSA) and 0.01% Tween. Non-specific binding sites were blocked by incubation with phosphate buffered saline supplemented with 1% BSA. Aliquots of 100 μ l each of sputum supernatant, rabbit polyclonal antibody, and goat antirabbit immunoglobulin conjugated with horseradish peroxidase were added sequentially. Assay wells were rinsed three times with buffer after incubation with each new reagent. Procedures were performed at room temperature and ELISA plates were shaken gently throughout. The reaction was developed using 100 μ l of substrate (0.4 mg/ml *o*-phenylenediamine in citrate phosphate buffer (pH 5.0) containing 4 μ l/ml of 3% hydrogen peroxide). Termination was achieved using 50 μ l of 4N sulphuric acid

and optical density read in a Dynatech MR 700 automated plate reader at 490 nm. Specimens were analysed in duplicate and unknown values read from standard curves constructed using recombinant TNF- α . The rabbit polyclonal antibody used suppressed the necrotic effect of recombinant TNF- α on a L-929 tumour cell line, thereby confirming its specificity (unpublished data).

STATISTICS

The distribution of the data was assessed by plotting normal probability scores. TNF- α concentration was log normally distributed while LTB₄ and the cysteinyl leukotrienes were normally distributed. The strength of the relationship was assessed using Pearson's correlation coefficient and significance was determined by linear regression analysis, using the least squares method. The 'Minitab' statistical software program was used to perform all analyses.

Results

Ten children were chronically colonised with mucoid strains of *Pseudomonas aeruginosa* only, one grew both *P. aeruginosa* and *Staphylococcus aureus*, two grew *S. aureus* only, and three had sterile sputum cultures. Mean (SD) FEV₁ of the group was 53 (15)% of predicted and FVC was 65 (14)% of predicted. Geometric mean TNF- α was 129.7 pg/ml with a 95% confidence interval of 48.2 to 348.3. Mean (SEM) LTB₄ was 97.8 (22.9) pmol/g and total cysteinyl leukotrienes were 60.9 (14.8) pmmol/g.

There was a significant positive correlation between TNF- α and both LTB₄ ($r=0.62$, $p<0.01$; fig 1) and the total cysteinyl leukotriene

Figure 2 Association between sputum TNF- α and severity of airflow obstruction.

Individual patient results ($n=16$)

Age (years)	Sputum	FEV ₁ (% predicted)	FVC (% predicted)	TNF- α (pg/ml)	LTB ₄ (pmol/g)	Total cysteinyl leukotrienes (pmol/g)
6	Sterile	71	72	49	56	98
8	Sterile	71	72	110	79	118
10	Sterile	63	65	388	263	26
9	<i>S aureus</i>	24	44	1065	268	65
6	<i>S aureus</i>	38	52	1988	129	135
9	Mucoid strains of <i>P aeruginosa</i> + <i>S aureus</i>	51	89	229	10	17
11	Mucoid strains of <i>P aeruginosa</i>	34	45	803	102	229
8	Mucoid strains of <i>P aeruginosa</i>	65	81	129	65	46
15	Mucoid strains of <i>P aeruginosa</i>	70	92	10	24	17
5	Mucoid strains of <i>P aeruginosa</i>	41	52	372	30	37
16	Mucoid strains of <i>P aeruginosa</i>	49	70	10	9	9
10	Mucoid strains of <i>P aeruginosa</i>	65	73	80	54	34
10	Mucoid strains of <i>P aeruginosa</i>	59	71	91	107	10
12	Mucoid strains of <i>P aeruginosa</i>	64	65	189	69	59
16	Mucoid strains of <i>P aeruginosa</i>	32	50	621	273	55
16	Mucoid strains of <i>P aeruginosa</i>	55	60	12	20	11

sputum content ($r=0.52$, $p<0.05$; fig 1) and a significant inverse relationship existed between TNF- α and FEV₁ ($r=-0.58$, $p=0.02$; fig 2) and FVC ($r=-0.62$, $p<0.01$; fig 2). A negative correlation was observed between sputum LTB₄ and FEV₁ ($r=-0.47$, $p=0.06$) and FVC ($r=-0.56$, $p=0.02$).

Individual patient details are shown in the table.

Discussion

The hypothesis that TNF- α may be orchestrating the exuberant inflammatory response observed in acute lung injury is supported by the observations that TNF- α is secreted after bolus administration of endotoxin in the porcine model of acute lung injury⁸ and that considerable quantities have been detected in the broncho-alveolar lavage fluid from patients with adult respiratory distress syndrome.⁹

Norman *et al* have demonstrated that concentrations of TNF- α are higher in plasma from patients with cystic fibrosis than in normal controls and that its concentration is increased during episodes of acute respiratory infection.¹⁰ It was not clear whether this represented a spillover from the inflamed airways or activation of blood monocytes as they passed through the pulmonary circulation. We have found TNF- α in cystic fibrosis sputa, during times of clinical stability, at concentrations that have been shown to produce neutrophil migration, respiratory burst, and degranulation in vitro.¹² This provides evidence in favour of the hypothesis that lung damage, due to chronic infection, is occurring during periods of apparent wellbeing. Normal children do not produce sputum and even during periods of acute respiratory infection, sputum specimens are notoriously difficult to obtain. Therefore, it was not possible to obtain a control group, thus we were denied comparative information about TNF- α and leukotriene concentrations in normal individuals.

Naturally occurring inhibitors of TNF- α are known to occur in serum¹¹; they have not yet been described in sputum. In the absence of a confirmatory bioassay, we can only speculate about the biological activity of the cytokine that we detected. However, the significant correla-

tion between TNF- α and the severity of airflow obstruction suggests that it was active. Moreover, the polyclonal antibody that we used neutralised the effect of recombinant TNF- α in vitro.

Cell wall lipopolysaccharides from Gram negative bacteria can induce cytokine production in vitro.¹² It is feasible that lipopolysaccharides derived from *P aeruginosa* are an important stimulus to TNF- α secretion in vivo and that this augments the massive influx of neutrophils observed within the airway of patients with cystic fibrosis. In addition, pyocyanin produced by *P aeruginosa* can delay the inactivation of LTB₄ by omega oxidation in vitro.¹³ This process, if present in vivo, may potentiate the cycle of neutrophil accumulation and activation. Although the study was not designed to address this issue in vivo, we did not identify any association between sputum pathogens and LTB₄ concentration (table).

Elastases, derived from neutrophils and mucoid strains of *P aeruginosa*, are present in cystic fibrosis secretions and may participate in the destructive process.¹⁴ They have the potential to degrade TNF- α which may explain why TNF- α was present, in lower than expected quantities, in three patients who were chronically colonised with mucoid strains of *P aeruginosa*. What is more difficult to explain is why the two patients colonised with *S aureus* had such high concentrations of TNF- α . However, *S aureus* is known to possess toxins and cell wall components that are capable of inducing TNF- α secretion by mononuclear phagocytes in vitro.^{15 16}

The leukotrienes are present in concentrations sufficient to exert potent biological effects on bronchial smooth muscle tone, mucous secretion, and airway inflammation.⁵ Unlike the ubiquitous cyclo-oxygenase pathway, 5-lipoxygenase activity is largely restricted to effector cells of the myeloid lineage. Though the precise cellular origin of leukotrienes in cystic fibrosis is not known, leucocytes are the leading candidate source. However, airway epithelia cannot be excluded and it has been recently reported that stimulated bovine epithelial cells can express chemotactic activity identical to that of LTB₄.¹⁷ Leukotrienes are not preformed or stored, and stimulation of 5-lipoxygenase is necessary for their synthesis. Therefore, they cannot be simply regarded as products of leucocyte degranulation or as a reflection of numbers of polymorphonuclear leucocytes. Neutrophils produce only LTB₄, and mast cells and eosinophils selectively produce the cysteinyl leukotrienes.¹⁸ TNF- α correlated directly with both LTB₄ and the cysteinyl leukotrienes, a finding that supports the concept that generalised 5-lipoxygenase priming by TNF- α occurs in vivo. Pulmonary macrophages can also produce large quantities of TNF- α and LTB₄ and much smaller quantities of LTC₄. Therefore, they cannot be excluded as the single source of all these mediators. Sputum leukotriene concentration correlates significantly with the Chrispin-Norman score of radiological abnormality.¹⁹ In the present study, LTB₄, which is itself a potent chemoattractant for neutrophils, also correlated with parameters of airflow obstruction,

suggesting that it too participates in airway inflammation.

Abnormalities of cyclo-oxygenase pathway products have also been described in cystic fibrosis. Prostaglandins and thromboxanes have important vascular and smooth muscle effects that may also contribute to the host inflammatory response.^{20,21} These findings suggest that excessive stimulation of phospholipase A₂ is occurring, and thereby increasing arachidonic acid availability for both 5-lipoxygenase and the cyclo-oxygenase pathways. In cystic fibrosis other intracellular metabolic functions appear disturbed and it is tempting to speculate that the cystic fibrosis gene subserves cellular functions other than the regulation of apical chloride channel permeability. Some authors have postulated that increased arachidonic acid availability is one component of the basic genetic defect in cystic fibrosis.²² However, the finding that recombinant TNF- α can prime human phospholipase A₂ to produce more lipid mediators in vitro²³ would be consistent with the concept that increased arachidonic acid turnover in cystic fibrosis is caused by a cytokine mediated upregulation of phospholipase A₂. The association we found between TNF- α concentration and disease severity suggests that this is an acquired response to chronic bacterial infection rather than indicative of an underlying genetic defect.

Correlation between variables does not prove a causal relationship and further investigation of this association will be necessary. However, our results support the hypothesis that TNF- α upregulates the production of the leukotrienes, and that they both do contribute to the pathophysiology of airways inflammation in cystic fibrosis. With the development of 5-lipoxygenase inhibitors, specific leukotriene receptor antagonists and cytokine antagonists, alternative therapeutic strategies may become available for selected patients with cystic fibrosis particularly those with an established cycle of bacterial colonisation and pulmonary inflammation.

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