Persistent increase in plasma and urinary leukotrienes after acute asthma

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Abstract
Leukotrienes may mediate bronchoconstriction in asthma. Cysteinyl leukotriene production rises in vivo after allergen challenge, but few reports describe leukotriene concentrations in clinical asthma or in children. Using high performance liquid chromatography/radioimmunoassay, plasma and urinary leukotrienes in asthmatic children (aged 5–10 years) were measured during an acute exacerbation (peak expiratory flow (PEF) <65%, n=10) and one month later (PEF 74–169%, n=9), and in non-atopic normal children (aged 1.3–13.2 years). In the asthmatics, geometric mean (95% confidence interval) plasma leukotriene B4 (LTB4) was 746 pg/ml (398 to 1403) acutely and 1026 pg/ml (662 to 1593) in remission, compared with 369 pg/ml (167 to 728) in the normal children (n=14). Plasma cysteinyl leukotrienes were low or undetectable, but urinary leukotriene E4 (LTE4) was higher in the asthmatics during an acute episode (210 pmol/mmol creatinine, 101 to 454) and at follow up (179 pmol/mmol, 110 to 293), compared with the normal children (98 pmol/mmol, 81 to 118, n=41). This persistent increase in plasma LTB4 and urinary LTE4 concentrations one month after a severe asthmatic episode suggests leukotriene production is related to chronic inflammation rather than to acute bronchoconstriction.

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It is increasingly accepted that products of lymphocytes of the Th2 subtype including interleukin (IL)-5 and IL-3 may regulate the inflammatory activity of eosinophils and mast cells within the asthmatic lung.1 These cells can generate a variety of mediators, toxic enzymes, and oxygen radicals which may account for the airflow obstruction, epithelial damage, and airway hyperresponsiveness of asthma.

Among these products are the lipid mediators cysteinyl leukotrienes (leukotriene C4 (LTC4), leukotriene D4 (LTD4), and leukotriene E4 (LTE4)), which abundant evidence implicates in airflow obstruction in asthma.2 They are potent inducers of bronchoconstriction,3 mucus hypersecretion,4 and airway oedema.5 LTE4 concentrations, used as a marker of whole body cysteinyl leukotriene production, rise after challenge of asthmatics with inhaled allergen6 and exercise.7 Cysteinyl leukotriene receptor antagonists inhibit both early and late bronchoconstrictor responses to allergen and block the associated increase in bronchial responsiveness,8 and they improve lung function and reduce symptoms in chronic asthma.9

In contrast, the possible role of leukotriene B4 (LTB4) in asthma is unclear. However, it is the most potent lipid chemotaxin known for neutrophils, which are implicated in sudden onset fatal asthma10 and in nocturnal asthma,11 and also chemotaxins monocytes, lymphocytes, and eosinophils.12 In vitro, LTB4 induces Th2 lymphocyte production of IL-5,13 which may promote eosinophilia in asthma and atopy, and augments the stimulatory effects of IL-4 on immunoglobulin E production by B lymphocytes.14 LTB4 can be generated by a range of cells within the lung, including mast cells and macrophages,15,16 and has been implicated in neutrophil infiltration after segmental allergen challenge in the human lung.17 LTB4 has been reliably detected in the bronchoalveolar lavage fluid of asthmatic subjects.18

Previous work by our group has shown a two to fivefold increased capacity for LTB4 and LTD4 generation in vitro by the peripheral blood polymorphonuclear leucocytes of stable atopic asthmatic subjects stimulated by calcium ionophore or formyl-met-leu-phe.19,20 In vivo, such an exaggerated leukotriene synthetic response to immunological stimulation within the asthmatic lung might contribute significantly to bronchoconstriction, chronic inflammation, and bronchial hyperresponsiveness.21

We aimed therefore to use combined high performance liquid chromatography (HPLC)/radioimmunoassay techniques to assay LTB4, LTD4, and LTE4 in plasma, and LTE4 in the urine, of asthmatic children admitted to hospital with an acute exacerbation, and at follow up at least one month later after clinical improvement. Attempts were made to reduce the possible confounding effects of anti-inflammatory medication, and concentrations were compared with those in a control group of normal children with no personal or family history of allergic disease.

Subjects and methods

CLINICAL CHARACTERISTICS OF SUBJECTS
Permission for the study (No B81/89) was obtained from the ethics committee of King’s College Hospital. Ten children with acute asthma (aged 5–10 years) were admitted with acute dyspnœa and wheezing. Peak expiratory flow (PEF) was <65% of predicted, with nine

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out of 10 having PEF <40% predicted. All had taken inhaled β2-agonists, but only one out of 10 was on inhaled corticosteroids, two were receiving sodium cromoglycate, and none were receiving theophyllines. All had a close family history of atopy, and skin prick tests were positive (two or more allergens) in four subjects tested. Blood and urine samples were taken before treatment with systemic steroids was begun.

Nine of the 10 asthmatic children provided blood and urine samples at follow up at least one month after the acute episode. All were well with PEF 74–169% of predicted. Although all had received systemic corticosteroid treatment for five days after admission, none had received systemic steroids for at least 25 days preceding the follow up visit. Only two out of nine children were receiving inhaled corticosteroids, and none had received theophyllines for at least 14 days.

Normal children (aged 1.3–13.2 years) with negative personal and family histories of atopy provided blood (n=14) or urine (n=41) as controls. All were healthy with no history of chronic respiratory disease and none were receiving medication.

SAMPLE COLLECTION
Blood (10 ml) was taken into a heparinised syringe containing the 5-lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA; 50 μM; Sigma) and the cysteinyi leukotriene bioconversion blockers L-serine-borate (30 mM) and L-cysteine (10 mM), to prevent generation or catabolism of leukotrienes in vitro. Urine was collected into a sterile container on ice and an aliquot sent for creatinine determination.

LEUKOTRIENE ASSAYS
Leukotrienes were assayed as described by us. Briefly, samples are spiked with tritiated (3H) leukotriene internal standards before methanol extraction and partial purification on octadecylsilane cartridges (Sep-Pak, Waters). Leukotrienes are separated by high performance liquid chromatography using a C18 column (Tecsphere 250×46 mm) with a methanol/water/acetic acid (75/25/0.1; pH 5.6) solvent system, and quantified by radioimmunoassay.

STATISTICAL ANALYSES
Power calculations on previous data from normal children suggested that differences in geometric mean urinary LTE4 concentrations of approximately twofold would be detectable with 80–90% probability in groups of 12–15 subjects each. No reliable data were available with which to perform similar calculations for plasma leukotriene comparisons. Plasma and urinary leukotriene concentrations approximated most closely to log10 normal distributions, and values are therefore given as the geometric mean and 95% confidence interval (CI). Comparisons between groups were made by paired or unpaired Student’s t tests on log normalised values.

Results

LTB4 CONCENTRATIONS IN PLASMA
Plasma LTB4 concentrations (fig 1) were above detection limits in all subjects except one normal. Geometric mean (95% CI) plasma LTB4 concentrations were 746 pg/ml (398 to 1403) in the asthmatic subjects during the acute exacerbation (n=10), twice that in the normal subjects (geometric mean 369 pg/ml, 95% CI 167 to 728; n=14); however, this did not reach statistical significance (p=0.097). Geometric mean plasma LTE4 concentrations rose further at follow up to 1026 pg/ml (662 to 1593; n=9), which was significantly higher than in the normal subjects (p=0.012).

CYSTEINYL LEUKOTRIENE CONCENTRATIONS IN PLASMA
Plasma concentrations of LTC4 and LTD4 were below detection limits (<50 pg/ml) in all asthmatic patients studied (n=6) both acutely and at follow up, and in all normal subjects studied (n=6; data not shown).

Plasma concentration of LTE4, however, were detected in 11 out of 14 normal subjects, and in all asthmatic patients both acutely and in remission (fig 2). In the asthmatics, geometric mean (95% CI) plasma LTE4 concentrations were 314 pg/ml (191 to 517) during the acute exacerbation (n=10) and 348 pg/ml (189 to 643) at follow up (n=9). Neither of these concentrations was significantly greater (p>0.3) than the plasma LTE4 concentration in normal subjects (geometric mean 232 pg/ml, 95% CI: 132 to 406; n=14). The high variability in the normal plasma LTE4 concentrations means that the power of the study to detect small group differences is relatively low. However, an enhancement of the same magnitude as that observed with LTB4 (2-9-fold),
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Overproduction later an acute normal than normal in the acute asthmatic episode, and were significantly higher than normal at the one month follow up. This supports the concept of an involvement of LTBA in chronic inflammation in asthma which may be up-regulated by an acute exacerbation.

Our previous findings that both LTBA and LTE4 are highly stable in whole blood in vitro, suggested these leukotrienes as the targets of choice for leukotriene measurement in the circulation. However, leukotrienes are generated in vivo in very small molar quantities, and biological fluids contain non-specific immunoreactivity that interferes with immunoassays. The importance of internal radio-labelled leukotriene standards, solid phase extraction on C18 cartridges, and HPLC to purify leukotrienes in biological fluids before immunoassay has been well documented.

Many early studies of leukotriene concentrations in plasma did not fulfil these methodological requirements, so that early reports of raised concentrations of cysteinyl leukotrienes and of LTBA in the plasma of asthmatics must now be treated with caution. In our studies LTC4 and LTD4 were undetectable (<50 pg/ml) in all subjects, but LTE4 and LTBA were detected in most normal and asthmatic subjects at concentrations similar to those found by other workers using HPLC/radioimmunoassay techniques.

However, venous plasma LTE4 was relatively low and highly variable, and failed to fully reflect the significantly increased cysteinyl leukotriene production in the asthmatic children that was observed in urinary LTE4 concentrations. Moreover, plasma LTE4 concentrations did not correlate with urinary LTE4. This lack of sensitivity suggests that plasma LTE4 measurements may not be useful as an adjunct to LTE4 urinalysis.

LTE4 concentrations in urine are used as a marker of whole body production of cysteinyl leukotrienes, and we attempted to relate plasma and urine LTE4 values in each subject group. In the normals, log10 plasma LTE4 and log10 urine LTE4 were not significantly correlated (r=-0.26, p=0.5; n=14). Similarly, in the asthmatic subjects, plasma and urine LTE4 concentrations correlated neither during the acute exacerbation (r=-0.061, p=0.9; n=10) nor at follow up (r=-0.053, p=0.9; n=9).

Discussion

We have demonstrated that production of cysteinyl leukotrienes, as reflected in urinary LTE4 concentrations, is significantly higher than normal in asthmatic children not only during an acute asthmatic episode, but also a month later when lung function has returned to normal. Overproduction of these potent bronchoconstrictor mediators in asthma is paradoxically therefore not directly related to acute bronchoconstriction, but may rather reflect an ongoing inflammatory process in the asthmatic lung with a role in bronchial hyperresponsiveness. Moreover, we have demonstrated that plasma values of the pro-inflammatory compound LTBA tended to be higher than in normals during the acute
increase in urinary LTE\textsubscript{4} in susceptible asthmatic adults after challenge with allergen, exercise, and aspirin. Relatively few studies have examined urinary LTE\textsubscript{4} excretion in relation to the variable lung function of clinical asthma. Taylor and colleagues found increased urinary LTE\textsubscript{4} in 20 adult asthmatics admitted to hospital for an acute exacerbation. Only eight of these asthmatics were re-examined at follow up, and in these subjects urinary LTE\textsubscript{4} concentrations had not fallen significantly, despite systemic corticosteroid and theophylline treatment resulting in a return to normal lung function. Increased urinary LTE\textsubscript{4} has also been found in a significant proportion of stable adult asthmatics. In our study, urinary LTE\textsubscript{4} concentrations were approximately double normal values both acutely and in remission in asthmatic children suggesting chronic overproduction of the cysteiny1 leukotrienes.

There is overwhelming evidence for chronic inflammation in the bronchial mucosa even in mild asthma. Involvement may be inflammatory processes and a variety of resident cells, including mast cells and macrophages, and infiltrating cells, such as eosinophils, which are capable of generating leukotrienes in response to IgE dependent stimulation. The source of the enhanced plasma LTB\textsubscript{4} and urinary LTE\textsubscript{4} we have observed cannot be ascribed with certainty to any one cell type. However, chronic overproduction of LTC\textsubscript{4} and LTB\textsubscript{4} by cells stimulated by persistent inhalation of environmental allergens might have profound effects by inducing bronchoconstriction and airway inflammation. The cysteiny1 leukotrienes may impede airflow by constricting bronchial smooth muscle, inducing mucus secretion, and promoting airway oedema, but in addition, LTD\textsubscript{4} has recently been shown to be a highly potent and specific chemotaxin for human eosinophils in vitro, and inhaled LTE\textsubscript{4} may also directly induce the eosinophil infiltration characteristic of the asthmatic lung. The putative source of LTD\textsubscript{4} is more problematic, as mast cells can generate only limited amounts, while we have shown bronchoalveolar lavage cells (alveolar macrophages) to have a markedly downregulated capacity for LTB\textsubscript{4} synthesis in vitro in mild asthma. Nevertheless, long term production of LTB\textsubscript{4} within the bronchial mucosa may cause infiltration of neutrophils and monocytes into the lung, and the chemotactic potency of LTB\textsubscript{4} towards eosinophils is often overlooked. Moreover, eosinophils primed by specific factors such as IL-5 respond readily to the non-specific chemotaxins LTB\textsubscript{4} and IL-8, and LTB\textsubscript{4} may itself promote the production of IL-5 from T lymphocytes.

Our work supports the concept that cysteiny1 leukotrienes may have a role in the chronic inflammation in the bronchial mucosa in asthma, and that this may underlie the early indications of an anti-inflammatory and steroid sparing effect of cysteiny1 leukotriene receptor antagonists in current clinical trials. The possibility of an involvement also of LTB\textsubscript{4} in chronic asthma in children strengthens the case for the further development of 5-lipoxygenase inhibitors which may counteract the effects of both classes of leukotriene. Further studies are called for to investigate the effects of 5-lipoxygenase inhibitors on leukotriene production in long term studies in health and disease.

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