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 chemoattracts 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 LTC₄ 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, LTC₄, LTD₄, and LTE₄ in the 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 dyspnoea 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 β_2 -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 μ M; Sigma) and the cysteinyl 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.²² Briefly, samples are spiked with tritiated (3 H) 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 (Techsphere 250 $\times$ 46 mm) with a methanol/water/acetic acid (75/25/0.01; 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 LTB₄ 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),

Figure 1 Concentrations of LTB₄ (log scale) in the plasma of asthmatic patients during an acute exacerbation and in remission, and in normal children. Horizontal bars indicate geometric mean and 95% CI; dotted line indicates detection limit of the assay. LTB₄ concentrations tended to be higher in the asthmatics acutely ($p=0.097$) than in the normals, and were significantly higher in the asthmatics in remission ($p=0.012$).

Figure 2 Concentrations of LTE₄ (log scale) in the plasma of asthmatic patients during acute exacerbation and in remission, and in normal children. Horizontal bars indicate geometric mean and 95% CI; dotted line indicates detection limit of the assay. There were no significant differences between the groups.

would be detectable in plasma LTE₄ concentrations with a power of approximately 75%; the probability of a significant type II error is therefore low.

URINARY LTE₄ CONCENTRATIONS

In contrast to the plasma LTE₄ results, a significant increase in urinary LTE₄ was apparent in the asthmatic subjects (fig 3). Geometric mean (95% CI) urine LTE₄ concentration was 219 pmol/mmol creatinine (101 to 454) during the acute exacerbation (n=10) and 179 pmol/mmol creatinine (110 to 293) at follow up (n=9), both significantly higher than in the normal children (geometric mean 97.7, 95% CI: 81 to 118; n=41) ($p=0.0025$ and $p=0.0085$ respectively).

As plasma and urinary LTE₄ concentrations may both reflect whole body production of cysteinyl leukotrienes, 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 LTB₄ tended to be higher than in normals during the acute

asthmatic episode, and were significantly higher than normal at the one month follow up. This supports the concept of an involvement of LTB₄ in chronic inflammation in asthma which may be up-regulated by an acute exacerbation.

Our previous findings that both LTB₄ and LTE₄ are highly stable in whole blood *in vitro*^{23 24} 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 radiolabelled leukotriene standards, solid phase extraction on C18 cartridges, and HPLC to purify leukotrienes in biological fluids before immunoassay has been well documented.^{25 26} Many early studies of leukotriene concentrations in plasma did not fulfil these methodological requirements, so that early reports of raised concentrations of cysteinyl leukotrienes²⁷⁻²⁹ and of LTB₄³⁰ 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 LTB₄ were detected in most normal and asthmatic subjects at concentrations similar to those found by other workers using HPLC/radioimmunoassay techniques.³¹ 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 because a fixed proportion (4-6%) of ³H-LTC₄ infused intravenously in man emerges as ³H-LTE₄ in the urine within four hours, irrespective of the dose administered.³² Several studies have described an

Figure 3 Concentrations of LTE₄ (log scale) in the urine of asthmatic patients during acute exacerbation and in remission, and in normal children. Horizontal bars indicate geometric mean and 95% CI; dotted line indicates detection limit of the assay. Urinary LTE₄ concentrations were significantly higher in the asthmatics both acutely ($p=0.003$) and in remission ($p=0.009$) compared with the normals.

increase in urinary LTE_4 in susceptible asthmatic adults after challenge with allergen,^{6 33 34} exercise,⁷ and aspirin.³⁵ Relatively few studies have examined urinary LTE_4 excretion in relation to the variable lung function of clinical asthma. Taylor and colleagues found increased urinary LTE_4 in 20 adult asthmatics admitted to hospital for an acute exacerbation.⁶ Only eight of these asthmatics were re-examined at follow up, and in these subjects urinary LTE_4 concentrations had not fallen significantly, despite systemic corticosteroid and theophylline treatment resulting in a return to normal lung function. Increased urinary LTE_4 has also been found in a significant proportion of stable adult asthmatics.³³ In our study, urinary LTE_4 concentrations were approximately double normal values both acutely and in remission in asthmatic children suggesting chronic overproduction of the cysteinyl leukotrienes.

There is overwhelming evidence for chronic inflammation in the bronchial mucosa even in mild asthma. Involved in this inflammatory process is 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_4 and urinary LTE_4 we have observed cannot be ascribed with certainty to any one cell type. However, chronic overproduction of LTC_4 and LTB_4 by cells stimulated by persistent inhalation of environmental allergens might have profound effects beyond immediate bronchoconstriction. The cysteinyl leukotrienes may impede airflow by constricting bronchial smooth muscle, inducing mucus secretion, and promoting airway oedema, but in addition, LTD_4 has recently been shown to be a highly potent and specific chemotaxin for human eosinophils in vitro,³⁶ and inhaled LTE_4 may also directly induce the eosinophil infiltration characteristic of the asthmatic lung.³⁷ The putative source of LTB_4 is more problematic, as mast cells can generate only limited amounts, while we have shown bronchoalveolar lavage cells (80% alveolar macrophages) to have a markedly down-regulated capacity for LTB_4 synthesis in vitro in mild asthma.³⁸ Nevertheless, long term production of LTB_4 within the bronchial mucosa may cause infiltration of neutrophils and monocytes into the lung, and the chemotactic potency of LTB_4 towards eosinophils^{12 39} is often overlooked. Moreover, eosinophils primed by specific factors such as IL-5 respond readily to the non-specific chemotaxins LTB_4 and IL-8,⁴⁰ and LTB_4 may itself promote the production of IL-5 from T lymphocytes.¹³

Our work supports the concept that cysteinyl 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 cysteinyl leukotriene receptor antagonists in current clinical trials. The possibility of an involvement also of LTB_4 in chronic asthma in children strengthens the case for the further development of 5-

lipoxygenase inhibitors which may counteract the actions 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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