Allergology International (2001) **50**: 21–28

Original Article

Sulfidopeptide leukotrienes, but not thromboxane B_2 or histamine, are elevated in sputum during exacerbation of asthma

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Abstract

Although sulfidopeptide leukotrienes (sLT) are considered to play an important role in the pathogenesis of asthma, their precise action has not been elucidated in asthmatics during exacerbation. In the present study, we examined sputum concentrations of sLT from asthmatic patients in order to determine whether sLT are actively involved in the exacerbation of asthma. We also examined sputum levels of thromboxane (TX) B_2 and histamine because these mediators are considered to be as important as sLT. The induced sputa by 3% hypertonic saline inhalation were treated with high-performance liquid chromatography and levels of sLT, TXB_2 and histamine were measured with enzyme immunoassay kits. These compounds tended to be elevated in asymptomatic asthmatic patients compared with healthy controls, but the differences were not significant. Levels of sLT and TXB_2 showed no difference between atopic and non-atopic patients, but histamine levels were higher in atopic patients than in non-atopic patients. However, sputa during the exacerbation contained significantly higher levels of sLT than those during the asymptomatic state. In contrast, neither histamine nor TXB_2 showed any changes with exacerbation. These results suggest that sLT may be one of the most potent for mounting the exacerbation of asthma.

Key words: asthma, histamine, sputum, sulfidopeptide leukotrienes, thromboxane B_2.

Introduction

Recently, it has been established that allergic inflammation is the most important in the pathogenesis of asthma. Many investigators have been analyzing the inflammatory status of airways in asthmatics using techniques such as bronchoalveolar lavage (BAL) and transbronchial lung biopsy (TBLB). However, because these methods are invasive, they are hardly repeated or performed during the exacerbation of asthma. Analysis of sputum is a well-established and commonly used technique for evaluating asthmatics.[1] Mediators and albumin levels were found to be higher in induced sputum compared with BAL fluid. However, mediators and albumin levels in sputum were closely correlated with those in BAL. In addition, the analysis of induced sputum revealed information qualitatively similar to that obtained by analysis of BAL.[2] Finally, many inflammatory cytokines have been measured in sputum from asthmatic patients.[3]

It is well known that leukotrienes (LT) are potent inflammatory mediators, producing acute bronchoconstriction and airway hyperresponsiveness to methacholine or histamine.[4–7] Increased numbers of eosinophils and neutrophils in the airway mucosa have been reported after the inhalation of LTE_4[8] or LTD_4.[9] However, analyses of these potent inflammatory mediators, sulfidopeptide-leukotrienes (sLT) such as LTC_4, LTD_4 and LTE_4, have been

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Received 5 November 1999. Accepted for publication 1 September 2000.

rarely performed in the sputum, partly because these lipid mediators are unstable and are rapidly metabolized, making their measurement difficult. These lipid mediators may serve as markers reflecting the clinical condition of asthma. We have examined expired nitric oxide (NO) from airways[10] and eosinophil cationic protein (ECP) in the peripheral blood as markers of airway inflammation in asthmatic patients, but none showed a relationship with asthma symptoms. Pranlukast, a new sLT receptor antagonist, has been reported to be remarkably effective in improving the mean peak expiratory flow rate (PEFR), forced expiratory volume in 1 s (FEV_1), and symptom score in a randomized double-blind placebo-controlled multicenter study in Japan,[11] Europe[12] and the US.[13] Therefore, we suspect that sLT are important mediators of asthma, affecting the severity of inflammation and airway hyperreactivity. Accordingly, we examined sLT levels in sputum obtained from asthmatic patients during exacerbation and asymptomatic status, as well as from healthy controls. Because it has been shown that seratrodast, a thromboxane (TX) A_2 receptor antagonist, could improve asthmatic symptoms,[14] we also investigated concentrations of TXB_2, a metabolite of TXA_2, and histamine in sputum.

METHODS

Subjects

Sixty-six patients with asthma and 12 healthy subjects were enrolled in this study (Table 1). Asthmatic patients were diagnosed on the basis of evidence of reversible airway obstruction and airway hypersensitivity and were treated in the Department of Allergy, National Minamiokayama Hospital. Healthy controls who participated in this study had no history of pulmonary, cardiovascular or nasal disorders. All subjects were non-smokers. Informed consent was obtained from all patients and healthy controls and the study was approved by the institutional review board.

Patients enrolled in this study were in a stable condition, with neither dyspnea nor wheeze. In some patients, sputa were also collected during exacerbation. Exacerbation was defined as a condition with symptoms, wheeze and dyspnea or 20% decrease of PEFR.

The mean (±SD) age of asthmatic patients was 50.3 ± 15.7 years, similar to controls, who were aged 46.8 ± 11.8 years. Of the asthmatic patients, 37 were atopic (20 male and 17 female; mean age 45.5 ± 16.7 years) and 29 were non-atopic (11 male and 18 female; mean age 56.1 ± 12.1 years). Thirteen atopic patients had mild disease, 18 had moderate disease and six had severe disease. One of the non-atopic asthmatics had mild disease, nine had moderate disease and 19 had severe disease. Patients who had allergic rhinitis, atopic dermatitis, allergic family history or high IgE antibody titer were considered atopic, while non-atopic patients were considered as those who had none of the above characteristics. The grade of severity was determined according to the Japanese Guideline for Asthma.[15]

Table 1 Characteristics of the subjects studied

	Asthmatics	Normal subjects
No. cases (male/female)	66 (31/35)	12 (10/2)
Age (years)	50.3 ± 15.7	46.8 ± 11.8
Type		
Atopic		
No. cases	37	
Age (years)	45.5 ± 16.7	
Non-atopic		
No. cases	29	
Age (years)	56.1 ± 12.1	
Lung function		
%VC	100.4 ± 21.7	
$\%FEV_1$	67.5 ± 10.6	

VC, vital capacity; FEV_1, forced expiratory volume in 1 s.

The majority of patients were on regular inhalation of beclomethasone dipropionate (300–1800 μg/day; 22 and 26 atopic and non-atopic patients, respectively) or oral administration of prednisolone (3–15 mg/day; five and 15 atopic and non-atopic patients, respectively) and theophylline or β_2-adrenoceptor agonists. The remaining patients were maintained on theophylline or β_2-adrenoceptor agonists without steroids (15 in the atopic and three in the non-atopic group).

Collection of sputum

Sputum was induced by inhalation of hypertonic saline (3%) according to established methods.[1,2] The reservoir of a nebulizer (Nebulizer Type A; Nissho, Osaka, Japan) was filled with 10 mL sterile 3% saline. After rinsing the mouth with water to avoid saliva contamination, each subject inhaled the nebulized solution for 30 min. Subjects were encouraged to cough throughout the procedure as needed, at least every 10 min, and the sputum was collected into clean plastic containers. If the sputum volume was insufficient after 30 min collection, 5% saline was inhaled for an additional 20 min. Further inhalation

was stopped even if the quantity of the collected sputum was still unsatisfactory. The volume of the collected sputum was recorded and immediately stored on ice to stop the metabolism of lipid mediators. Stopping enzyme was not used in this study because our method to analyze sLT, described later, was designed to measure both LTC_4 and its metabolites. A 1 mL sputum sample was diluted four-fold with ethanol and stored at –80°C for analysis of sLT and TXB_2. Another 1 mL sputum was diluted three-fold with isotonic saline and stored at –80°C for histamine assay. A small amount of the remainder was taken for preparing a May–Grunwald–Giemsa stained smear for the cell differential.

Analysis of LT and TX

Analysis of sLT and TXB_2 was performed according to the modified three-stage procedure (purification, separation and quantitation), as described previously.[16–18] Briefly, the sample was centrifuged at 1500 *g* for 10 min at 4°C to remove debris and precipitated protein and the supernatant was collected. After evaporation in a vacuum, the supernatant was reconstituted with 6 mL methanol and purified on C18 Sep-Pak cartridges (Waters Co., Massachusetts, USA). Leukotriene- and TX-containing materials were eluted in methanol, evaporated to dryness and stored at –80°C for application to reverse-phase high-performance liquid chromatography (HPLC; SCL-10A; Shimazu, Kyoto, Japan). The HPLC was performed using 5 μm C18 analytical and guard columns with a mobile phase (47% acetonitrile with 1 mmol/L sodium 1-octanesulfonate and 2 mmol/L KH_2PO_4 and 3.38 mg/mL phosphoric acid) at a flow rate of 1 mL/min. Using a fraction collector (FCR-10A; Shimazu), separation and collection of LTC_4, LTD_4, LTE_4 and TXB_2 was undertaken. Measurements of sLT were performed using whole fractions of LTC_4, LTD_4 and LTE_4 with a Peptide–Leukotriene EIA KIT (Cayman Chemical, Ann Arbor, MI, USA). We did not measure each fraction of LTC_4, LTD_4 and LTE_4 because LTC_4 is rapidly metabolized to LTD_4. TXB_2 was measured in collected TXB_2 fractions with an EIA Kit (Cayman Chemical). The recovery rates of these lipid mediators were between 75 and 80%. Results are expressed in ng/mL of sputum and were corrected by the quantity of albumin in the sputum.

Analysis of histamine

Analysis of histamine was performed as reported recently.[3] The diluted sputum was homogenized for 10 min at room temperature. After centrifugation at 75 000 *g* for 30 min at 4°C, the supernatant was collected and stored at –80°C until measurement with an EIA Kit (Immunotech, Marseilles, France). Results were corrected by albumin concentration in the sputum and expressed in nmol/L. Albumin was measured in the histamine supernatant using a COBAS MIRA analyzer (Roche Diagnostic, Tokyo, Japan).

Statistical analysis

Data are expressed as the geometric mean ± geometric SD. Data were statistically analyzed by analysis of variance (ANOVA) using STATISTICA (StatSoft JAPAN, Tokyo, Japan). A probability of 5% or less was considered statistically significant.

RESULTS

Sputum cell differentials

For cell differentials, we subtracted the number of squamous cells from the number of whole cells in the sputum and counted 500 cells, because squamous cells in the sputum sample represented salivary contamination. As shown in Fig. 1, the corrected cell differentials showed a significantly higher percentage of eosinophils ($P < 0.02$) in all sputum samples from asthmatic patients (10.02 ± 8.16%) compared with those from normal subjects (0.28 ± 0.49%). The percentage of eosinophils in the sputum was significantly higher in asthmatics during exacerbation (11.44 ± 10.11%; $P < 0.01$) and also in asymptomatic asthmatics (8.66 ± 5.65%; $P < 0.02$) compared with that in normal controls. However, there was no significant difference in the percentage of other cell types in exacerbated and asymptomatic patients compared with normal controls in the present study.

Sulfidepeptide–leukotriene, thromboxane and histamine

We compared the concentrations of sLT (Fig. 2a) in the sputum from asymptomatic asthmatics and normal controls (1.02 ± 1.93 and 0.43 ± 0.30 ng/mL, respectively) and there was no significant difference. Concentrations of TXB_2 (Fig. 2b) in the sputum showed a similar tendency in asymptomatic asthmatics and normal controls (1.11 ± 2.65 and 0.27 ± 0.17 ng/mL, respectively) and there was no significant difference between the two groups. Histamine concentrations (Fig. 2c) in the

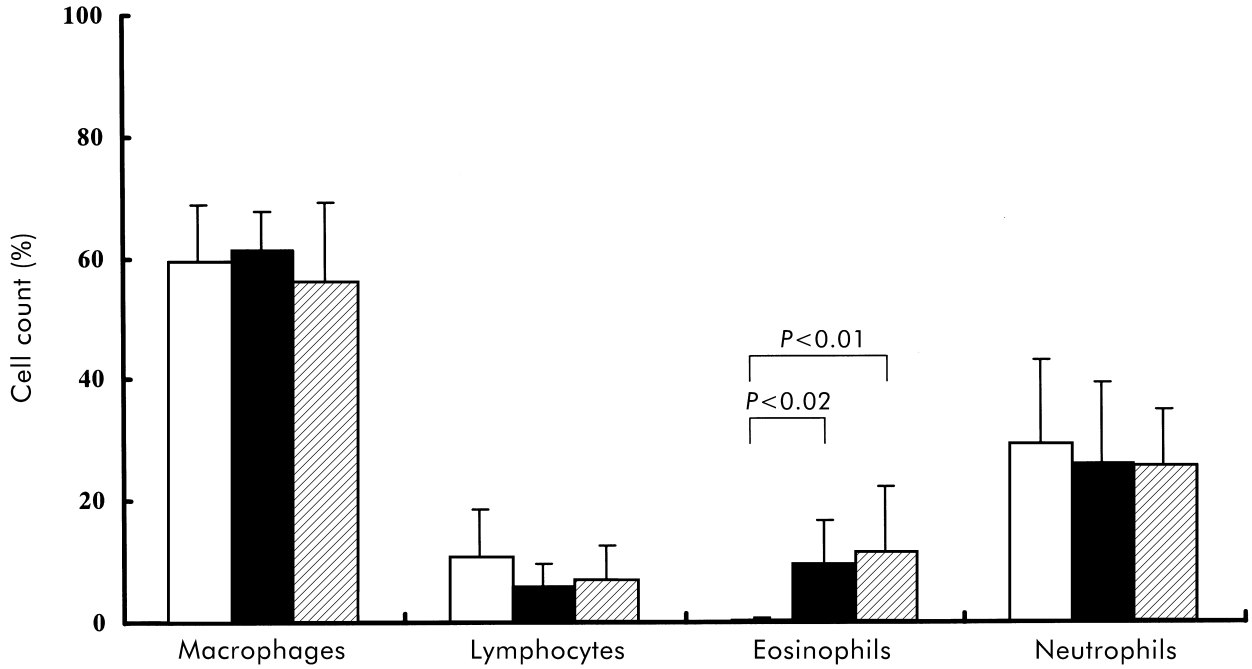

Fig. 1 Differential cell count in sputum from normal subjects (□) and asthmatics (■, asymptomatic; ▨, during exacerbation). The percentage of eosinophils was increased in asthmatics with or without exacerbation compared with normal subjects. Eosinophils were further increased during exacerbation. No significant difference was observed in macrophages, lymphocytes or neutrophils.

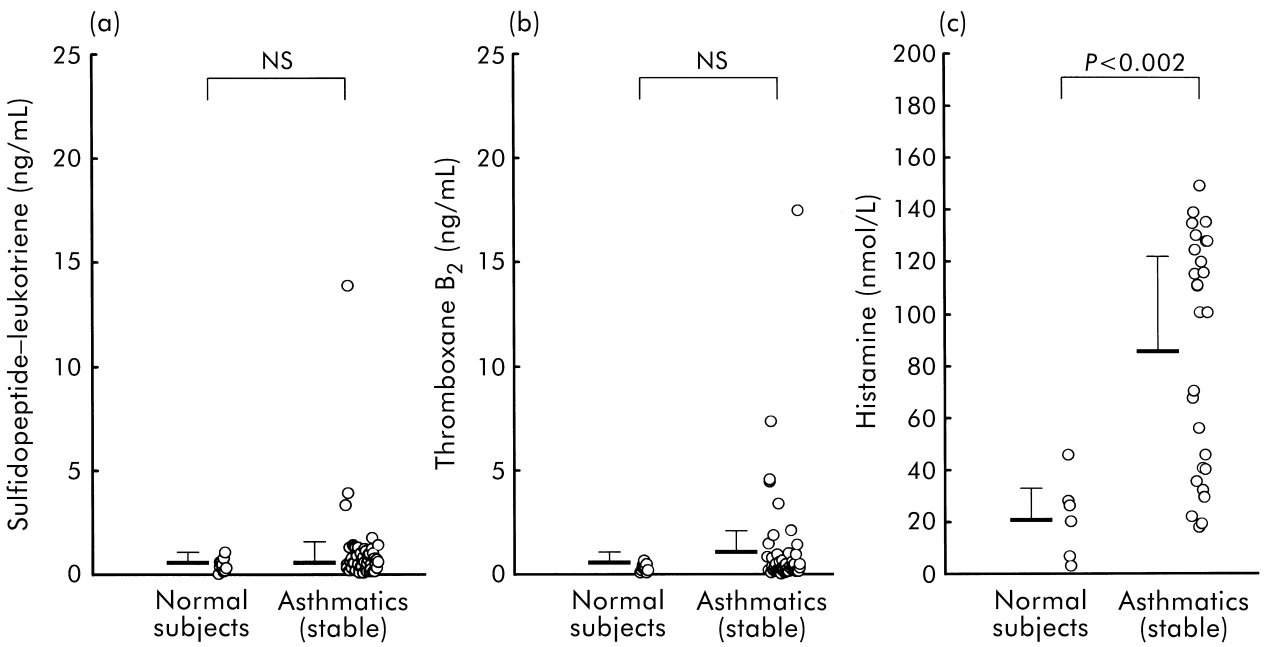

Fig. 2 Concentrations of (a) sulfidopeptide leukotrienes (sLT), (b) thromboxane (TX) B_2 and (c) histamine in sputum from normal subjects (n = 12) and asthmatics without symptoms (n = 53). Measurements were performed several times in asthmatics. The sLT levels in sputum from asthmatics without symptoms were not significantly higher than those in normal controls. Levels of TXB_2 showed no significant difference. Histamine levels showed a significant increase in asthmatics. Symbols indicate individual data points, while the bars are the mean ± SD.

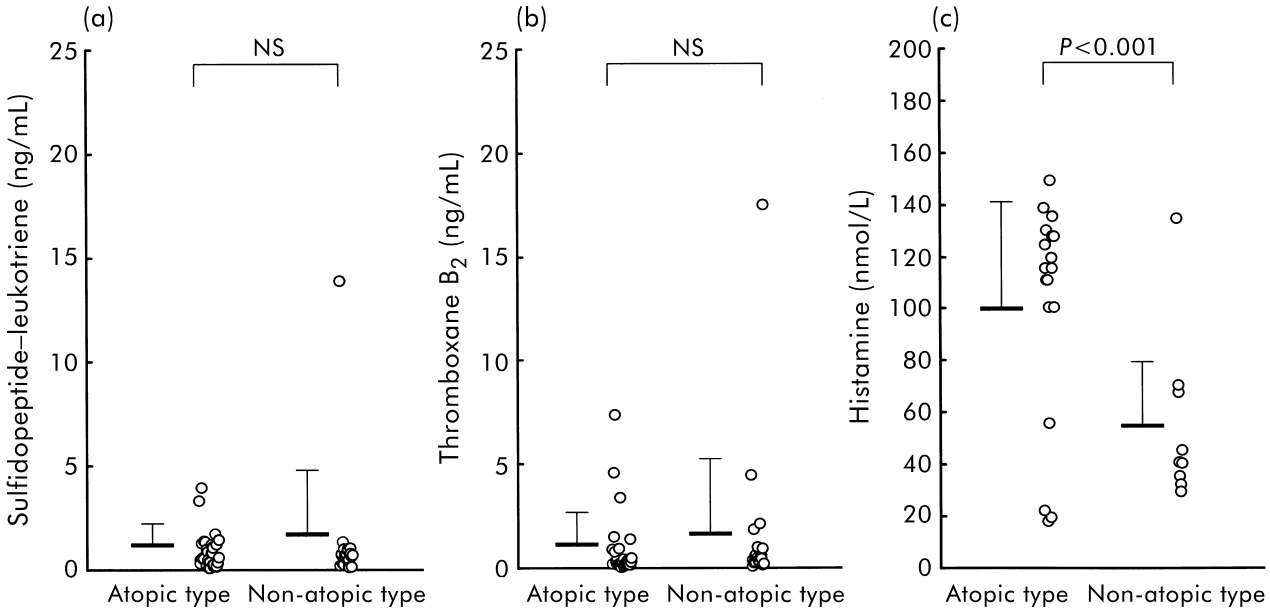

Fig. 3 Comparisons of (a) sulfidopeptide leukotrienes (sLT), (b) thromboxane (TX) B_2 and (c) histamine levels in sputum between atopic ($n = 37$) and non-atopic ($n = 29$) patients. Only histamine levels showed a significant difference and were higher in atopic than in non-atopic subjects ($P < 0.001$).

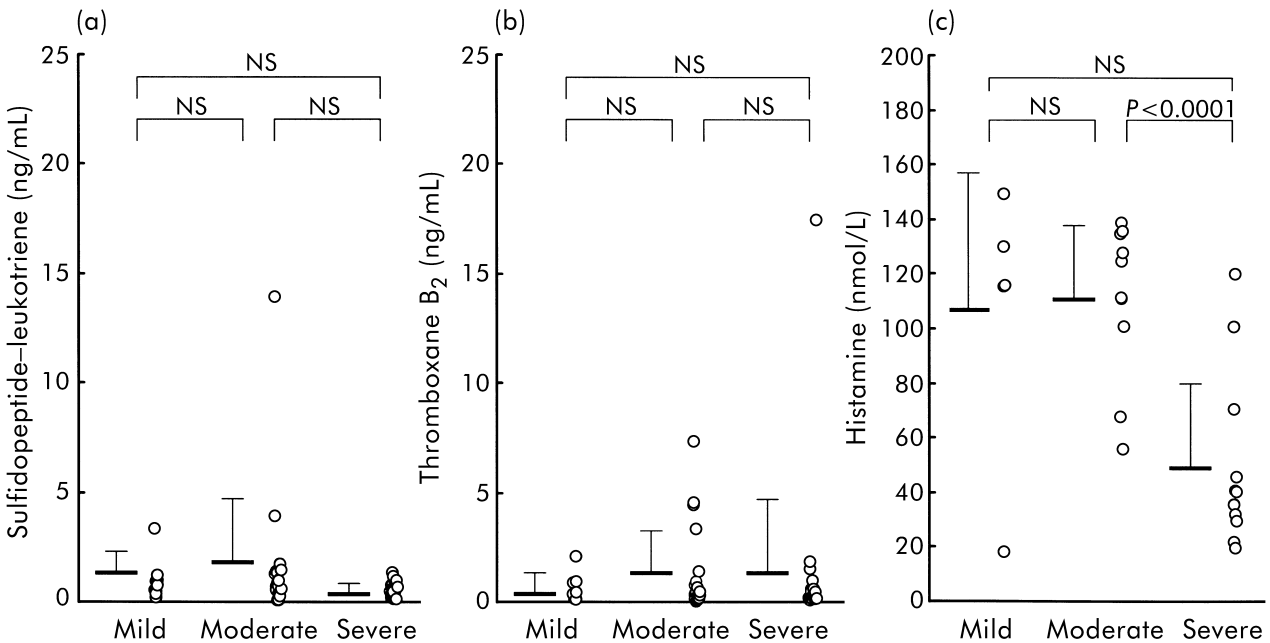

Fig. 4 Comparison of (a) sulfidopeptide leukotrienes (sLT), (b) thromboxane (TX) B_2 and (c) histamine levels in sputum according to severity of asthma. Levels of sLT and TXB_2 in the sputum showed no significant difference. However, histamine levels in sputum showed a significant decrease in severe cases compared with moderate cases ($P < 0.0001$). Data are the mean ± SD, NS, not significant.

sputum from asthmatics (85.64 ± 44.81 nmol/L) were significantly higher compared with normal controls (21.47 ± 15.45 nmol/L; $P < 0.002$).

We then compared these mediators between atopic and non-atopic patients. The sLT levels (Fig. 3a) in the sputum from atopic and non-atopic patients were

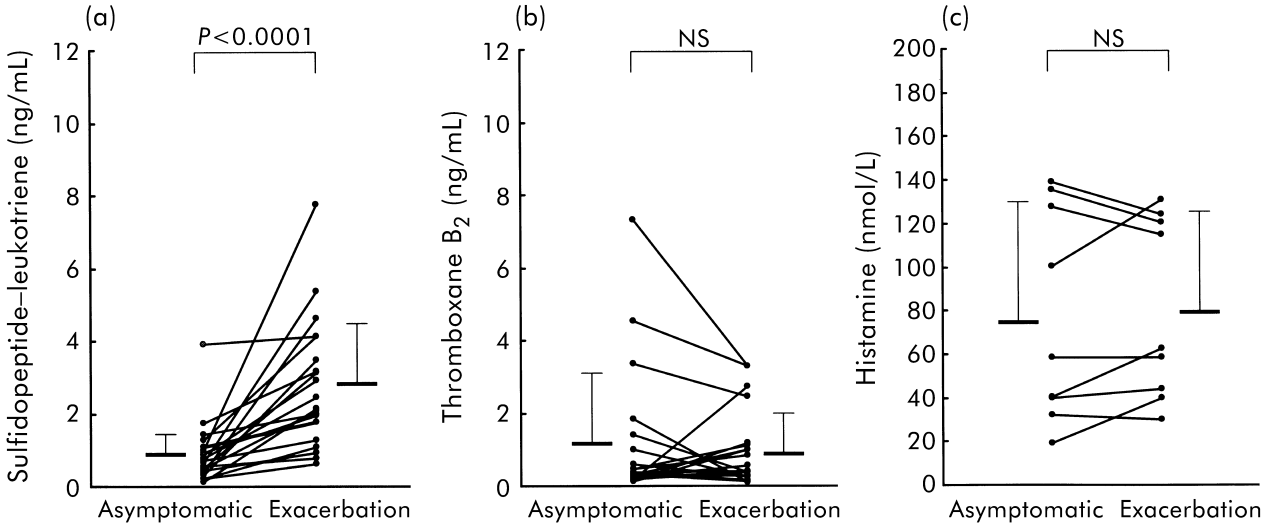

Fig. 5 Comparisons of (a) sulfidopeptide leukotrienes (sLT), (b) thromboxane (TX) B_2 and (c) histamine levels in the sputum of asymptomatic asthmatics compared with the exacerbation state. Levels of sLT were elevated in exacerbation ($P < 0.0001$). However, TXB_2 and histamine levels showed no significant change. Data are the mean ± SD.

0.86 ± 0.87 and 1.26 ± 2.91 ng/mL, respectively. There was no significant difference between these two groups. As for TXB_2 levels (Fig. 3b), similar results were obtained with 0.81 ± 1.52 and 1.55 ± 3.78 ng/mL in the atopic and non-atopic groups, respectively. However, histamine concentrations were significantly higher in the atopic group (101.03 ± 42.44 nmol/L) compared with the non-atopic group (54.87 ± 33.18 nmol/L), as shown in Fig. 3c ($P < 0.001$).

These mediators in the sputum were also evaluated according to the severity of asthma (mild, moderate, or severe). The sLT levels were 0.98 ± 0.89 ng/mL in the mild group, 1.55 ± 2.95 ng/mL in the moderate group and 0.53 ± 0.35 ng/mL in the severe group. Differences were not significant among these three groups (Fig. 4a). In addition, there was no significant difference in TXB_2 levels among the mild (0.54 ± 0.61 ng/mL), moderate (1.26 ± 1.95 ng/mL) and severe (1.22 ± 3.66 ng/mL) groups (Fig. 4b). In contrast, there was a significant difference in histamine levels (Fig. 4c) only between the moderate (112.03 ± 27.70 nmol/L) and severe (50.25 ± 32.75 nmol/L) groups ($P < 0.0001$).

There was no difference in sLT levels between normal subjects and asthmatics without symptoms. We then investigated sLT levels within the same individual asthmatics when their pulmonary functions were stable compared with acute exacerbation (20% decrease in PEFR with wheeze and dyspnea). As shown in Fig. 5a, sLT levels were significantly elevated during exacerbation (2.78 ± 1.78 ng/mL; $P < 0.0001$) compared with those obtained when patients were asymptomatic (0.88 ± 0.83 ng/mL). However, TXB_2 levels did not change in association with exacerbation (1.00 ± 1.06 ng/mL) compared with the stable state (1.19 ± 1.85 ng/mL; Fig. 5b). Histamine levels also did not show any change with exacerbation (Fig. 5c; 76.87 ± 48.40 ng/mL with exacerbation *vs* 80.46 ± 41.10 ng/mL in the stable state).

Discussion

Research into airway inflammation using sputum has been established and there are many reports using this technique. Fahy *et al*. have shown changes in the type and quantity of inflammatory cells in sputum following inhalation of hypertonic saline after an allergen challenge.[19,20]

In this study, using these established methods with sputum, we demonstrated an elevated level of sLT in the sputum during exacerbation of asthma. In contrast, TXB_2 and histamine were not elevated during exacerbation of asthma. These results suggest that sLT may play a more important role in worsening symptoms of asthma compared with TX and histamine. However, these results do not directly indicate that sLT are the most important factors in allergic inflammation in the airway, because sLT were not significantly elevated in the stable state, the condition in which allergic inflammation was still present. It is known that inflammatory cells in the airway can

produce LTs.[21] We have previously reported elevated levels of LTC_4 and LTB_4 in BAL fluid in the late asthmatic response following inhalation of house dust allergen.[22] In addition, increased LTC_4 levels in BAL fluid from atopic asthmatics after allergen challenge have been reported.[23] In contrast, sLT receptor antagonists, such as pranlukast, montelukast and zafirlukast, have been shown to improve FEV_1 in patients with chronic persistent asthma,[24] to inhibit allergen-induced bronchoconstriction,[25] to produce marked clinical effects[12,26] and to prevent airway obstruction induced by lysine–aspirin in aspirin-sensitive asthmatics.[27] Our results and the results of these reports suggest that sLT may play a more important role in the acute asthmatic reaction, such as an exacerbation, and after antigen challenge than in the stable persistent inflammatory state. In contrast, TX may play a more effective role compared with sLT in baseline bronchial hyperresponsiveness. Some reports have shown that the TXA_2 receptor antagonist seratrodast was effective in asthmatics[14] and that 4 days administration of seratrodast at 40 mg/day reduced bronchial hyper-responsiveness in patients with asthma.[28] In contrast, a few reports have shown that sLT attenuates baseline bronchial hyper-responsiveness.[29,30]

In this study, only sLT levels, not TXB_2 or histamine levels, in the sputum were elevated during exacerbation compared with asymptomatic disease. There are several possible explanations for this increase in sLT. First, sLT are formed by 5-lipoxygenase (5-LO), while TX is produced by cyclo-oxygenase. Because the synthesizing enzymes are different, sLT and TX may be not always be produced in parallel; indeed, not only a decrease in sLT but also an increase in TX in BAL fluid was observed after administration of the 5-LO inhibitor zileuton.[31] Another possible explanation, as mentioned before, is that sLT are more important factors in the acute asthmatic reaction compared with baseline inflammation, while TX may be more involved in baseline allergic inflammation.[14,28] Another explanation is that a process progressing to exacerbation may be variable depending on individual asthmatics. For example, sLT may be the main factor in one patient, while TXB_2 may be important in another. Indeed, cases in whom sLT levels in the sputum were low and TXB_2 levels were high and cases with high sLT levels and low TXB_2 levels were observed in our study.

Histamine concentrations were significantly higher in atopic patients. Histamine is known to be an important factor in atopic disorders. However, sLT and TXB_2 levels were not different in atopic and non-atopic patients. It is not surprising that sLT and TX do not relate to atopic reactions because mast cells predominate in this reaction. In contrast, sLT and TX are supposed to play roles in eosinophil inflammation. Another significant change, the lower histamine level in patients with severe disease, may be explained by frequent administration of corticosteroids in such patients. However, corticosteroids may also decrease the levels of sLT and TXB_2. It is possible that corticosteroids only control increased sLT and TXB_2 levels to normal range.

In conclusion, our results suggest that sLT contribute to the pathogenesis of asthma or, at least, that sLT exacerbate asthma more potently than histamine and TXA_2. This fact explains why sLT receptor antagonists can reduce asthma symptoms and improve PEF and FEV_1, while histamine receptor antagonists do not.

Acknowledgments

The authors thank Masami Takeyama (Department of Allergy, National Minamiokayama Hospital) for performing the EIA assays and Dr Robert G Townley ((Allergic Disease Center, Creighton University, Omaha, NB, USA) for his helpful suggestions.

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