分岐と内径の関係が生体においてどのようになっているのかを初めて観察可能になり、肺局所における主幹部から細部にわたる気道系の形態や機能，とくに末梢気道の力学的状態を解析する技術の開発に繋がると考える。

E．結論

現在の３次元ＣＴ撮影で観察可能な気道内径は 1mm であるが、健常者と喘息患者では、全く異なる気管支の分岐レベルで内径 1mm を呈することが明らかとなった。また、サルブタモール吸入は可視範囲の全ての気道を拡張することが明らかになった。

G．研究発表

１．論文発表

① G. Tamura, Y. Suda. Airway Dilatation after Inhalation of short-acting β-agonist. N Engl J Med, 2005 (in press).

２．学会発表

なし

H．知的財産権の出願・登録状況

１．特許取得

なし。

２．実用新案登録

なし。

３．その他

なし

アレルギー性気道炎症の新たな制御機構の解明

分担研究者　岩本逸夫
千葉大学医学部アレルギー・膠原病内科助教授

研究協力者　中島裕史、高取宏昌、加々美新一郎、広瀬晃一、玉地智宏
（千葉大学医学部アレルギー・膠原病内科）

研究要旨

気管支喘息の気道過敏性の病態解明と早期診断法、治療法の確立には、アレルギー性気道炎症の制御機構の分子レベルでの解明が必須である。これまで我々は、Stat6 に加え、Stat5a がアレルギー性気道炎症の惹起に必須であること、さらに Stat5a が Th2 細胞分化に関与する転写因子であることを直接的に明らかにしてきた。本研究では、1）Stat6 非依存性 Th2 細胞分化における Stat5a の役割、及び2）Stat5a による SOCS3 発現誘導を介した Th1 細胞分化抑制機構を明らかにすることを研究目的とした。Stat5a/Stat6 ダブル欠損 T 細胞では、Stat6 欠損 T 細胞よりも強い Th2 細胞分化障害を示し、Th2 細胞分化は殆ど認められなかった。持続活性型 Stat5a を Stat6 欠損 T 細胞に過剰発現させても Th2 細胞分化は回復しなかった。Stat5a/Stat6 ダブル欠損マウスにおける抗原誘発気道好酸球及びリンパ球浸潤は、Stat6 欠損マウスよりも著明に減弱していた。Stat5a-/- CD4 陽性 T 細胞では、野生型 CD4 陽性 T 細胞に比して IL-12 刺激による Stat4 リン酸化及び Th1 細胞分化が亢進していた。さらに、Stat5a-/-CD4 陽性 T 細胞では、SOCS3 の発現が低下していた。そして、持続活性型 Stat5a は SOCS3 promoter を活性化した。Stat5a-/-CD4 陽性 T 細胞に SOCS3 を発現させると Th1 細胞分化が抑制され Th2 細胞分化は増強された。

本研究により、Stat5a は Stat6 非依存的に Th2 細胞分化を誘導し、アレルギー性気道炎症を増強することが明らかにされた。そのメカニズムとして Stat5a は、SOCS3 の発現を誘導し、IL-12-Stat4 シグナルを抑制することにより Th1 細胞分化を抑制する。これらの結果は、Stat5a シグナルの制御によるアレルギー性気道疾患治療の可能性を示唆する。

A．研究目的

気管支喘息は慢性のアレルギー性気道炎症を病態とし、その結果気道過敏性の亢進と気道閉塞が惹起される。アレルギー性気道炎症は、Th2 細胞の選択的活性化により惹起され、Th2 細胞と好酸球を主体とする炎症細胞浸潤、粘液細胞の増加、気道浮腫、さらには気道リモデリングにより特徴づけられる。したがって、気管支喘息の気道過敏性の病態解析とそれに基づく効果的かつ根本的な治療法の確立には、アレルギー性気道炎症の成立機序及びその制御機構の分子レベルでの解明が必須である。これまで Th2 細胞の分化とアレルギー性気道炎症の惹起には Stat6 が重要な役割を演じていることが知られているが、最近我々は、IL-2、IL-5、IL-7 をはじめ多くのサイトカインにより活性化される Stat5a がアレルギー性気道炎症の惹起に必須であること、さらに Stat5a が Th2 細胞分化に関与する転写因子であることを直接的に明らかにした（Blood 2001）。

本研究では、1）Stat6 非依存性 Th2 細胞分化における Stat5a の役割、及び2）Stat5a による SOCS3 発現誘導を介した Th1 細胞分化抑制機構を明らかにした。

B．方法

1）Stat5a による Stat6 非依存性 Th2 細胞分化機構の解析：

a）野生型マウス、Stat5a 欠損マウス、Stat6 欠損マウス、及び Stat5a/Stat6 ダブル欠損マウスを作製し、脾臓 T 細胞の増殖能、脾臓 T 細胞を抗 CD3 抗体と外因性サイトカインで刺激した際の T 細胞分化、サイトカイン産生を ELISA 法

及び細胞内サイトカイン染色法を用いて解析した。b) Stat6 欠損 T 細胞にレトロウイルスを用いて持続活性型 Stat5a を発現させた際の T 細胞分化に対する影響を解析した。c) Stat5a/Stat6 ダブル欠損マウスにおけるアレルギー性気道炎症を他の 3 群のそれと比較検討した。

2)Stat5a による Th1 細胞分化抑制機構の解析:

a) 野生型及び Stat5a-/- CD4 陽性 T 細胞における IL-12 依存性 Th1 細胞分化、IL-12 刺激による Stat4 のリン酸化、及び IL-12/Stat4 シグナルの抑制分子である SOCS3 の発現を比較検討した。b) SOCS3 promoter に対する持続活性型 Stat5a 及び持続活性型 Stat6 発現の効果を検討した。c) Stat5a-/- CD4 陽性 T 細胞の Th1/Th2 分化に対する SOCS3 発現の効果を検討した。

C. 結果及び D. 考察

1) Stat6 欠損 T 細胞では Th2 細胞分化が強く障害されたが、依然野生型 T 細胞の 25-30%認められた。Stat5a/Stat6 ダブル欠損 T 細胞では、Stat6 欠損 T 細胞よりも強い Th2 細胞分化障害を示し、Th2 細胞分化は殆ど認められなかった。T 細胞増殖能については、各マウス間で有意な差が認められなかった。持続活性型 Stat5a を Stat6 欠損 T 細胞に過剰発現させても Th2 細胞分化は回復しなかった。Stat5a/Stat6 ダブル欠損マウスにおける抗原誘発気道好酸球及びリンパ球浸潤は、Stat6 欠損マウスよりも著明に減弱していた。しかし杯細胞の増加は、両者とも殆ど認められなかった。

2) Stat5a-/- CD4 陽性 T 細胞では Th1 細胞分化が亢進しており、抗 IFN-γ抗体により Th2 細胞分化が誘導された。Stat5a-/- CD4 陽性 T 細胞では、野生型 CD4 陽性 T 細胞に比して IL-12 刺激による Stat4 リン酸化及び Th1 細胞分化が亢進していた。さらに、Stat5a-/-CD4 陽性 T 細胞では、IL-12 による Stat4 リン酸化を抑制する SOCS3 の発現が低下していた。そして、持続活性型 Stat5a は SOCS3 promoter を活性化した。クロマチン免疫沈降法により、Stat5a が CD4 陽性 T 細胞の SOCS3 promoter に結合することが確認された。Stat5a-/-CD4 陽性 T 細胞に SOCS3 を発現させると Th1 細胞分化が抑制され Th2 細胞分化は増強された。

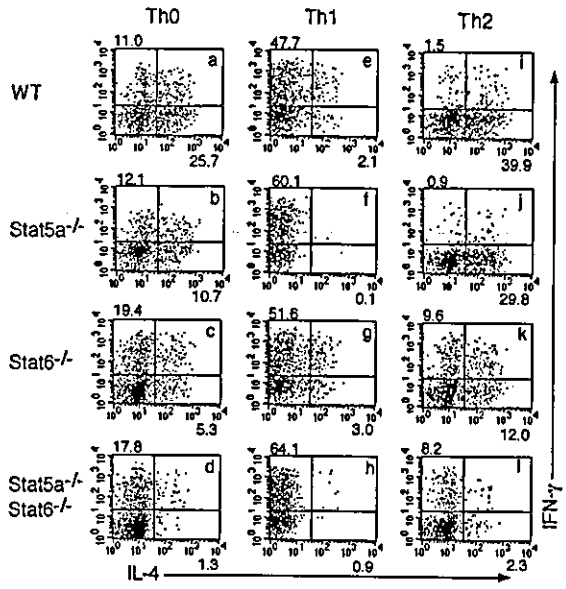

図 1. Stat5a/Stat6 ダブル欠損 T 細胞における Th2 細胞分化障害

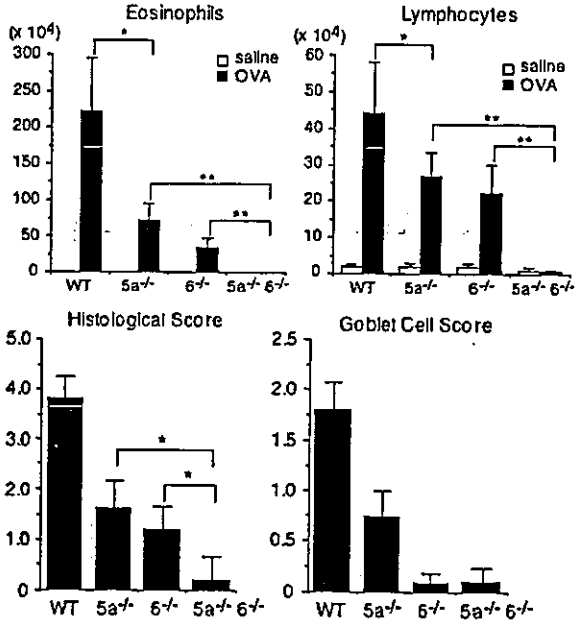

図 2. Stat5a/Stat6 ダブル欠損における抗原誘発気道好酸球とリンパ球浸潤及び杯細胞増加が著明に低下している

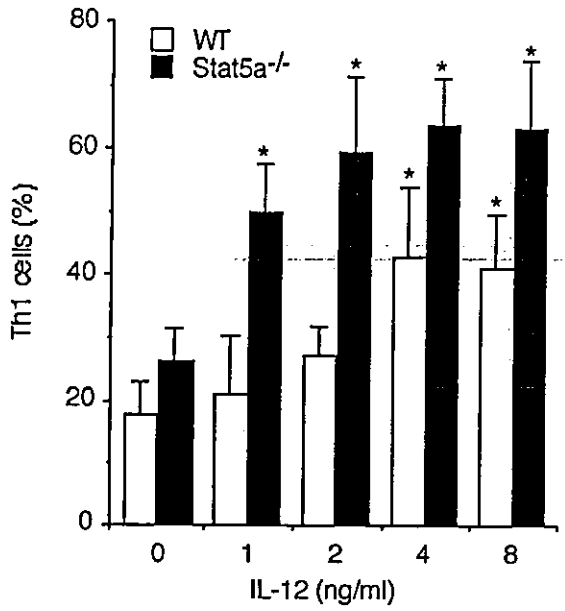

図３．Stat5a-/- CD4 陽性 T 細胞では IL-12 による Th1 細胞分化が亢進している

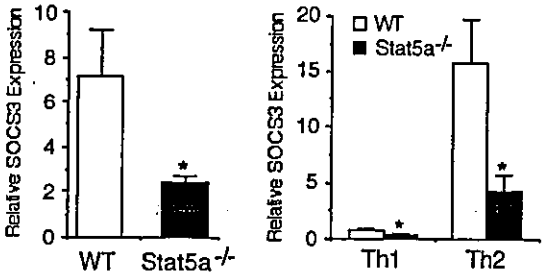

図４．Stat5a-/-CD4 陽性 T 細胞では SOCS3 の発現が低下している

E．結論

本研究により、Stat5a は Stat6 非依存的に Th2 細胞分化を誘導し、アレルギー性気道炎症を増強することが明らかにされた。そのメカニズムとして Stat5a は、SOCS3 の発現を誘導し、IL-12-Stat4 シグナルを抑制することにより Th1 細胞分化を抑制する。これらの結果は、Stat5a シグナルの制御によるアレルギー性気道疾患治療の可能性を示唆する。

F．健康危険情報

なし

G．研究発表

1．論文発表

1. Maezawa Y, Nakajima H, Seto Y, Suto A, Kumano K, Kubo S, Karasuyama H, Saito Y, Iwamoto I. IgE-dependent enhancement of Th2 cell-mediated allergic inflammation in the airways. Clin. Exp. Immunol. 135: 12-18, 2004.

2. Mori Y, Hirose K, Suzuki K, Nakajima H, Seto Y, Ikeda K, Shimoda K, Nakayama K, Saito Y, Iwamoto I. Tyk2 is essential for IFN-α-induced gene expression in mast cells. Int. Arch. Allergy Immunol. 134:25-29, 2004.

3. Takatori H, Nakajima H, Hirose K, Kagami S-i, Tamachi T, Suto A, Suzuki K, Saito Y, Iwamoto I. Indispensable role of Stat5a in Stat6-independent Th2 cell differentiation and allergic airway inflammation. J. Immunol. 174:3734-3740, 2005.

4. Takatori H, Nakajima H, Kagami S-i, Hirose K, Suto A, Suzuki K, Kubo M, Yoshimura A, Saito Y, Iwamoto I. Stat5a inhibits IL-12-induced Th1 cell differentiation through the induction of SOCS3 expression. J. Immunol. 174:4105-4112, 2005.

5. Ikeda K, Nakajima H, Suzuki K, Watanabe N, Kagami S-i, Iwamoto I. Stat5a is essential for the proliferation and survival of murine mast cells. Int. Arch. Allergy Immunol. in press.

2．学会発表

1. H. Nakajima, S-i. Kagami, H. Takatori, I. Iwamoto. (2004) Cytokine signaling in airway inflammation. The 6th Asia-Pacific Congress of Allergology and Clinical Immunology, Abstract p19.

H．知的財産権の出願・登録状況

なし

III. 研究成果の刊行に関する一覧表

1) Higashi N, Taniguchi M, Mita H, et al. Clinical features of asthmatic patients with increased urinary leukotriene E4 excretion. J Allergy Clin Immunol. 113:277-283, 2004.

2) Higashi N, Mita H, Taniguchi M, et al. Urinary eicosanoid and tyrosine derivative concentrations in patients with vasculitides. J Allergy Clin Immunol. 114:1353-8, 2004.

3) Mita H, Higashi N, Taniguchi M, Higashi A, Akiyama K. Increase in urinary leukotriene B4 glucuronide concentration in patients with aspirin-intolerant asthma after intravenous aspirin challenge. Clin Exp Allergy. 34:1262-9, 2004.

4) Mita H, Higashi N, Taniguchi M, Higashi A, Kawagishi Y, Akiyama K. Urinary 3-bromotyrosine and 3-chlorotyrosine concentrations in asthmatic patients: lack of increase in 3-bromotyrosine concentration in urine and plasma proteins in aspirin-induced asthma after intravenous aspirin challenge. Clin Exp Allergy. 34:931-8, 2004.

5) Horiguchi S, Okamoto Y, Chazono Z, Sakurai D, Kobayashi K. Expression of membrane-bound CD23 in nasal mucosal B cells from patients with perennial allergic rhinitis. Ann Allergy Asthma Immunol. 95:286-291, 2005.

6) Tamura G, Suda Y. Airway dilatation after inhalation of short-acting β-agonist. N Engl J Med. (in press)

7) Maezawa Y, Nakajima H, Seto Y, Suto A, Kumano K, Kubo S, Karasuyama H, Saito Y, Iwamoto I. IgE-dependent enhancement of Th2 cell-mediated allergic inflammation in the airways. Clin. Exp. Immunol. 135:12-18, 2004.

8) Mori Y, Hirose K, Suzuki K, Nakajima H, Seto Y, Ikeda K, Shimoda K, Nakayama K, Saito Y, Iwamoto I. Tyk2 is essential for IFN-α-induced gene expression in mast cells. Int. Arch. Allergy Immunol. 134:25-29, 2004.

9) Takatori H, Nakajima H, Hirose K, Kagami S-i, Tamachi T, Suto A, Suzuki K, Saito Y, Iwamoto I. Indispensable role of Stat5a in Stat6-independent Th2 cell differentiation and allergic airway inflammation. J. Immunol. 174:3734-3740, 2005.

10) Takatori H, Nakajima H, Kagami S-i, Hirose K, Suto A, Suzuki K, Kubo M, Yoshimura A, Saito Y, Iwamoto I. Stat5a inhibits IL-12-induced Th1 cell differentiation through the induction of SOCS3 expression. J. Immunol. 174:4105-4112, 2005.

11) Ikeda K, Nakajima H, Suzuki K, Watanabe N, Kagami S-i, Iwamoto I. Stat5a is essential for the proliferation and survival of murine mast cells. Int. Arch. Allergy Immunol. (in press)

Clinical features of asthmatic patients with increased urinary leukotriene E4 excretion (hyperleukotrienuria): Involvement of chronic hyperplastic rhinosinusitis with nasal polyposis

Noritaka Higashi, MD,[a,b] Masami Taniguchi, MD,[a] Haruhisa Mita, PhD,[a] Yukio Kawagishi, MD,[a,c] Toyota Ishii, MD,[a] Ai Higashi, MD,[a,b] Mitsuhiro Osame, MD,[b] and Kazuo Akiyama, MD[a] *Kanagawa, Kagoshima, and Toyama, Japan*

Background: The urinary leukotriene E4 (U-LTE4) concentration is significantly increased in patients with aspirin-intolerant asthma (AIA). However, the relationship between the clinicopathogenetic factors of asthma and the U-LTE4 concentration remains undetermined.

Objective: We sought to examine the clinical features of asthmatic patients with increased excretion levels of U-LTE4 (hyperleukotrienuria).

Methods: We measured the U-LTE4 concentrations in 137 asthmatic patients (including 64 patients with AIA) who were in clinically stable condition. A U-LTE4 concentration of 150 pg/mg creatinine or greater (mean U-LTE4 + 3 SDs of normal healthy control subjects) was indicative of hyperleukotrienuria.

Results: The basal concentration of U-LTE4 was significantly higher in the patients with AIA than in those with aspirin-tolerant asthma (ATA; median, 227.2 vs 90.3 pg/mg creatinine; $P < .01$). Compared with normal leukotrienuria in the patients with AIA, hyperleukotrienuria in the patients with AIA was associated with older age and decrease in pulmonary function. On the other hand, compared with normal leukotrienuria in the patients with ATA, hyperleukotrienuria in the patients with ATA was associated with severe asthma and chronic hyperplastic rhinosinusitis with nasal polyposis (CHRS/NP), which are well-known symptoms of the aspirin triad, as well as hypereosinophilia and anosmia. The patients with ATA with CHRS/NP excreted U-LTE4 at significantly high concentrations. There were significant decreases in the U-LTE4 concentrations before and after the sinus surgery in both the AIA and ATA groups ($P < .05$).

Conclusion: Cysteinyl leukotrienes are not strictly associated with aspirin intolerance itself but rather with clinical features, such as CHRS/NP, that are similar to those seen in AIA. CHRS/NP might be involved in cysteinyl leukotriene overproduction in asthmatic patients. (J Allergy Clin Immunol 2004;113:277-83.)

Key words: Aspirin-intolerant asthma, urinary leukotriene E4, chronic hyperplastic rhinosinusitis with nasal polyposis

Cysteinyl leukotrienes (cys-LTs), namely leukotriene C4 (LTC4), LTD4, and LTE4, play an extremely important role in the pathophysiology of asthma.[1] Cys-LTs cause potent bronchoconstriction, mucosal edema, and increased mucus secretion within the airways of asthmatic patients.[2,3] LTE4 has been identified as a major metabolite of LTC4,[4,5] and urinary LTE4 (U-LTE4) is now considered as the most reliable analytic parameter for monitoring the endogenous synthesis of cysLTs.[6,7] Previous studies have shown that the U-LTE4 concentration is useful in demonstrating cys-LT release in vivo during allergen challenge[8,9] and acute exacerbation of asthma.[10,11] Moreover, even in clinically stable conditions, basal urinary excretion levels of LTE4 in patients with aspirin-intolerant asthma (AIA) are significantly higher than those in patients with aspirin-tolerant asthma (ATA).[12,13] The cellular source of cys-LTs is as yet unknown, but it possibly includes eosinophils and mast cells.[14,15] It also remains unclear which step in the pathway is responsible for cys-LT overproduction in patients with AIA. Cowburn et al[16] reported that the number of cells expressing LTC4 synthase (LTC4S) is higher in the bronchial mucosa of patients with AIA than in that of patients with ATA and that the amount of cys-LTs in bronchoalveolar lavage fluid is strongly correlated with the number of LTC4S-positive cells. In addition, Sanak et al[17,18] found that the polymorphism (ie, A to C transversion at 444 nucleotides upstream of the ATG translation start site) in the promoter region of the *LTC4S* gene is associated with the development of AIA in a Polish population. However, this polymorphism of the *LTC4S* gene might not be the probable mechanism underlying cys-LT overproduction in patients with AIA. Van Sambeek et al[19] reported that the C allele in the *LTC4S* gene is not correlated with the phenotype of AIA in the United States and that this polymorphism does not affect the transcriptional activity of the *LTC4S* gene. More recently, Kawagishi et al[20] also reported similar findings that there is no clear association between this polymorphism of the *LTC4S* gene and the aspirin intolerance or the enzymatic activity of the *LTC4S* gene in eosinophils. There has been no

From [a]the Clinical Research Center, National Sagamihara Hospital, Kanagawa; [b]The Third Department of Internal Medicine, Kagoshima University School of Medicine, Kagoshima; and [c]The First Department of Internal Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama.

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Reprint requests: Noritaka Higashi, MD, Karolinska Institutet, Stockholm SE 171 77, Sweden.

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Abbreviations used
AIA: Aspirin-intolerant asthma
ATA: Aspirin-tolerant asthma
CHRS/NP: Chronic hyperplastic rhinosinusitis with nasal polyposis
cys-LT: Cysteinyl leukotriene
LTC4S: Leukotriene C4 synthase
LTE4: Leukotriene E4
U-LTE4: Urinary leukotriene E4

report demonstrating that the basal concentration of U-LTE4 differs between wild-type A homozygotes and variant C allelic carriers in patients with AIA[18,20] or in patients with ATA.[20] The clinicopathogenetic factors associated with the increased excretion levels of U-LTE4 in asthmatic patients have not been determined. In our study we evaluated the clinicopathogenetic factors that might be associated with the increased excretion level of U-LTE4 in asthmatic patients and demonstrated that the cys-LT overproduction is associated with some clinical features also observed in AIA, such as chronic hyperplastic rhinosinusitis with nasal polyposis (CHRS/NP).

METHODS

Subjects

The hospital-based case-control study was conducted from June 1998 through March 2002. The subjects of this study were 64 nonsmoking asthmatic outpatients with AIA (age range, 21-79 years; mean age, 53.3 years; 23 male and 41 female patients) and 73 control asthmatic outpatients who tolerated aspirin well (ATA; age range, 21-80 years; mean age, 51.2 years; 38 male and 35 female patients; Table I). The diagnosis of asthma was based on the American Thoracic Society criteria.[21] Asthma severity was classified on the basis of daily medication regimen and response to treatment according to the Global Initiative for Asthma guidelines.[22] All the patients with AIA have been proved to have histories of severe bronchoconstriction and nasal symptoms after ingestion of at least 2 different nonsteroidal anti-inflammatory drugs or have had a positive reaction to aspirin systemic challenge.[20] All the patients with ATA have had a negative reaction to the aspirin challenge. All the patients were in clinically stable condition. None of the patients had complications of cystic fibrosis, immotile cilia syndrome, or autoimmune diseases, and none of them had an upper respiratory tract infection in the 6 weeks preceeding the study. Thirty-five healthy volunteers without subjective symptoms or objective findings of diseases, including asthma and allergic rhinitis, were also enrolled in this study as healthy control subjects (age range, 25-81 years; mean age, 50.1 years; 23 male and 12 female subjects). Permission to conduct the study was obtained from the Ethics Committee of the National Sagamihara Hospital, and all the patients who participated in the study provided informed consent.

Study design

On entry into this study, the patients were documented according to a detailed structured questionnaire. The age at onset of asthma was determined as accurately as possible. In case of uncertainty, the earliest respiratory symptoms, including cough, wheezing, and episodic dyspnea, were taken into account. Nasal symptoms were also assessed. Self-assessment of olfactory disturbance was performed by the patients using an analog scale for rating symptoms from 0 (none) to 4 points (anosmia) by evaluating the degree at which the disturbance interfered with their everyday life. When a 4-point (anosmia) score was obtained, we reconfirmed the presence of anosmia by using cards or smell bottles containing cigarettes, coffee, deodorants, and cloves, as previously described but with some modifications.[23] Subjective assessment of olfactory disturbance in asthmatic patients with seasonal allergic rhinitis was performed during the off season of pollen allergens. The diagnosis of CHRS/NP[24-26] was assessed by an independent observer on the basis of clinical histories, rhinoscopic findings, and sinus radiographic or computed tomographic scans. In this article CHRS/NP is determined by evaluating the clinical symptoms of chronic rhinosinusitis with evidence of bilateral sinus mucosal thickening on radiographic studies in conjunction with nasal polyposis.[27-30] In contrast, infectious chronic rhinosinusitis,[31] as determined by means of clinical definition, such as the presence of facial pain, purulent drainage, or an extensive air fluid level on sinus radiographs, was an exclusion criterion.

We collected blood and urine samples from the patients, and then pulmonary function tests were performed between 9 and 11 AM. The number of eosinophils in peripheral blood was determined by using a standard automated cell counter. Because treatment with β_2-agonists or with anti-inflammatory drugs, including oral or inhaled corticosteroids, sodium cromoglycate, or oral leukotriene receptor antagonists themselves, does not affect U-LTE4 levels,[32] these medications were not withheld at the time of urine sample collection in this study.

To reconfirm the involvement of CHRS/NP in the increase in the excretion level of U-LTE4, we compared the LTE4 concentration in urine between before and after the elective endoscopic surgery for CHRS/NP (3-4 weeks after the surgical treatment), which is not part of the goal of this study. The subjects in the reconfirmatory study were 7 patients with AIA (age range, 37-68 years; mean age, 52.9 years; 2 male and 5 female patients) and 8 patients with ATA (age range, 22-71 years; mean age, 48.4 years 5 male and 3 female patients). Before and after the endoscopic surgery for CHRS/NP (sinus surgery), all these patients were in clinically stable condition, with no changes in asthma symptoms or the kind and dose of medication. Treatments included only inhaled steroids, leukotriene receptor antagonists, and single use of short-acting β_2-agonists but not systemic corticosteroid administration.

Measurements

We measured the LTE4 concentration in spot urine by using a method previously described.[20,33-35] The U-LTE4 concentration is expressed as picograms per milligram of creatinine.

Analysis of data

Demographic characteristics are expressed as means ± SD. The U-LTE4 concentration is shown as the median and the range. U-LTE4 concentrations in the AIA, ATA, and healthy control groups were first compared by using the Kruskal-Wallis test. When a significant difference was found, the Mann-Whitney *U* test with the Bonferroni correction for comparison between groups was performed. U-LTE4 concentrations in the patients with different clinical asthma severity levels were compared by using the same statistical analyses described above. U-LTE4 concentrations in patients with CHRS/NP and those with normal sinuses were compared with the Mann-Whitney *U* test. Similarly, U-LTE4 concentrations in patients with AIA and ATA were compared with the Mann-Whitney *U* test. U-LTE4 concentrations before and after the sinus surgery were compared by using the Wilcoxon *t* test.

In this study the U-LTE4 concentration was the basis for classifying the patients into those showing either increased excretion levels of U-LTE4 (hyperleukotrienuria) or nonincreased excretion levels of U-LTE4 (normal leukotrienuria). A U-LTE4 concentration of

TABLE I. Demographic characteristics of the patients with AIA and those with ATA

	AIA	ATA	*P* value
No.	64	73	
Male/female sex	23/41	38/35	NS
Age (y), mean (SD)	53.3 (13.5)	51.2 (15.2)	NS
Onset age (y), mean (SD)	38.1 (14.0)	37.3 (17.7)	NS
Atopic status, n (%)	32 (50.0)	51 (69.9)	<.05
Blood eosinophil (×10⁶/mL), median (range)	585 (5-3520)	430 (20-4160)	NS
Serum IgE-RIST (IU/mL), median (range)	114 (14-1390)	233 (15-2290)	<.05
U-LTE4 (pg/mg creatinine), median (range)	227.2 (30.9-1465.7)	90.3 (16.5-915.8)	<.01
Pulmonary function tests			
FEV_1 (mL), mean (SD)	2043 (757)	2100 (796)	NS
FEV_1 (% predicted), mean (SD)	77.3 (19.8)	80.7 (21.5)	NS
Clinical features			
Severity			NS
Intermittent, n (%)	5 (7.8)	10 (13.7)	
Mild persistent, n (%)	12 (18.8)	23 (31.5)	
Moderate persistent, n (%)	17 (26.6)	17 (23.3)	
Severe persistent, n (%)	30 (46.9)	23 (31.5)	
CHRS/NP, n (%)	43* (71.7)	27† (40.3)	<.001
Anosmia, n (%)	41‡ (67.2)	16§ (24.2)	<.001
Treatment			
Oral systemic CS, n (%)	22 (34.4)	11 (15.1)	<.05
LTRAs, n (%)	11 (17.2)	18 (24.7)	NS
Dose of inhaled CS (mg/d), median‖	800	800	NS

NS, Not significant; *RIST*, radioimmunosorbent test; *CS*, corticosteroids.
*n = 60 and ‡n = 61, patients with AIA.
†n = 67 and §n = 66, patients with ATA.
‖Dose of inhaled corticosteroids is the median daily dose in beclomethasone equivalent units. Beclomethasone is assumed to be equipotent with budesonide and has a potency half that of fluticasone.

greater than 150 pg/mg creatinine, which corresponds to the mean U-LTE4 plus 3 SDs of the healthy control subjects, indicated hyperleukotrienuria, and a U-LTE4 concentration of less than 100 pg/mg creatinine (mean U-LTE4 + 1 SD of the healthy control subjects) indicated normal leukotrienuria. We compared the clinical features between asthmatic patients with hyperleukotrienuria and those with normal leukotrienuria. The differences in demographic characteristics between these 2 groups were analyzed by using the unpaired Student *t* test and the χ^2 test. Relationships were analyzed by using the Spearman rank correlation test. *P* values of less than .05 were regarded as statistically significant.

RESULTS

Table I shows the clinical characteristics of the patients with AIA and those with ATA. These 2 groups were well matched for age, sex, severity of asthma, respiratory function, and doses of both inhaled steroids and leukotriene receptor antagonists. In the ATA group, however, both the frequency of atopic asthmatic patients (69.9% vs 50.0%, $P < .05$) and the level of serum IgE on radioimmunosorbent testing (median, 233 vs 114 IU/mL; $P < .05$) were significantly higher than those in the AIA group. The frequency of the complication of CHRS/NP was higher in the patients with AIA than in the patients

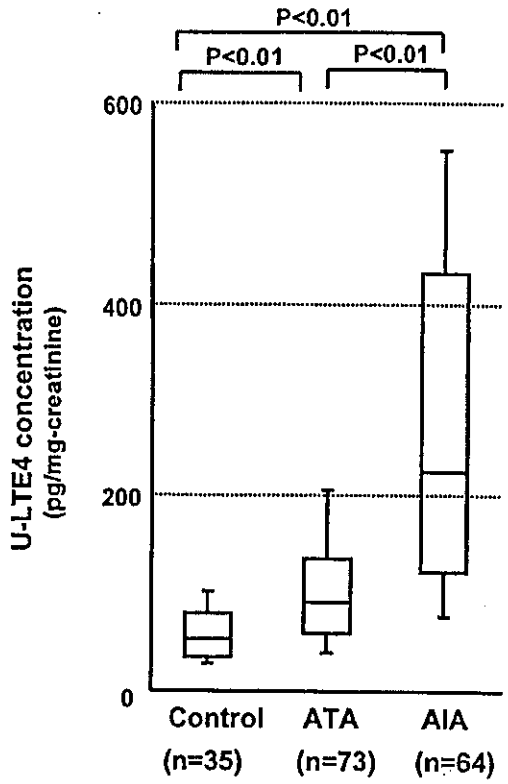

FIG 1. U-LTE4 concentration (in picograms per milligram of creatinine) in patients with AIA, patients with ATA, and healthy control subjects. U-LTE4 concentration in each group is expressed as median plus percentile (10%, 25%, 75%, and 90%).

with ATA (71.7% vs 40.3%, $P < .001$). In addition, the frequency of anosmia was higher in the patients with AIA (frequency, 67.2% vs 24.2%; $P < .001$).

The basal excretion level of U-LTE4 was significantly higher in patients with AIA than in those with ATA (median, 227.2 vs 90.3 pg/mg creatinine; $P < .01$; Fig 1 and Table I). There was no significant difference in the U-LTE4 concentration between the sexes for these 2 groups (data not shown). When U-LTE4 concentrations between these 2 groups with different clinical asthma severity levels were compared, the U-LTE4 concentrations in patients with AIA with moderate or severe persistent asthma were significantly higher than those in patients with ATA with the same severity of asthma ($P < .01$ and $P < .05$, respectively; Fig 2). Table II shows the clinical characteristics of patients with hyperleukotrienuria and those with normal leukotrienuria in each group. The frequency of the patients with AIA with hyperleukotrienuria was 67.2%. Compared with the patients with AIA with normal leukotrienuria, the patients with AIA with hyperleukotrienuria were significantly older (mean, 56.1 vs 44.8 years, $P < .05$) and had a lower pulmonary function (mean FEV_1, 1907 vs 2401 mL; $P < .05$). In contrast, the frequency of patients with ATA with hyperleukotrienuria was 21.9%, and patients with ATA with hyperleukotrienuria had more severe asthma ($P < .05$) and a higher frequency of anosmia (frequency, 50.0% vs 13.9%; $P < .05$). In addition, 81.3% of the

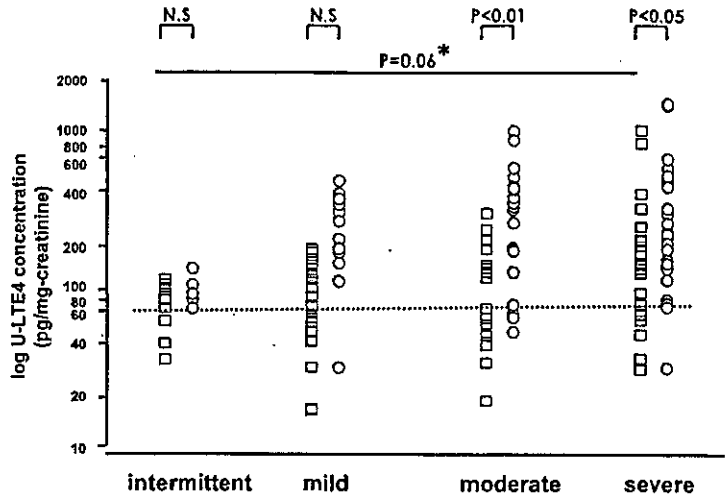

FIG 2. U-LTE4 concentration (in picograms per milligram of creatinine) in patients with AIA and patients with ATA classified according to clinical severity of asthma. U-LTE4 concentration is expressed by using the log scale. Patients with ATA and patients with AIA are denoted by an *open square* and an *open circle*, respectively. The *dotted line* indicates the mean level of U-LTE4 in healthy control subjects. *U-LTE4 concentrations in the patients with ATA with different clinical asthma severity levels were compared by using the Kruskal-Wallis test.

patients with ATA with hyperleukotrienuria appeared to have CHRS/NP compared with 22.9% of the patients with ATA with normal leukotrienuria ($P < .001$). The number of eosinophils in peripheral blood was greater in the patients with ATA with hyperleukotrienuria (median, 860/μL vs 330/μL, $P < .01$). Female patients with ATA with hyperleukotrienuria showed a higher U-LTE4 concentration than male patients with ATA (median, 258.7 vs 184.3 pg/mg creatinine; $P < .05$).

Between the 2 groups with hyperleukotrienuria, the frequency of atopic status was significantly less in the patients with AIA (frequency, 48.8% vs 87.5%; $P < .05$). In contrast, between the 2 groups with normal leukotrienuria, anosmia had a greater frequency in the patients with AIA (frequency, 50.0% vs 13.9%; $P < .05$).

When we examined the correlations between the U-LTE4 concentration and other clinical parameters, a statistically nonsignificant but noteworthy correlation between the U-LTE4 concentration and clinical asthma severity level in the patients with ATA was found ($P = .06$, Fig 2). In addition, U-LTE4 concentrations in the patients with ATA with CHRS/NP (n = 27) were higher than those in the patients with ATA with normal sinuses (n = 30; median, 148.0 vs 81.2 pg/mg creatinine; $P < .05$; Fig 3). In contrast, there were 43 patients with CHRS/NP and only 8 with normal sinuses in the AIA group. No significant correlations between the U-LTE4 concentration and clinical parameters, such as asthma severity or the complication of CHRS/NP, were observed in the AIA group. There was a significant decrease in the U-LTE4 concentration after the sinus surgery in 15 asthmatic patients (median, 221.3 vs 72.3 pg/mg creatinine; $P < .01$). Six of 9 asthmatic patients with hyperleukotrienuria before the sinus surgery were not classified into the hyperleukotrienuria group after the sinus surgery. Fig 4 shows significant decreases in the U-LTE4 concentrations before and after the sinus surgery in both the AIA and ATA groups (median, 322.7 pg/mg creatinine [before] vs 98.2 pg/mg creatinine [after], $P < 0.05$, for the AIA group; 93.4 pg/mg creatinine [before] vs 80.9 pg/mg creatinine [after], $P < .05$, for the ATA group). Before the sinus surgery, the U-LTE4 concentrations of both groups were not significantly different. Similarly, the U-LTE4 concentrations of both groups after the sinus surgery showed no significant difference. All the nasal polyp and sinus sections revealed a diffused and marked infiltration of eosinophils, leading to the diagnosis of CHRS/NP.[36-38]

DISCUSSION

There is accumulating evidence that supports the central role of cys-LTs as mediators of inflammatory diseases.[1] An increase in the U-LTE4 concentration has been observed not only in asthma but also in autoimmune diseases, including rheumatoid arthritis[39] and systemic lupus erythematosus.[40] However, the relationship between the clinicopathogenetic factors of asthma and U-LTE4 concentration remains undetermined. We have demonstrated for the first time that there are some important clinicopathogenetic factors associated with increased excretion levels of U-LTE4 (ie, hyperleukotrienuria) in asthmatic patients. Hyperleukotrienuria in patients with AIA (67.2% of patients with AIA in this study) was associated with older age and decrease in pulmonary function. On the other hand, hyperleukotrienuria in patients with ATA (21.9% of the patients with ATA in this study) was associated with severe asthma and CHRS/NP, which are well-known symptoms of the aspirin triad,[41] as well as hypereosinophilia and anosmia. Conversely, the U-LTE4 concentration in the patients with ATA with CHRS/NP was

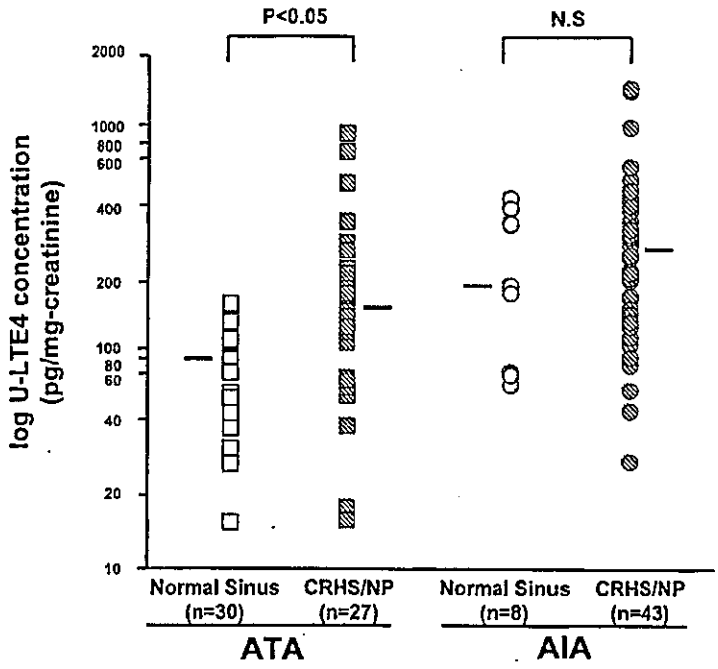

FIG 3. U-LTE4 concentration (in picograms per milligram of creatinine) in patients with CHRS/NP and those with normal sinuses. U-LTE4 concentration is expressed by using the log scale. The *horizontal bars* indicate medians. Patients with ATA and patients with AIA are denoted by an *open square* and an *open circle*, respectively.

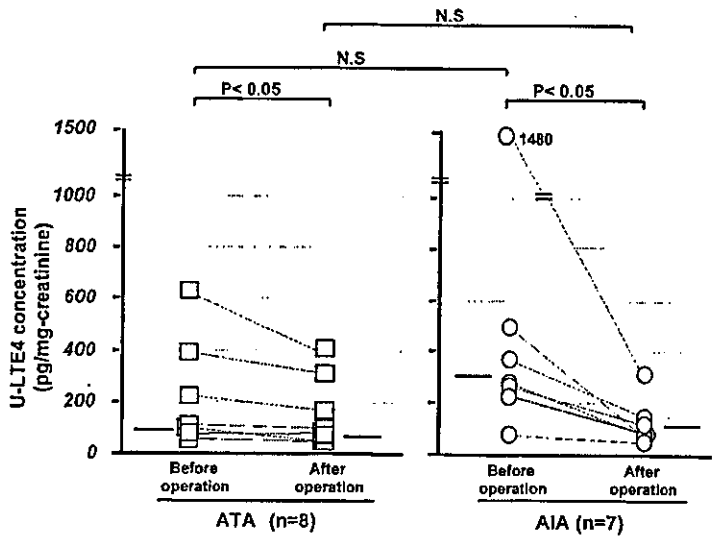

FIG 4. A significant decrease in the U-LTE4 concentration between before and after the endoscopic surgery of rhinosinusitis with nasal polyposis. *Horizontal bars* indicate medians. Patients with ATA and patients with AIA are denoted by *squares* and *circles*, respectively.

higher than that in the patients with ATA with normal sinuses. In addition, a statistically nonsignificant but noteworthy correlation between the U-LTE4 concentration and clinical asthma severity in patients with ATA was found. In support of our findings, ten Brinke et al[42] have suggested a direct relationship between hyperplastic rhinosinusitis and lower airway inflammation in severe asthma, and they have suggested that extensive rhinosinusitis is associated with adult-onset asthma, aspirin intolerance, and nasal polyposis.

Taken together, these findings led us to the following generalizations. First, cys-LT overproduction is not strictly associated with aspirin intolerance itself but is associated with clinical features, such as CHRS/NP, that are similar to those of AIA. Second, CHRS/NP might play an important role in cys-LT overproduction in asthmatic

TABLE II. Demographic characteristics of each group

	AIA			ATA				
	Hyperleuko-trienuria	Normal leukotrienuria	P_{AIA} (h-n)	Hyperleuko-trienuria	Normal leukotrienuria	P_{ATA} (h-n)	PG(h-h)	PG(n-n)
No.	43	12		16	40			
Male/female sex	16/27	3/9	NS	6/10	21/19	NS	NS	NS
Age (y), mean (SD)	56.1 (13.0)	44.8 (11.4)	<.05	52.1 (12.2)	49.2 (16.1)	NS	NS	NS
Onset age (y), mean (SD)	39.2 (14.1)	32.3 (15.9)	NS	39.4 (16.9)	33.5 (17.1)	NS	NS	NS
Atopic status (n)	21 (48.8)	7 (58.3)	NS	14 (87.5)	23 (57.5)	NS (.07)	<.05	NS
Blood eosinophil ($\times 10^6$/mL), median (range)	535 (12-3520)	560 (5-2460)	NS	860 (200-4163)	330 (20-930)	<.01	NS	NS
Serum IgE-RIST (IU/mL), median (range)	114 (14-1390)	119 (15-600)	NS	314 (44-2290)	162 (15-2050)	NS	NS	NS
Pulmonary function tests								
FEV_1 (mL), mean (SD)	1907 (746)	2401 (622)	<.05	2117 (923)	2308 (788)	NS	NS	NS
FEV_1 (% predicted), mean (SD)	73.5 (19.5)	91.1 (18.8)	<.05	81.1 (26.3)	84.1 (20.4)	NS	NS	NS
Clinical features								
Severity			NS			<.05	NS	NS
Intermittent, n (%)	0 (0)	3 (25.0)		0 (0)	8 (20.0)			
Mild persistent, n (%)	9 (20.9)	1 (8.3)		2 (12.5)	15 (37.5)			
Moderate persistent, n (%)	12 (27.9)	4 (33.3)		4 (25.0)	8 (20.0)			
Severe persistent, n (%)	22 (51.2)	4 (33.3)		10 (62.5)	9 (22.5)			
CHRS/NP, n (%)	30* (75.0)	6 (50.0)	NS	13 (81.3)	8† (22.9)	<.001	NS	NS
Anosmia, n (%)	28* (70.0)	6 (50.0)	NS	8 (50.0)	5‡ (13.9)	<.05	NS	<.05
Treatment								
Oral systemic CS, n (%)	12 (27.9)	4 (33.3)	NS	1 (6.3)	6 (15.0)	NS	NS	NS
LTRAs, n (%)	12 (27.9)	4 (33.3)	NS	3 (18.8)	9 (22.5)	NS	NS	NS
Dose of inhaled CS (mg/d), median§	800	950	NS	800	800	NS	NS	NS

*n = 40, patients with AIA with hyperleukotrienuria.
†n = 35 and ‡n = 36, patients with ATA with normal leukotrienuria.
§Dose of inhaled corticosteroids is the median daily dose in beclomethasone equivalent units. Beclomethasone is assumed to be equipotent with budesonide and has a potency half of that of fluticasone.
P(h-n), Significant differences between asthmatic patients with hyperleukotrienuria and those with normal leukotrienuria; *PG*, significant differences between the 2 groups; *PG(h-h)*, significant differences between the 2 groups with hyperleukotrienuria; *PG(n-n)*, significant differences between the 2 groups with normal leukotrienuria; *NS*, not significant; *RIST*, radioimmunosorbent test; *CS*, corticosteroids.

patients. The precise pathophysiologic mechanism underlying hyperleukotrienuria remains obscure. We attempted to reconfirm the involvement of CHRS/NP in the pathogenesis of hyperleukotrienuria, and thus we compared the U-LTE4 concentrations between before and after the sinus surgery. We demonstrated for the first time that there is a significant decrease in the U-LTE4 concentrations after the sinus surgery, suggesting that sinonasal tissue might be one of the main tissues that produce cys-LTs in asthmatic patients. Surgical resection of both large nasal polyps and extensive hyperplastic sinonasal mucosa contributes to the termination of the vicious cycle in which cys-LTs and chemotactic factors produced in the sinonasal tissue[43] promote the chemotaxis of eosinophils.[44] More recently, it has been reported that cys-LT concentrations were significantly higher in sinus tissues obtained from patients with CHRS/NP than in healthy sinus tissues and noneosinophilic sinus tissues.[45] Thus the complication of severe CHRS/NP might affect the U-LTE4 concentration, and sinonasal tissues might be one of the main tissues that produce cys-LTs in asthmatic patients. Interestingly, we reconfirmed the decrease in U-LTE4 concentration after surgery for CHRS/NP (72.4 pg/mg creatinine to 32.8 pg/mg creatinine) in only one nonasthmatic patient. Because U-LTE4 has a detection limit to serve as a biomarker of airway inflammation,[32,35] it is necessary to carry out further investigations with a large number of patients. Considering the high recurrence rate of nasal polyposis in patients with AIA,[46] we should examine whether those with CHRS/NP recurrence have significant increases in U-LTE4 levels over time and whether aspirin intolerance will develop in patients with ATA with hyperleukotrienuria exhibiting clinical characteristics similar to those of patients with AIA.

In conclusion, our study showed, for the first time, a close relationship between the U-LTE4 concentration and clinical features of asthmatic patients. We suggest the possibility that cys-LTs are not strictly associated with aspirin intolerance itself but with clinical features such as CHRS/NP that are similar to those of AIA. In particular, our study suggests the involvement of CHRS/NP in cys-LT overproduction in asthmatic patients.

REFERENCES

1. Drazen JM. Cysteinyl leukotrienes. In: Busse WW, Holgate ST, editor. Asthma and rhinitis. 2nd ed. Oxford: Blackwell Scientific Publications; 2000. p. 1014-26.
2. Lewis RA, Austen KF, Soberman RJ. Leukotrienes and other products of the 5-lipoxygenase pathway. N Engl J Med 1990;323:645-55.
3. Henderson WR Jr. The role of leukotrienes in inflammation. Ann Intern Med 1994;121:684-97.
4. Maltby NH, Taylor GW, Ritter JM, Moore K, Fuller RW, Dollery CT. Leukotriene C4 elimination and metabolism in man. J Allergy Clin Immunol 1990;85:3-9.
5. Verhagen J, Bel EH, Kijne GM, Sterk PJ, Bruynzeel PL, Veldink GA, et al. The excretion of leukotriene E4 into urine following inhalation of leukotriene D4 by human individuals. Biochem Biophys Res Commun 1987;148:864-8.
6. Asano K, Lilly CM, O'Donnell WJ, Israel E, Fischer A, Ransil BJ, et al. Diurnal variation of urinary leukotriene E4 and histamine excretion rates in normal subjects and patients with mild-to-moderate asthma. J Allergy Clin Immunol 1995;96:643-51.
7. Manning PJ, Rokach J, Malo JL, Ethier D, Cartier A, Girard Y, et al. Urinary leukotriene E4 levels during early and late asthmatic responses. J Allergy Clin Immunol 1990;86:211-20.
8. Sladek K, Dworski R, Fitzgerald GA, Buikus KL, Block FJ, Mamey SR Jr, et al. Allergen-stimulated release of thromboxane A2 and leukotriene E4 in humans. Effect of indomethacin. Am Rev Respir Dis 1990;141:1441-5.
9. Kumlin M, Dahlen B, Bjorck T, Zetterstrom O, Granstrom E, Dahlen SE. Urinary excretion of leukotriene E4 and 11-dehydro-thromboxane B2 in response to bronchial provocations with allergen, aspirin, leukotriene D4, and histamine in asthmatics. Am Rev Respir Dis 1992;146:96-103.
10. Drazen JM, O'Brien J, Sparrow D, Weiss ST, Martins MA, Israel E, et al. Recovery of leukotriene E4 from the urine of patients with airway obstruction. Am Rev Respir Dis 1992;146:104-8.
11. Oosaki R, Mizushima Y, Kawasaki A, Mita H, Akiyama K, Kobayashi M. Correlation among urinary eosinophil protein X, leukotriene E4, and 11-dehydrothromboxane B_2 in patients with spontaneous asthmatic attack. Clin Exp Allergy 1998;28:1138-44.
12. Christie PE, Tagari P, Ford-Hutchinson AW, Charlesson S, Chee P, Arm JP, et al. Urinary LTE4 concentrations increase after aspirin challenge in aspirin-sensitive asthmatic subjects. Am Rev Respir Dis 1991;143:1025-9.
13. Oosaki R, Mizushima Y, Mita H, Shida T, Akiyama K, Kobayashi M. Urinary leukotriene E4 and 11-dehydro-thromboxane B_2 in patients with aspirin-sensitive asthma. Allergy 1997;52:470-3.
14. Nasser SM, Pfister R, Christie PE, Sousa AR, Barker J, Schmitz-Schumann M, et al. Inflammatory cell populations in bronchial biopsies from aspirin-sensitive asthmatic subjects. Am J Respir Crit Care Med 1996;153:90-6.
15. Sousa AR, Lams BEA, Pfistar R, Christie PE, Schmitz M, Lee TH. Expression of interleukin-5 and granulocyte-macrophage colony-stimulating factor in aspirin-sensitive and non-aspirin-sensitive asthmatic airways. Am J Respir Crit Care Med 1997;156:1384-9.
16. Cowburn AS, Sladek K, Soja J, Adamek L, Nizankowska E, Szczeklik A, et al. Profound overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. J Clin Invest 1998;101:1-13.
17. Sanak M, Simon HU, Szczeklik A. Leukotriene C4 synthase promoter polymorphism and risk of aspirin-induced asthma. Lancet 1997;350:1599-600.
18. Sanak M, Pierzchalska M, Bazan-Socha S, Szczeklik A. Enhanced expression of the leukotriene C(4) synthase due to overactive transcription of an allelic variant associated with aspirin-intolerant asthma. Am J Respir Cell Mol Biol 2000;23:290-6.
19. Van Sambeek R, Stevenson DD, Baldasaro M, Lam BK, Zhao J, Yoshida S, et al. 5' flanking region polymorphism of the gene encoding leukotriene C4 synthase does not correlate with the aspirin-intolerant asthma phenotype in the United States. J Allergy Clin Immunol 2000;106:72-6.
20. Kawagishi Y, Mita H, Taniguchi M, Maruyama M, Oosaki R, Higashi N, et al. Leukotriene C4 synthase promoter polymorphism in Japanese patients with aspirin-induced asthma. J Allergy Clin Immunol 2002;109:936-42.
21. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. Am Rev Respir Dis 1987;136:225-44.
22. NHLBI/WHO workshop report. Global strategy for asthma management and prevention. Bethesda, Md: National Institute of Health, National Health, Lung and Blood Institute; 2002. Publication no. 02-3659.
23. Doty RL, Shaman P, Dann M. Development of the University of Pennsylvania Smell Identification Test: a standardized microencapsulated test of olfactory function. Physiol Behav 1984;32:489-502.
24. Hamilos DL, Leung DY, Wood R, Bean DK, Song YL, Schotman E, et al. Eosinophil infiltration in nonallergic chronic hyperplastic sinusitis with nasal polyposis (CHS/NP) is associated with endothelial VCAM-1 upregulation and expression of TNF-α. Am J Respir Cell Mol Biol 1996;15:443-50.
25. Kaliner MA, Osguthorpe JD, Fireman P. Sinusitis: bench to bedside. J Allergy Clin Immunol 1997;99(suppl):S829-48.
26. Mygind N, Dahl R, Bachert C. Nasal polyposis, eosinophil dominated inflammation, and allergy. Thorax 2000;55(suppl):S79-83.
27. Eichel BS. A proposal for staging system for hyperplastic rhinosinusitis based on the presence or absence of intranasal polyposis. Ear Nose Throat J 1999;78:262-8.
28. Newman LJ, Platts-Mills TAE, Phillips CD, Hazen KC, Gross CW. Chronic sinusitis: relationship of computed tomographic findings to allergy, asthma, and eosinophilia. JAMA 1994;271:363-7.
29. Bresciani M, Paradis L, Des Rouches A, Vernhet H, Vachier I, Godard P, et al. Rhinosinusitis in severe asthma. J Allergy Clin Immunol 2001;107:73-80.
30. Pfister R, Lutolf M, Schapowal A, Glatte B, Schmitz M, Menz G. Screening for sinus disease in patients with asthma: a computed tomography controlled comparison of A-mode ultrasonography and standard radiography. J Allergy Clin Immunol 1994;94:804-9.
31. Wald ER. Microbiology of acute and chronic sinusitis in children and adults. Am J Med Sci 1998;316:13-20.
32. Sampson AP, Pizzichini E, Bisgaard H. Effects of cysteinyl leukotrienes and leukotriene receptor antagonists on markers of inflammation. J Allergy Clin Immunol 2003;111(suppl):S49-61.
33. Kumlin M. Analytical methods for the measurement of leukotrienes and other eicosanoids in biological samples from asthmatic subjects. J Chromatogr A 1996;725:29-40.
34. Mita H, Oosaki R, Mizushima Y, Kobayashi M, Akiyama K. Efficient method for the quantitation of urinary leukotriene E4: extraction using an Empore C18 disk cartridge. J Chromatogr B Biomed Sci Appl 1997;692:461-6.
35. Higashi N, Taniguchi M, Mita H, Osame M, Akiyama K. A comparative study of eicosanoid concentrations in sputum and urine in patients with aspirin-intolerant asthma. Clin Exp Allergy 2002;32:1484-90.
36. Harlin SL, Ansel DG, Lane SR, Myers J, Kephart GM, Gleich GJ. A clinical and pathologic study of chronic sinusitis: the role of the eosinophil. J Allergy Clin Immunol 1988;81:867-75.
37. Demoly P, Crampette L, Mondain M, Campbell AM, Lequeux N, Enander I, et al. Assessment of inflammation in noninfectious chronic maxillary sinusitis. J Allergy Clin Immunol 1994;94:95-108.
38. Ferguson BJ. Eosinophilic mucin rhinosinusitis a distinct clinicopathological entity. Laryngoscope 2000;110:799-813.
39. Fauler J, Thon A, Tsikas D, von der Hardt H, Frolich JC. Enhanced synthesis of cysteinyl leukotrienes in juvenile rheumatoid arthritis. Arthritis Rheum 1994;37:93-7.
40. Hackshaw KV, Voelkel NF, Thomas RB, Westcott JY. Urine leukotriene E4 levels are elevated in patients with active systemic lupus erythematosus. J Rheumatol 1992;19:252-8.
41. Samter M, Beers RF. Intolerance to aspirin: clinical studies and consideration of its pathogenesis. Ann Intern Med 1968;68:875-83.
42. ten Brinke A, Grootendorst DC, Schmidt JT, De Bruine FT, van Buchem MA, Sterk PJ, et al. Chronic sinusitis in severe asthma is related to sputum eosinophilia. J Allergy Clin Immunol 2002;109:621-6.
43. Minshall EM, Cameron L, Lavigne F, Leung DY, Hamilos D, Garcia-Zepada EA, et al. Eotaxin mRNA and protein expression in chronic sinusitis and allergen-induced nasal responses in seasonal allergic rhinitis. Am J Respir Cell Mol Biol 1997;17:683-90.
44. Laitinen LA, Laitinen A, Haahtela T, Vilkka V, Spur BW, Lee TH. Leukotriene E4 and granulocytic infiltration into asthmatic airways. Lancet 1993;341:989-90.
45. Steinke JW, Bradley D, Arango P, Crouse CD, Frierson H, Kountakis SE, et al. Cysteinyl leukotriene expression in chronic hyperplastic sinusitis-nasal polyposis: importance to eosinophilia and asthma. J Allergy Clin Immunol 2003;111:342-9.
46. Jantti-Alanko S, Holopainen E, Malmberg H. Recurrence of nasal polyps after surgical treatment. Rhinology 1989;8:59-64.

Urinary eicosanoid and tyrosine derivative concentrations in patients with vasculitides

Noritaka Higashi, MD, PhD,[a,b] Haruhisa Mita, PhD,[a] Masami Taniguchi, MD, PhD,[a] Naomi Turikisawa, MD, PhD,[a] Ai Higashi, MD,[a,b] Yoshinori Ozawa, MD, PhD,[a] Shigeto Tohma, MD, PhD,[a] Kimiyoshi Arimura, MD, PhD,[b] and Kazuo Akiyama, MD[a]
Sagamihara, Kanagawa, and Kagoshima, Japan

Background: Vasculitides are classified on the basis of the type of cell involved, namely, eosinophilic vasculitides such as Churg-Strauss syndrome (CSS) and noneosinophilic vasculitides. However, knowledge on inflammatory mediators and oxidative tissue damage associated with vasculitides is insufficient.
Objective: We measured the urinary concentrations of inflammatory mediators and tyrosine derivatives to assess biomarkers associated with the pathophysiology of vasculitides.
Methods: Urine was collected from 9 patients with CSS during acute exacerbation and during clinical remission, 24 patients with rheumatoid arthritis in stable condition, and 8 patients with vasculitis diseases (VDs) during acute exacerbation. Leukotriene E_4 (LTE_4), 9α,11β prostaglandin F_2, and eosinophil-derived neurotoxin (EDN) concentrations were determined by enzyme immunoassay. 3-Bromotyrosine (BrY) and 3-chlorotyrosine (ClY) concentrations were determined by gas chromatography-mass spectrometry.
Results: The urinary LTE_4, EDN, BrY, and ClY concentrations were significantly higher in the patients with CSS during acute exacerbation than in healthy control subjects and, except for urinary ClY concentration, significantly decreased during clinical remission. The urinary EDN and BrY concentrations were significantly higher in patients with CSS during acute exacerbation than in patients with VD during acute exacerbation. Only urinary LTE_4 concentration was significantly different between the patients with rheumatoid arthritis in stable condition and the patients with VD during acute exacerbation.
Conclusion: Oxidative tissue damage caused by eosinophil peroxidase is a pathophysiological characteristic of eosinophil-associated diseases such as CSS. Urinary LTE_4 concentration may reflect a pathophysiological event involved in eosinophilic and noneosinophilic vasculitides. Cysteinyl-leukotriene pathways are potential therapeutic targets for small-vessel vasculitides. (J Allergy Clin Immunol 2004;114:1353-8.)

Key words: Churg-Strauss syndrome, vasculitides, 3-bromotyrosine, 3-chlorotyrosine, leukotriene E_4

Abbreviations used
ANCA: Antineutrophil cytoplasmic autoantibody
BrY: 3-Bromotyrosine
ClY: 3-Chlorotyrosine
cr: Creatinine
CSS: Churg-Strauss syndrome
cysLT: Cysteinyl-leukotriene
EDN: Eosinophil-derived neurotoxin
EPO: Eosinophil peroxidase
HC: Healthy control
HOBr: Hypobromous acid
LT: Leukotriene
MPA: Microscopic polyangiitis
PG: Prostaglandin
RA: Rheumatoid arthritis
TA: Temporal arteritis
VD: Vasculitis disease
WG: Wegener granulomatosis

Eosinophils possess a wide range of biological properties. Namely, eosinophils release proteins, inflammatory cytokines, and mediators, such as eicosanoids and platelet-activating factors, and can cause tissue injury by releasing a spectrum of toxic products. Eosinophil peroxidase (EPO)[1-3] also resides in a matrix of cytoplasmic granules and is one of the most abundant proteins in eosinophils.[4] EPO plays a role in mediating the host-defense mechanism, such as the destruction of invading parasites and the pathological damage of host tissue by oxidizing intermediates. Briefly, activated eosinophils generate superoxide (O_2^-) by using a membrane-associated nicotinamide adenine dinucleotide phosphate oxidase,[5] and its dismutation product, H_2O_2.[1] By using H_2O_2 as a cosubstrate, EPO in eosinophils generates a halogenating oxidant, which is a potent reactive, cytotoxic, and diffusible species. Despite the fact that the plasma chloride (Cl^-) concentration is 1000-fold higher than that of bromide (Br^-), interestingly, the major product of the EPO-H_2O_2 system is hypobromous acid (HOBr): $Br^- + H_2O_2 + H^+ \rightarrow HOBr + H_2O$.[6] *In vitro*, HOBr reacts with primary amines to form N-mono-bromamines, and it converts tyrosine to 3-bromotyrosine (BrY).[7,8] Similarly, myeloperoxidase, a structurally and functionally distinct enzyme produced by neutrophils, monocytes, and certain tissue macrophages,[9] also contributes to inflammatory tissue injury. Neutrophils selectively use Cl^- in plasma to

From [a]the Clinical Research Center, National Sagamihara Hospital, Sagamihara, Kanagawa; and [b]the Third Department of Internal Medicine, Kagoshima University School of Medicine, Sakuragaoka, Kagoshima.
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Reprint requests: Noritaka Higashi, MD, Scheeles väg1, PO Box 287, National Institute of Environmental Medicine, Karolinska Institutet, SE-171 77 Stockholm, Sweden. E-mail: Noritaka.Higashi@imm.ki.se.
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TABLE I. Demographic characteristics of patients with CSS

Male/female sex	2/7
Age, y, mean (SD)	51.8 (15.2)
Age at onset, y, mean (SD)	
Asthma onset	42.3 (14.3)
CSS onset	49.8 (14.5)
Blood eosinophil $\times 10^6$/L, median (range)	
During acute exacerbation	6590 (2510-17,880)
During remission	70 (10-700)
IgE-radioimmunosorbent test, IU/mL, median (range)	467 (18-3360)
Cumulative organ involvement, n	
Mononeuritis multiplex	9
Lung	9
Sinus	7
Kidney/urinary tract	7
Heart	4
Skin	2
Mediastinum	1

generate chlorinating oxidants.[10-12] Thus, 3-chlorotyrosine (ClY) is considered to be a selective marker of myeloperoxidase-catalyzed oxidation, whereas BrY is that of EPO-catalyzed oxidation.[13]

The characteristic feature of Churg-Strauss syndrome (CSS),[14,15] an eosinophilic necrotizing vasculitis,[16] is hypereosinophilia in blood and tissues, such as those of the lungs, gastrointestine, nerves, and kidneys. The extent of eosinophilia commonly reflects clinical disease activity.[14,15] Previous studies demonstrated that the seromarkers of the activation of eosinophils, such as eosinophil cationic protein and eosinophil-derived neurotoxin (EDN), can predict a relapse.[17] However, measurement of these seromarkers is not applicable to patients with noneosinophilic small-vessel vasculitides, such as Wegener granulomatosis (WG) and microscopic polyangiitis (MPA). Considering the oxidative reaction-associated myeloperoxidase in the human artery,[12,18] we hypothesized that measuring both BrY and ClY may serve as a powerful method for estimating oxidative tissue damage and the relative contributions of eosinophils versus neutrophils *in vivo*. Furthermore, the high levels of antibody against myeloperoxidase-specific antineutrophil cytoplasmic autoantibody (ANCA) are observed in patients with myeloperoxidase-ANCA–related vasculitides such as WG and MPA.[19,20] Because ANCA-activated neutrophils can adhere to and destroy endothelial cells *in vitro*, ANCA is considered to inhibit the inactivation of myeloperoxidase, resulting in tissue damage.[21,22] In addition, Mayatepek and Lehmann[23] demonstrated a high urinary leukotriene (LT) E_4 concentration in patients with Kawasaki disease, which is the most common childhood vasculitis. However, there has been little experimental evidence to substantiate the close relationship between cysteinyl-leukotriene (cysLT) and vasculitides. According to the National Institutes of Health workshop report,[24] there is no objective evidence that CSS is actually caused by LT receptor antagonists. Because there have been no comparative studies of urinary eicosanoid concentrations and clinical characteristics, we aimed to characterize the profiles of eicosanoid, BrY, and ClY concentrations in patients with systemic small-vessel vasculitides, including CSS.

METHODS

Subjects

The subjects of this study were 9 patients with CSS (age range, 27-75 years; mean age, 51.8 years; 2 male and 7 female), 8 patients with vasculitis diseases (VDs) during acute exacerbation (age range, 50-77 years; mean age, 66.0 years; 2 male and 6 female), 24 patients with rheumatoid arthritis (RA) in a clinically stable condition (age range, 39-77 years; mean age, 60.1 years; 6 male and 18 female), and 8 healthy control (HC) subjects (age range, 27-55 years; mean age, 37.3 years; 4 male and 4 female). CSS was diagnosed according to the 1990 American College of Rheumatology criteria and the 1992 Chapel Hill definition.[25,26] Three patients had recurrent CSS. The mean age at onset of CSS was 49.8 (Table I). Histopathological confirmations, such as necrotizing vasculitis, extravascular necrotizing granulomas, and/or hypereosinophilia in extravascular tissues, were present in all 9 patients with CSS. Clinical examinations and staging included the lung function test, chest radiography, bronchoalveolar lavage test, echocardiography, radioisotope scintigraphy, otorhinolaryngologic and neurologic examinations, and laboratory screening for ANCA.[27] During the acute exacerbation of CSS, all patients with CSS showed vasculitis symptoms involving multiple organs, such as eosinophilic pneumonia and cardiopathy, in addition to peripheral hypereosinophilia (mean %, 58.1%) and mononeuritis multiplex, as shown in Table I. After intensive immunosuppressive therapies with drugs including systemic corticosteroids (n = 5 with intravenous administration of 1000 mg/d methylprednisolone for 3 days; n = 9 with 30-40 mg/d prednisolone), cyclosporine (n = 5, 50-100 mg/d), and/or intravenous immunoglobulin[27] (n = 7), all 9 patients with CSS were clinically in a disease remission phase, maintained at a dose of 5 to 25 mg/d prednisolone (n = 7) in addition to cyclosporine (n = 4, 50-100 mg/d) at the time of follow-up examination. The mean percentage of peripheral eosinophils was 2.3%. The duration between acute exacerbation and remission was 6.1 ± 2.5 months.

The patients with VD had an acute exacerbation of vasculitis accompanied by autoimmune diseases, such as MPA,[26] WG,[26,28] temporal arteritis (TA, giant-cell arteritis),[29] and RA[30] (Table II). The patients with VD were diagnosed on the basis of clinical and laboratory examination findings, such as the presence of ANCA (n = 4) and immunocomplex C1q. Pathological vasculitis was confirmed in 5 of 8 patients. In contrast, the stable RA group, composed of 24 patients with RA in a stable condition, was a comparative control for patients with an acute exacerbation of VD. Nine patients received systemic corticosteroid (mean prednisolone dose, 4.2 mg/d), whereas 12 patients received methotrexate therapy. None of the patients had an upper respiratory tract infection in the 4 weeks preceding the study. Permission to conduct the study was obtained from the Ethics Committee of the National Sagamihara Hospital, and all of the patients who participated gave their informed consent.

Measurements

Spot urine was collected between 9:00 and 11:00 AM from patients with CSS during acute exacerbation and during clinical remission, patients with VD during acute exacerbation, patients with stable RA,

TABLE II. Demographic characteristics of patients with VD

Male/female sex	2/6
Age, y, mean (SD)	66.0 (8.4)
White blood cells $\times 10^6$/L	9453 (3024)
Neutrophil %, mean (SD)	81.0 (8.7)
Eosinophil %, mean (SD)	2.1 (1.9)
C-reactive protein, mg/dL, mean (SD)	9.7 (6.6)
C1q, mg/mL, median (range)*	5.0 (1.6-29.6)
Underlying diseases, n	
RA	3
MPA	1
MPA, PSS, and Sjogren syndrome	1
WG	1
Mixed-connective tissue disease and RA	1
TA and Behcet disease	1
Cumulative vasculitis-associated symptoms, n	
Mononeuritis multiplex	5
Skin ulcer	2
Scleritis	2
RA nodule/TA nodule	3/1
Pathological findings, n	5
Obstructive vasculitis/necrotizing vasculitis	3/2

*n = 7.

and HC subjects. In particular, in the cases of acute exacerbations of CSS and VD, urine was collected before intensive immunosuppressive therapy. We determined the urinary concentrations of LTE_4 (Cayman, Ann Arbor, Mich), 9α,11β prostaglandin (PG) F_2 (Cayman), which corresponds to the PGD_2 metabolite, and EDN (MBL, Nagoya, Japan) by enzyme immunoassay as previously reported.[31] The urinary concentrations of BrY and ClY, the selective markers of EPO-catalyzed and myeloperoxidase-catalyzed oxidations, respectively, were determined by gas chromatography-mass spectrometry by using ^{13}C-labeled compounds as internal standards, as reported elsewhere.[32] Briefly, after the addition of $^{13}C_6$-BrY (50 ng) and $^{13}C_6$-ClY (30 ng) to 2 mL urine, BrY and ClY were extracted with 25% methanol by using a reverse-phase column and then converted to the corresponding heptafluorobutyryl *tert*-butyldimethylsilyl derivatives.[33,34] BrY and ClY concentrations were determined by using Shimadzu gas chromatography-mass spectrometry QP2010 (Kyoto, Japan) equipped with a SPD-5 capillary column (15 m; 0.25-mm internal diameter; 0.25-μm film thickness; Supelco, Bellefonte, Pa) in the negative ion chemical ionization mode with methane as the reagent gas. BrY and ClY concentrations were determined by measuring the fragment ions at mass-to-charge ratio (m/z) 489.10 for endogenous compounds and m/z 495.15 for the internal standards. Urinary LTE_4, 9α,11βPGF_2, EDN, BrY, and ClY concentrations were normalized to urinary creatinine (cr) concentration.

Analysis of data

Demographic characteristics are expressed as means ± SDs. The urinary eicosanoid, EDN, BrY, and ClY concentrations are expressed on a log scale in the figures. These urinary concentrations in the 4 groups (CSS during acute exacerbation, VD during acute exacerbation, stable RA, and HC groups) were first compared by using the Kruskal-Wallis test. When a significant difference was found, the Mann-Whitney *U* test with the Bonferroni correction for comparison between groups was performed. The urinary concentrations of the 5 biomarkers in CSS patients during acute exacerbation and clinical remission were compared by using the Wilcoxon *t* test. Relationships were analyzed by using the Spearman rank correlation test. *P* values of less than .05 were regarded as statistically significant.

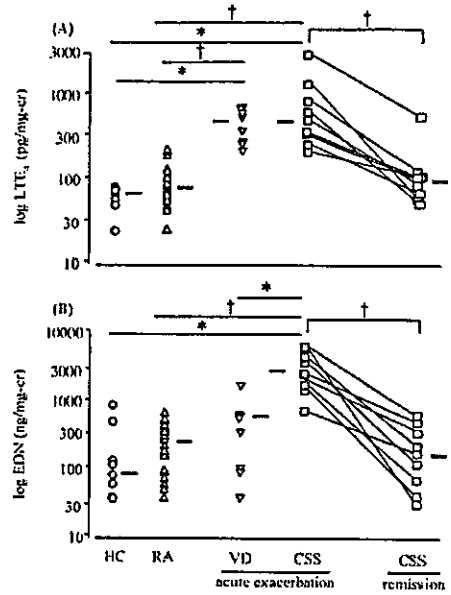

FIG 1. Urinary LTE_4 (A) and EDN (B) concentrations in each group. Urinary concentrations are expressed by using the log scale. Patients with CSS, VD, and RA and HC subjects are denoted by *closed squares, closed triangles, open triangles,* and *open circles,* respectively. *Horizontal bars* indicate medians. *$P < .05$; †$P < .01$.

RESULTS

As shown in Fig 1, the urinary LTE_4 concentration was significantly higher in the patients with CSS during acute exacerbation (median, 449.6 pg/mg-cr) than in the patients with stable RA (median, 79.3 pg/mg-cr; $P < .01$) and the HC subjects (67.5 pg/mg-cr; $P < .05$). A significantly higher urinary EDN concentration was observed in the patients with CSS during acute exacerbation (2404.0 ng/mg-cr) than in the patients with VD (432.3 ng/mg-cr; $P < .05$), the patients with stable RA (296.7 ng/mg-cr; $P < .01$), and the HC subjects (94.9 ng/mg-cr; $P < .05$), respectively. Fig 2 shows the urinary BrY and ClY concentrations in each group. The urinary BrY concentration was significantly higher in the patients with CSS during acute exacerbation (182.6 ng/mg-cr) than in the patients with stable RA (36.8 ng/mg-cr; $P < .01$) and the HC subjects (25.2 ng/mg-cr; $P < .05$). The urinary ClY concentration was significantly higher in the patients with CSS during acute exacerbation (6.1 ng/mg-cr; $P < .05$), the patients with VD during acute exacerbation (9.2 ng/mg-cr; $P < .05$) and the patients with stable RA (4.7 ng/mg-cr; $P < .05$) than in the HC subjects (1.2 ng/mg-cr). No significant difference in urinary 9α,11βPGF_2 concentration was observed among the 4 groups (data was not shown). Next, we examined the correlation between these urinary parameters and the involvement of vasculitis. As shown in Figs 1 and 2, there were significant differences in urinary LTE_4, EDN, and BrY concentrations in the patients with CSS during acute exacerbation and clinical remission (median, for LTE_4, 449.6 pg/mg-cr vs 91.2 pg/mg-cr;

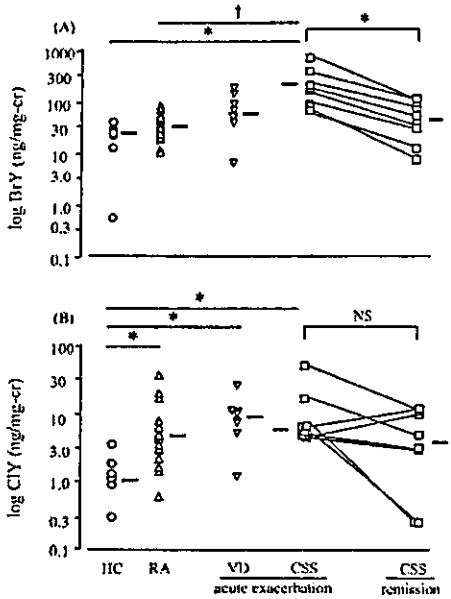

FIG 2. Urinary BrY (A) and ClY (B) concentrations in each group. Results are expressed as in Fig 1. *$P < .05$; †$P < .01$.

$P < .01$; for EDN, 2404.0 ng/mg-cr vs 151.7 ng/mg-cr; $P < .01$; for BrY, 182.6 ng/mg-cr vs 44.4 ng/mg-cr; $P < .05$). Only urinary LTE_4 concentration was significantly different between the patients with stable RA and the patients with VD during acute exacerbation (median, 79.3 pg/mg-cr vs 434.0 pg/mg-cr; $P < .01$). No correlation was found among these 5 urinary markers in any of the 4 groups.

DISCUSSION

Leukotriene E_4 has been identified as a major metabolite of LTC_4, and urinary LTE_4 concentration is now considered the most appropriate analytical parameter for monitoring the endogenous synthesis of cysLTs.[35] In this study, we demonstrated for the first time that urinary LTE_4 concentration was significantly higher in patients with CSS during acute exacerbation than in HC subjects, and significantly decreased during clinical remission. It is most interesting to note that the increased urinary LTE_4 concentration in patients with VD during acute exacerbation was observed despite relatively low EDN concentrations. Recent studies have demonstrated a close relationship between cysLTs and vascular events. Sjöström et al[36] demonstrated that microsomal glutathione S-transferase 2, a distant homologue of LTC_4 synthase, is a critical enzyme present in vascular walls for LTC_4 biosynthesis, originating from the transfer of LTA_4 from granulocytes to endothelial cells. In addition, an increased urinary LTE_4 concentration was observed in patients with ischemic heart diseases.[37-39] Taking these findings together, transcellular biosynthesis among mononuclear cells and endothelial cells plays an important role in the cysLT overproduction in vasculitides. Aspirin intolerance is also characterized by a cysLT overproduction profile.[40-42] In particular, the clinical features of CSS are quite similar to those of the aspirin intolerance phenotype—namely, bronchial asthma, eosinophilic sinusitis, and hypereosinophilia. We previously demonstrated that basal urinary LTE_4 concentration in patients with asthma is higher than that in HC subjects, and that basal urinary LTE_4 concentration in asthmatic patients with eosinophilic sinusitis is higher than that in asthmatic patients without eosinophilic sinusitis.[40] In addition, we preliminarily confirmed in this study that markedly high urinary LTE_4 concentrations (167.6, 188.3, and 199.0 pg/mg-cr) were observed in 3 patients with nonvasculitic eosinophil diseases (acute eosinophilic pneumonia, episodic eosinophilic angioedema, and bronchial asthma with hypereosinophilia). However, despite markedly high percentages of blood eosinophils (mean, 34.6%), the extents of increase in urinary LTE_4 concentrations in these 3 patients with nonvasculitic eosinophil diseases were relatively smaller than in patients with CSS. Thus, particularly in CSS, eosinophilic vasculitides may be involved in cysLT overproduction in addition to eosinophilic pneumonia and sinusitis. Transcellular biosynthesis among endothelial cells and LTC_4 synthase–positive cells, including eosinophils, plays a key role in the mechanism underlying cysLT production in CSS. The vicious cycle, in which cysLTs promote the progenitor effect of LTC_4-producing cells,[43] possibly contributes to the further increased production of cysLTs in patients with an acute exacerbation of vasculitides. At least, this study demonstrated that urinary LTE_4 concentration as a new biomarker determined by a noninvasive methodology possibly contributes to the early diagnosis of small-vessel vasculitides.

In this study, we determined the urinary concentrations of 2 halogenated oxidation products—that is, BrY and ClY. BrY is considered a candidate marker of eosinophil activation,[13,32] ClY of neutrophil and monocyte activation. BrY and ClY concentrations in biological samples such as bronchoalveolar lavage fluid[13,44] and sputum[45] have been determined. Thus, we hypothesized that the adaptation of this methodology is expected to identify oxidative tissue damage and the involvement of specific inflammatory cells in vasculitides and hypereosinophilia.[46,47] In the patients with CSS, the urinary BrY concentration significantly increased during acute exacerbation and decreased during clinical remission. We previously demonstrated the significantly higher urinary BrY and ClY concentrations in patients with asthma than in the HC subjects.[32] Similarly, 3 patients with nonvasculitic eosinophil diseases described previously also showed high urinary BrY concentrations (97.4, 122.9, and 62.0 ng/mg-cr). Thus, these findings suggest that the oxidative tissue damage caused by activated eosinophils is a pathophysiological characteristic of eosinophil-associated diseases such as bronchial asthma and CSS. In contrast, we also analyzed the time course of the concentrations of urinary tyrosine derivatives in 2 patients with severe anaphylactic shock. This additional analysis showed normal urinary tyrosine derivative concentrations despite

marked increases in urinary $9\alpha,11\beta PGF_2$ and LTE_4 concentrations during severe anaphylactic shock, suggesting that the effect of activated mast cells on urinary tyrosine derivative concentration (data not shown) is negligible or limited. Moreover, these results strongly suggest that BrY and ClY are preferentially produced by activated eosinophils and neutrophils/monocytes, respectively. Interestingly, 2 deceased patients with VD showed the highest concentration of urinary BrY (145.6 and 196.2 ng/mg-cr), and 1 of them also had methotrexate-induced pneumonitis.[48] There is clear evidence linking eosinophils and methotrexate-induced lung injury. The blood and tissue eosinophilia are often found in patients with methotrexate-induced pneumonitis.[49] Considering that the patients with VD in our study are heterogeneous, further investigations of a large number of patients with VD are required to determine whether urinary BrY concentration may be a useful prognostic predictor of vasculitides and methotrexate-induced pneumonitis. There was no significant difference in urinary ClY concentration among the patients with RA, VD, and CSS, suggesting that urinary ClY concentration may be a less sensitive biochemical indicator of noneosinophilic oxidative tissue damage. Urinary BrY and ClY concentrations may not be directly associated with the pathogenesis of vasculitides. However, we must consider that the stable and major metabolites of BrY and ClY in urine have not yet been elucidated. Other candidate biomarkers for oxidative tissue damage in vasculitides may be total nitrite and nitrate ($NO_2^- + NO_3^-$) concentration[50] and 3-nitro-4-hydroxyphenyacetic acid concentration[51] in urine.

In conclusion, although urinary LTE_4 concentration failed to discriminate these 2 eosinophilic and noneosinophilic vasculitides, urinary LTE_4 concentration may be used as a sensitive biomarker for monitoring the pathophysiological events involved in vasculitides. These data suggest that cysLT pathways may be new therapeutic targets for small-vessel vasculitides. In addition, we demonstrated for the first time that oxidative tissue damage caused by activated eosinophils is a pathophysiological characteristic of eosinophil-associated allergic diseases such as bronchial asthma and CSS.

REFERENCES

1. Jong EC, Henderson WR, Klebanoff SJ. Bactericidal activity of eosinophil peroxidase. J Immunol 1980;124:1378-82.
2. Ten RM, Pease LR, McKean DJ, Bell MP, Gleich GJ. Molecular cloning of the human eosinophil peroxidase: evidence for the existence of a peroxidase multigene family. J Exp Med 1989;169:1757-69.
3. Sakamaki K, Tomonaga M, Tsukui K, Nagata S. Molecular cloning and characterization of a chromosomal gene for human eosinophil peroxidase. J Biol Chem 1989;264:16828-36.
4. Carlson MG, Peterson CG, Venge P. Human eosinophil peroxidase: purification and characterization. J Immunol 1985;134:1875-9.
5. Klebanoff SJ, Locksley RM, Jong EC, Rosen H. Oxidative response of phagocytes to parasite invasion. Ciba Found Symp 1983;99:92-112.
6. Weiss SJ, Test ST, Eckmann CM, Roos D, Regiani S. Brominating oxidants generated by human eosinophils. Science 1986;234:200-3.
7. Thomas EL, Bozeman PM, Jefferson MM, King CC. Oxidation of bromide by the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase: formation of bromamines. J Biol Chem 1995;270: 2906-13.
8. Wu W, Chen Y, d'Avignon A, Hazen SL. 3-Bromotyrosine and 3,5-dibromotyrosine are major products of protein oxidation by eosinophil peroxidase: potential markers for eosinophil-dependent tissue injury *in vivo*. Biochemistry 1999;38:3538-48.
9. Podrez EA, Abu-Sound HM, Hazen SL. Myeloperoxidase-generated oxidants and atherosclerosis. Free Radic Biol Med 2000;28:1717-25.
10. Weiss SJ, Klein R, Slivka A, Wei M. Chlorination of taurine by human neutrophils: evidence for hypochlorous acid generation. J Clin Invest 1982;70:598-607.
11. Foote CS, Goyne TE, Lehrer RI. Assessment of chlorination by human neutrophils. Nature 1983;301:715-6.
12. Hazen SL, Hsu FF, Mueller DM, Crowley JR, Heinecke JW. Human neutrophils employ chloride gas as an oxidant during phagocytosis. J Clin Invest 1996;98:1283-9.
13. Wu W, Samoszuk MK, Comhair SA, Thomassen MJ, Farver CF, Dweik RA, et al. Eosinophils generate brominating oxidants in allergen-induced asthma. J Clin Invest 2000;105:1455-63.
14. Lanham JG, Elkon KB, Pusey CD, Hughes GR. Systemic vasculitis with asthma and eosinophilia: a clinical approach to the Churg-Strauss syndrome. Medicine 1984;63:65-81.
15. Guillevin L, Cohen P, Gayraud M, Lhote F, Jarrousse B, Casassus P. Churg-Strauss syndrome: clinical study and long-term follow-up of 96 patients. Medicine 1999;78:26-37.
16. Jennette JC, Wilkman AS, Falk RJ. Anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis and vasculitis. Am J Pathol 1989;35:921-30.
17. Schmitt WH, Csernok E, Kobayashi S, Klinkenborg A, Reinhold-Keller E, Gross WL. Churg-Strauss syndrome: serum markers of lymphocyte activation and endothelial damage. Arthritis Rheum 1998;41:445-52.
18. Hazen SL, Heinecke JW. 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J Clin Invest 1997;99:2075-81.
19. van der Woude FJ, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, et al. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. Lancet 1985;1:425-9.
20. Falk RJ, Jenette JC. Anti-neutrophil cytoplasmic antibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotising and crescenteric glomerulonephritis. N Engl J Med 1988;318:1651-7.
21. Savige J, Gillis D, Benson E, Davies D, Esnault V, Falk RJ, et al. International Consensus Statement on Testing and Reporting of Anti-neutrophil Cytoplasmic Antibodies (ANCA). Am J Clin Pathol 1999; 111:507-13.
22. Sundy JS, Haynes BF. Pathogenic mechanisms of vessel damage in vasculitis syndromes. Rheum Dis Clin North Am 1995;21:861-81.
23. Mayatepek E, Lehmann WD. Increased generation of cysteinyl leukotrienes in Kawasaki disease. Arch Dis Child 1995;72:526-7.
24. Weller PF, Plaut M, Taggart V, Trontell A. The relationship of asthma therapy and Churg-Strauss syndrome: NIH workshop summary report. J Allergy Clin Immunol 2001;108:175-83.
25. Masi AT, Hunder GG, Lie JT, Michel BA, Bloch DA, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Churg-Strauss syndrome (allergic granulomatosis and angiitis). Arthritis Rheum 1990;33:1094-100.
26. Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, et al. Nomenclature of systemic vasculitis: proposal of an International Consensus Conference. Arthritis Rheum 1994;37:187-92.
27. Tsurikisawa N, Taniguchi M, Saito H, Himeno H, Ishibashi A, Suzuki S, et al. Treatment of Churg-Strauss syndrome with high-dose intravenous immunoglobulin. Ann Allergy Asthma Immunol 2004;92:80-7.
28. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Arthritis Rheum 1990;33: 1101-7.
29. Hunder GG, Bloch DA, Michel BA, Stevens MB, Arend WP, Calabrese LH, et al. The American College of Rheumatology 1990 criteria for the classification of giant cell arteritis. Arthritis Rheum 1990;33:1122-8.

30. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
31. Higashi N, Taniguchi M, Mita H, Osame M, Akiyama K. A comparative study of eicosanoid concentration in sputum and urine in the patients with aspirin-intolerant asthma. Clin Exp Allergy 2002;32:1484-90.
32. Mita H, Higashi N, Taniguchi M, Higashi A, Kawagishi Y, Akiyama K. Urinary 3-bromotyrosine and 3-chlorotyrosine concentrations in asthmatic patients: lack of increase in 3-bromotyrosine concentration in urine and plasma proteins in aspirin-induced asthma after aspirin challenge. Clin Exp Allergy 2004;34:931-8.
33. Frost MT, Halliwall B, Moore KP. Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. Biochem J 2000;345:453-8.
34. Gaut JP, Byun J, Tran HD, Heinecke JW. Artifact-free quantification of free 3-chlorotyrosine, bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionization gas chromatography mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry. Anal Biochem 2002;300:252-9.
35. Kumlin M. Measurements of leukotrienes in the urine: strategies and applications. Allergy 1997;52:124-35.
36. Sjöström M, Jakobsson PJ, Heimburger M, Palmblad J, Haeggström JZ. Human umbilical vein endothelial cells generate leukotriene C_4 via microsomal glutathione S-transferase type 2 and express the $CysLT_1$ receptor. Eur J Biochem 2001;268:2578-86.
37. Sampson AP. Leukotrienes in cardiovascular disease. Clin Exp Allergy Rev 2001;1:170-4.
38. Brezinski DA, Nesto RW, Serhan CN. Angioplasty triggers intracoronary leukotrienes and lipoxin A4: impact of aspirin therapy. Circulation 1992; 86:56-63.
39. Szczeklik A, Nizankowska E, Mastalerz L, Bochenek G. Myocardial ischemia possibly mediated by cysteinyl leukotrienes. J Allergy Clin Immunol 2002;109:572-3.
40. Higashi N, Taniguchi M, Mita H, Kawagishi Y, Ishii T, Higashi A, et al. Clinical features of asthmatic patients with increased urinary leukotriene E4 excretion (hyperleukotrienuria): involvement of chronic hyperplastic rhinosinusitis with nasal polyposis. J Allergy Clin Immunol 2004;113: 277-83.
41. Szczeklik A, Stevenson DD. Aspirin-induced asthma: advances in pathogenesis, diagnosis and management. J Allergy Clin Immunol 2003;111:913-21.
42. Mastalerz L, Setkowicz M, Sanak M, Szczeklik A. Hypersensitivity to aspirin: common eicosanoid alterations in urticaria and asthma. J Allergy Clin Immunol 2004;113:771-5.
43. Laitinen LA, Laitinen A, Haahtela T, Vilkka V, Spur BW, Lee TH. Leukotriene E4 and granulocytic infiltration into asthmatic airways. Lancet 1993;341:989-90.
44. MacPherson JC, Comhair SA, Erzurum SC, Klein DF, Lipscomb MF, Kavuru MS, et al. Eosinophils are a major source of nitric oxide-derived oxidants in severe asthma: characterization of pathways available to eosinophils for generating reactive nitrogen species. J Immunol 2001; 166:5763-72.
45. Aldridge RE, Chan T, van Dalen CJ, Senthilmohan R, Winn M, Venge P, et al. Eosinophil peroxidase produces hypobromous acid in the airways of stable asthmatics. Free Radic Biol Med 2002;33:847-56.
46. Holgate ST. The epidemic of allergy and asthma. Nature 1999;402:B2-4.
47. Weller PF. Eosinophils: structure and functions. Curr Opin Immunol 1994;6:85-90.
48. Sato E, Camhi SL, Koyama S, Robbins RA. Methotrexate stimulates lung fibroblasts and epithelial cells to release eosinophil chemotactic activity. J Rheumatol 2001;28:502-8.
49. Cooper JA Jr, White DA, Matthay RA. Drug-induced pulmonary disease, part 1: cytotoxic drugs. Am Rev Respir Dis 1986;133:321-40.
50. Evereklioglu C, Özbek E, Çekmen M, Mehmet N, Duygulu N, Ozkiris A, et al. Urinary nitric oxide levels are increased and correlated with plasma concentrations in patients with Behçet's disease: is it a new urinary activity marker? Nephrology (Carlton) 2003;8:231-8.
51. Mani AR, Pannala AS, Orie NN, Ollosson R, Harry D, Rice-Evans CA, et al. Nitration of endogenous para-hydroxyphenylacetic acid and the metabolism of nitrotyrosine. Biochem J 2003;374:521-7.

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Increase in urinary leukotriene B_4 glucuronide concentration in patients with aspirin-intolerant asthma after intravenous aspirin challenge

H. Mita, N. Higashi, M. Taniguchi, A. Higashi and K. Akiyama

Clinical Research Center, National Sagamihara Hospital, Kanagawa, Japan

Summary

Background Aspirin challenge of aspirin-intolerant asthma (AIA) patients causes a significant increase in leukotriene E_4 (LTE_4) concentration in urine. However, knowledge on leukotriene B_4 (LTB_4) generation in patients with AIA is insufficient. Recent research has demonstrated that exogenously administered LTB_4 is excreted as glucuronide into the urine in human healthy subjects.

Objective The purpose of this study is to estimate urinary LTB_4 glucuronide (LTBG) concentration in the clinically stable condition in healthy subjects and asthmatic patients and to investigate changes in urinary LTBG concentration in patients with AIA after aspirin challenge.

Methods A provocation test was performed by intravenous aspirin challenge. After urine was hydrolysed by β-glucuronidase, the fraction containing LTB_4 was purified by high-performance liquid chromatography and LTB_4 concentration was quantified by enzyme immunoassay. Urinary LTBG concentration was calculated as the difference between the concentration obtained with hydrolysis and that without hydrolysis.

Results (1) After hydrolysis, the presence of urinary LTB_4 was verified by gas chromatography-mass spectrometry-selected ion monitoring. (2) The urinary LTBG concentration was significantly higher in the asthmatic patients than in the healthy subjects (median, 5.37 pg/mg creatinine [range 1.2–13] vs. 3.32 pg/mg creatinine [range, 0.14–10.5], $P = 0.0159$). (3) The patients with AIA ($n = 7$), but not those with aspirin-tolerant asthma ($n = 6$), showed significant increases in LTBG and LTE_4 excretions after aspirin challenge. (4) When the concentrations after aspirin challenge were analysed simultaneously, a significant linear correlation was observed between urinary LTBG concentration and urinary LTE_4 concentration in patients with AIA (Spearman's rank correlation test, $r = 0.817$, $P = 0.0003$).

Conclusion LTBG is present in human urine, albeit at a concentration lower than urinary LTE_4. In addition to a marked increase in cysteinyl-leukotriene production, aspirin challenge induced LTB_4 production in AIA patients.

Keywords aspirin-intolerant asthma, leukotriene B_4 glucuronide, leukotriene B_4, leukotriene E_4

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Introduction

Aspirin and related non-steroidal anti-inflammatory drug ingestion precipitates severe bronchoconstriction in a subset of patients with bronchial asthma. This syndrome is referred to as aspirin-intolerant asthma (AIA). Because these drugs inhibit cyclooxygenase, an enzyme responsible for the formation of PGs and thromboxane, it has been assumed that cyclooxygenase has a pivotal role in the initiation of the intolerance reaction, and an abnormality in arachidonic acid metabolism may be responsible in the onset of the reaction [1]. However, the mechanism underlying the intolerance reaction remains unknown. AIA patients show an increased basal production of cysteinyl-leukotrienes (cys-LTs) even under a clinically stable condition, as evidenced by an increased excretion of leukotriene E_4 (LTE_4) into the urine [2–6]. Following aspirin administration, the patients show a further increase in urinary LTE_4 excretion [2, 7, 8]. These findings suggest that cys-LTs play a considerable role in the pathogenesis of AIA [1, 9]. Based on studies in which eicosanoids and chemical mediators were measured in biological fluids, it is most plausible that mast cells, but not eosinophils, are activated in patients with AIA after aspirin challenge [10, 11]. However, it has been impossible to determine definitely the type of cell that generates leukotriene C_4 (LTC_4) in patients with AIA after aspirin challenge despite extensive investigation.

Leukotriene B_4 (LTB_4), which is a product of another branch of the LT pathway, is a chemotactic factor for eosinophils and neutrophils [12]. LTB_4 also stimulates the adhesion of neutrophils to endothelial cells and causes degranulation and superoxide anion generation in neutrophils [13]. *In vivo* LTC_4 production has been estimated by measuring urinary LTE_4 concentration. On the other hand, the method of estimating LTB_4 production has not been established yet, because an easily accessible metabolite has

Correspondence: Haruhisa Mita, Clinical Research Center, National Sagamihara Hospital, 18-1 Sakuradai, Sagamihara, Kanagawa 228-8522, Japan. E-mail address: h-mita@sagamihara-hosp.gr.jp

not been identified in spite of extensive studies [14]. A recent study has demonstrated that LTB_4 glucuronide (LTBG) and 20-carboxy-LTB_4 appear in the urine after an intravenous injection of a relatively large dose of LTB_4 and that these compounds may be used as a marker for the whole-body production of LTB_4 [15, 16]. To date, there have been no studies of the measurement of these compounds in urine from humans who did not receive exogenous LTB_4. The purpose of this study was to establish a method of LTBG quantification and to measure urinary LTBG concentration in order to assess *in vivo* LTB_4 production in humans. In addition, we have measured urinary LTBG concentration after aspirin challenge in AIA patients in order to better understand the relationship between arachidonic acid metabolism and the pathogenesis of AIA.

Materials and methods

Measurement of urinary LTBG concentration

Siliconized glass tubes, polypropylene tubes and polypropylene pipettes were used throughout the study. Two urine aliquots were withdrawn from the stored urine samples, which had been collected during visits to the hospital in the morning (between 9:00 and 12:00 hours). One urine aliquot (1 mL) was subjected to enzymatic hydrolysis and the other urine aliquot (1 mL) was used for the quantification of intact urinary LTB_4 without hydrolysis. Each urine sample was loaded on an LC18 column (Supelco, Bellefonte, PA, USA). The column was washed with distilled water, and LTB_4 and its glucuronide were eluted with 3 mL of methanol. The methanol extract was concentrated under reduced pressure and dissolved in 1 mL of 0.1 M phosphate buffer (pH 7.0). The solution was incubated with 200 U of β-glucuronidase (G7646, Sigma, St Louis, MO, USA) at 37 °C overnight. After extraction using an Empore C18 disk cartridge (3M, St Paul, MN, USA), LTB_4 was purified by high-performance liquid chromatography (HPLC). HPLC was performed on a NOVA-PAK C18 column (Waters, Milford, MA, USA) with a solvent mixture of methanol-distilled water-acetic acid (65 : 35 : 0.1, v/v/v) containing 0.1% EDTA (pH adjusted to 5.4 with ammonium hydroxide) at a flow rate of 1.0 mL/min at 37 °C [17]. LTB_4 and LTE_4 were eluted at 10.1 and 11.1 min, respectively, and two distinct peaks were obtained. The fraction with a retention time corresponding to that of LTB_4 was collected and LTB_4 was recovered from eluates with Empore C18 disk cartridge. The method using Empore C18 disk cartridge is rapid, yields reproducible results. LTB_4 concentration was determined by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA). The value obtained for the sample treated with β-glucuronidase was subtracted from the amount of LTB_4 to obtain the amount of LTBG. The concentrations were expressed as picogram per milligram of creatinine.

LTB_4 identification by gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM)

LTB_4 was converted to its pentafluorobenzyl ester di-trimethylsilyl ether derivative as reported previously [18]. When the derivative was analysed by GC-MS in the negative-ion chemical ionization mode using methane as reagent gas, the mass spectrum of the derivative revealed two prominent ions. The ion at *m/z* 479 was formed as a base ion following the elimination of the pentafluorobenzyl fragment from the molecular ion, and this ion further fragmented to the ion at *m/z* 389 following the loss of the trimethylsilyl alcohol fragment. Due to thermal rearrangement, the retention time of the ion at *m/z* 389 was slightly longer than that at *m/z* 479 [18]. The mass spectrum was measured using Shimadzu GC-MS QP2010 (Kyoto, Japan) equipped with an SPD-5 capillary column (15 m, 0.25 mm internal diameter, 0.25 μm film thickness; Supelco). The ion source and interface temperatures were set at 250 °C. The initial column temperature was maintained at 150 °C for 2 min and then increased to 300 °C at 10 °C/min. The retention time of the derivative was about 9.6 min under these conditions.

Measurement of recovery rates of LTB_4

When we measured the recovery rates of LTB_4 using a ^{3}H-labelled compound, radioactivity remained even when the ^{3}H-labelled compound was decomposed after purification by chromatography, as long as the ^{3}H label remained intact. That is, radioactivity would not identify the ^{3}H-labelled compound itself and it is impossible to measure the correct recovery rates of LTB. Therefore, we used the following method of measuring recovery rate. (1) We added 10 pmol each of deuterium-labelled LTB_4 (d4-LTB_4, Cayman Chemical) and LTB_4 to the urine. We then extracted them with ethyl acetate at pH 1.0 and converted them to pentafluorobenzyl di-trimethylsilyl ether derivatives. The peak areas of the derivatives were measured by GC-MS-NCI at *m/z* 479 and at *m/z* 483, which corresponded to fragment ions derived from LTB_4 and d4-LTB_4, respectively. We assumed that the ratio of the peak area of *m/z* 483 to that of *m/z* 479 is *A*. (2) We added 10 pmol of LTB_4 to the urine, which was then purified using all the methods, and after the purification, 10 pmol of d4-LTB_4 was added. We measured the ratio of the peak area of *m/z* 483 to that for *m/z* 479 of the urine sample, and assumed that if the ratio of the peak area of *m/z* 483 to that for *m/z* 479 is *B*, we could calculate the recovery rate (%) as $B \times 100/A$.

Characteristics of subjects

The study group consisted of 18 bronchial asthmatic patients (seven females, 11 males), which excluded AIA patients, and 18 healthy subjects (11 females, seven males). The mean ages were 52.8 ± 15.6 years for the asthmatic patients and 48.1 ± 13.6 years for the healthy subjects. The asthmatic patients were classified into 13 atopic and five non-atopic. All the asthmatic patients were in a clinically stable condition. None of the asthmatic patients were receiving systemic corticosteroids or a 5-lipoxygenase inhibitor, and 17 out of the 18 asthmatic patients were using inhalation steroids (median, 800 μg equivalent to BDP/day; range, 400–2400 μg). Asthma severity was classified as follows: mild persistent asthma in three patients, moderate persistent asthma in nine patients, and severe persistent asthma in six patients.