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Urinary concentrations of 15-epimer of lipoxin A_4 are lower in patients with aspirin-intolerant compared with aspirin-tolerant asthma

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Summary

Background Although an abnormality in arachidonic acid metabolism may be responsible for aspirin-intolerant asthma (AIA), there is little knowledge about the concentrations of urinary lipoxin A_4 (LXA_4) and the 15-epimer of LXA_4 (15-epi-LXA_4) in relation to asthma severity in AIA subjects.

Objective The purpose of this study is to estimate urinary LXA_4 and the 15-epimer concentrations to investigate lipoxins in AIA.

Methods In this study, we examined AIA, aspirin-tolerant asthma (ATA) and healthy control groups. The AIA and ATA groups were subdivided into the severe asthma and non-severe asthma subgroups. Urinary LXA_4, 15-epi-LXA_4 and leukotriene E_4 (LTE_4) were quantified using enzyme immunoassay after separating these compounds using high-performance liquid chromatography.

Results The urinary LXA_4 concentration was significantly lower than the 15-epi-LXA_4 concentration in the asthmatic subjects. The AIA group showed significantly lower urinary 15-epi-LXA_4 ($P < 0.01$) and higher urinary LTE_4 concentrations ($P < 0.05$) than the ATA group. Comparison of 15-epi-LXA_4 concentrations between the severe asthmatic and non-severe asthmatic subjects in the AIA and ATA groups revealed that the decreased 15-epi-LXA_4 concentration may be related to aspirin intolerance, but not asthma severity. Receiver operator characteristic curves demonstrated that the concentration ratio of LTE_4 to 15-epi-LXA_4 was superior to 15-epi-LXA_4 concentration and LTE_4 concentration as a predictive factor for aspirin intolerance.

Conclusions and Clinical Relevance We have demonstrated for the first time that urinary 15-epi-LXA_4 concentration is significantly higher than LXA_4 concentration in both the AIA and ATA groups. 15-Epi-LXA_4 concentration was significantly lower in the AIA group with an increased urinary LTE_4 concentration than in the ATA group. An imbalance between proinflammatory cysteinyl-leukotrienes and anti-inflammatory 15-epi-LXA_4 may be involved in AIA pathogenesis.

Keywords 15-epimer of lipoxin A_4, aspirin-intolerant asthma, asthma severity, leukotriene E_4, lipoxin A_4

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Introduction

The clinical syndrome aspirin-intolerant asthma (AIA) is characterized by aspirin/non-steroidal anti-inflammatory drug (NSAID) intolerance, bronchial asthma and chronic rhinosinusitis with nasal polyposis [1]. Several clinicoepidemiologic studies [2–4] have demonstrated that AIA is one of the common risk factors for the development of severe asthma. An imbalanced eicosanoid production is presumably associated with the degree of airflow obstruction and asthma severity in AIA subjects, because eicosanoids have both beneficial and detrimental effects on the lung.

We hereby declare no financial relationship with any technological and/or pharmaceutical manufacturer that has interest in the subject matter or materials discussed in this manuscript.

Measurement of urinary concentrations of mediators demonstrated significant differences in eicosanoid concentrations between AIA and aspirin-tolerant asthma (ATA) subjects. The basal urinary concentration of leukotriene (LT) E_4 is significantly higher in AIA subjects [5], and aspirin provocation increases the urinary concentrations of LTE_4 and LTB_4 glucuronide in AIA subjects [6]. We have recently reported that the urinary concentration of prostaglandin E_2 (PGE_2), an indicator of PGE_2 biosynthesis in the kidney, is significantly lower in AIA subjects [7], although Mastalerz et al. have not observed the difference in the basal concentration of urinary PGE_2 metabolites between AIA and ATA subjects [8]. The concentrations of urinary PGE_2 metabolites differently decreased following the administration of aspirin or celecoxib between AIA and ATA subjects [8].

Production of lipoxin (LX), which is an anti-inflammatory compound produced by cell-to-cell interaction (Fig. 1), has been examined in AIA and ATA subjects using whole blood after stimulation with calcium ionophore in some studies. AIA is characterized by a lower capacity for biosynthesis of lipoxin A_4 (LXA_4) [9] and a significantly lower LXA_4 concentration in activated whole blood [10, 11], sputum [12] and bronchoalveolar lavage fluid (BALF) [13] from subjects with severe asthma than from those with milder asthma. On the other hand, the 15-epimer of LXA_4 (15-epi-LXA_4), which exerts about a twofold stronger inhibitory effect on granulocyte adhesion than LXA_4 [14], is also produced by cell-to-cell interaction involving acetylated cyclooxygenase (COX)-2 and 5-lipoxygenase [15] in the presence of aspirin or by potential endogenous acetylating agents [16] (Fig. 1). AIA subjects show a decreased expression level of the COX-2 transcript in nasal polyps [17] or bronchial epithelial cells [18], which may result in a suppressed generation of 15-epi-LXA_4. There has been only one study demonstrating the presence of LXA_4 in urine using enzyme immunoassay (EIA) without separation; however, it was found that the antibody for LXA_4 cross-reacts with 15-epi-LXA_4 [19].

In this study, we quantified urinary LXA_4 and 15-epi-LXA_4 after separating these compounds using high-performance liquid chromatography (HPLC). This method enabled us to precisely determine whether LXA_4 is present in urine samples or whether the observed concentrations resulted from the cross-reactivity of the antibody with 15-epi-LXA_4. Furthermore, we examined the relationship among LXA_4 or 15-epi-LXA_4 concentration, asthma severity and intolerance to aspirin/NSAIDs.

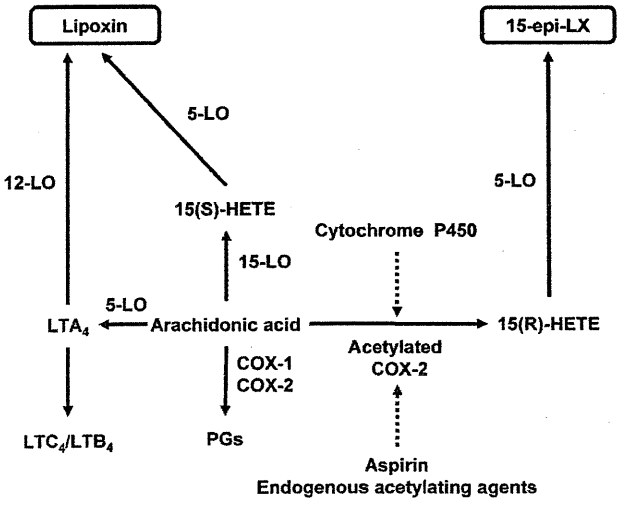

Fig. 1. Pathway for production of lipoxin (LX), 15-epimer of LX (15-epi-LX) and other eicosanoids. Leukotriene (LT) A_4, which is a product of arachidonic acid oxygenation by 5-lipoxygenase (LO), is a precursor of LTC_4 and LTB_4. Arachidonic acid is converted to prostaglandins (PGs) and thromboxane by cyclooxygenase 1 (COX-1) or COX-2. 15 (S)-hydroxyeicosatetraenoic acid (HETE) is produced from arachidonic acid by 15-LO, and the (R) enantimer of 15-HETE is formed by cytochrome P-450 or COX-2 acetylated either by exogenous aspirin or by endogenous acetylating agents. LX is produced from either 15 (S)-HETE by 5-LO or LTA_4 by 12-LO. 15-epi-LX is produced from 15 (R)-HETE by 5-LO. The racemic mixture of 15-HETE is also non-enzymatically formed by oxidation of arachidonic acid.

Materials and methods

Subjects and study design

Subjects with asthma, who were classified into the AIA and ATA groups, were enrolled in this study. Adult asthmatic subjects were recruited from among the outpatients of Sagamihara National Hospital (Table 1). The diagnosis of asthma was based on the American Thoracic Society criteria [20]. AIA patients were subjected to the single-blind provocation test to confirm aspirin sensitivity during the 2 years before the study by a previously described method [5]. They were intravenously administered doubling doses of lysine aspirin (12.5–200 mg equivalent to aspirin). Asthmatic subjects showing a decrease in forced expiratory volume in 1 s ($FEV_{1.0}$) by 20% or greater as compared with the baseline were assigned to the AIA group. Subjects with respiratory tract infection within 6 weeks, ischaemic cardiovascular diseases or renal or liver dysfunction were excluded from the study. None of the subjects of the groups received aspirin, NSAIDs or COX-2-selective inhibitors for at least 6 months prior to urine collection. Healthy control subjects were non-atopic healthy hospital staff members; none of them were taking any medication. Permission to conduct this study was obtained from the Ethics Committee of Sagamihara National Hospital, St. Marianna University School of

Table 1. Clinical characteristics of subjects

		ATA		AIA		
	Control	Non-severe	Severe	Non-severe	Severe	*P* value
No. of subjects (female), *n*	10 (2)	16 (6)		15 (11)		
		9 (3)	7 (3)	5 (4)	10 (7)	
Age, years*	46.3 (17.3)	59.2 (20.3)		53.9 (16.0)		NS
		54.8 (23.0)	64.9 (16.1)	51.8 (26.7)	55.0 (8.8)	
Duration of asthma, years*	NA	12.3 (13.2)		13.1 (10.9)		NS
		8.3 (12.1)	17.3 (13.8)	13.4 (14.6)	12.9 (9.5)	
Peripheral eosinophils (cells/mm^3)*	NM	415.0 (332.7)		630.2 (404.4)		NS
		362.9 (325.1)	488.0 (366.6)	685.5 (417.7)	608.1 (419.7)	
FEV_1 (% predicted),%*	NM	88.0 (20.1)		81.7 (16.9)		NS
		96.4 (15.6)	77.1 (21.0)	92.9 (15.4)	78.9 (17.0)	
Treatments						
Inhaled corticosteroids (ICS), *n*	NA	11		14		
		4	7	4	10	
Daily ICS dose among treated†	NA	400 (100–1300)		1600 (800–1800)		NS
(mcg, budesonide equivalent)		0 (0–400)	1600 (600–1800)	800 (200–1000)	1600 (1600–2000)	
Oral corticosteroids, *n*	NA	2		3		
		0	2	0	3	
LTRAs, *n*	NA	8		7		NS
		3	5	2	5	

*Values are means (SD).

†Values are medians (interquartile range).

AIA, aspirin-intolerant asthma; ATA, aspirin-tolerant asthma; NS, not significant; $FEV_{1.0}$, forced expiratory volume in 1 s; LTRA, leukotriene receptor antagonist; NA, not applicable; NM, not measured.

Medicine, and all the subjects gave their written informed consent.

Asthma severity

Asthma severity was determined on the basis of the criteria developed by the Severe Asthma Research Program (SARP) [4, 10]. The subjects were followed up for at least 6–12 months with dose titration of drugs in accordance with the level of control of asthma. The asthmatic subjects were further classified into the severe asthma and non-severe asthma subgroups, as defined on the basis of the SARP criteria.

Quantification of LTE_4, LXA_4 and 15-epi-LXA_4 in urine

Urine samples were collected between 9:00 and 11:00 AM, and aliquots were stored at −40°C until analysis. Urinary LTE_4 concentration was quantified using EIA after purification using HPLC, as reported previously [21]. Before urine collection, peripheral blood was collected from the subjects to count the number of eosinophils.

Urinary LXA_4 and 15-epi-LXA_4 concentrations were measured using EIA (Neogen Corp. Lexington, KY, USA) after separation using HPLC. Briefly, urine (2 mL) was passed through an Empore C18 disk cartridge (SD type), and both LXA_4 and 15-epi-LXA_4 were eluted from the cartridge using 0.5 mL of methanol. HPLC was performed on a Discovery RP Amide C16 column (Supelco, Park Bellefonte, PA, USA) using a solvent mixture of methanol : distilled water : acetic acid (55 : 45 : 0.1, v/v/v) at a flow rate of 1.0 mL/min at 37°C. The fractions corresponding to the retention times of LXA_4 (approximately 18.3 min) and 15-'epi-LXA_4 (approximately 19.6 min) were collected (Fig. 2). After the fractions were diluted with distilled water, the fraction was loaded onto an Empore C18 cartridge. LXA_4 and 15-epi-LXA_4 were recovered from the cartridge using 0.5 mL of methanol. The methanol extracts were stored at −40°C, and LXA_4 and 15-epi-LXA_4 concentrations were determined using the corresponding EIA kits within 1 or 2 weeks. The concentrations were expressed as pg/mg of creatinine.

When using authentic LXA_4 and 15-epi-LXA_4 (0.4 ng), which were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and Merck KGaA (Darmstadt, Germany), respectively, the overall recovery of LXA_4 and 15-epi-LXA_4 by our method including extraction with an Empore C18 cartridge and purification by HPLC was about 70%. The detection limit of the LXA_4 and 15-epi-LXA_4 EIA kits was 20 pg/mL. The 15-epi-LXA_4 EIA kit showed very little cross-reactivity with LXA_4 (3%). The LXA_4 EIA kit showed 24% cross-reactivity with 15-epi-LXA_4.

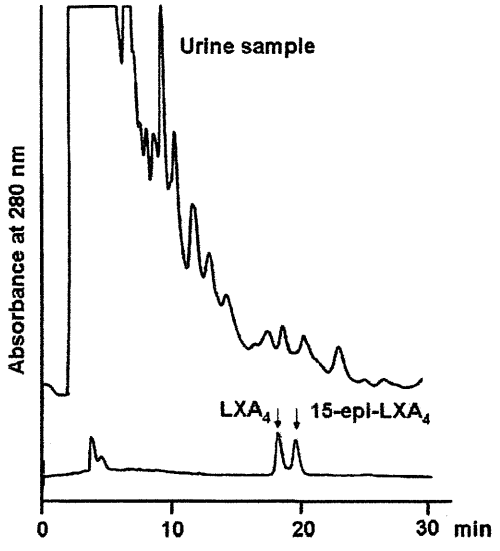

Fig. 2. Separation of extract of urine (upper) and authentic lipoxin A_4 (LXA_4) and 15-epimer of LXA_4 (15-epi-LXA_4) (lower 5.0 ng each) using high-performance liquid chromatography.

Statistical analyses

For samples with LXA_4 and 15-epi-LXA_4 concentrations below the lowest detection limits, the data were expressed as half the detection limit in statistical analyses. Data are expressed as median and inter-quartile range or mean and SD. The Kruskal–Wallis test was performed for comparison among the three groups in addition to post hoc tests. Between-group comparisons were performed using non-parametric Mann–Whitney *U*-test. Correlation analysis was performed using Spearman's rank correlation coefficient. Comparison of categorical variables was performed using Fisher's exact probability test. Differences were considered significant when the *P* value was less than 0.05. Receiver-operating characteristic (ROC) curves were constructed using JMP 8.0 software (SAS Institute, Cary, NC, USA).

Results

Clinical characteristics of subjects

Table 1 shows the clinical characteristics of the subjects enrolled in this study. There were no significant differences in age, duration of asthma, eosinophil count and spirometry baseline between the ATA and AIA groups.

After the provocation test, broncho-constriction was induced in AIA subjects at cumulative doses of 87.5 mg in 11 subjects, 187.5 mg in two subjects and 387.5 mg in two subjects. Several subjects experienced symptoms such as abdominal pain, flushing, cough, nasal obstruction, tearing, nausea and chest tightness.

No significant difference was observed in $FEV_{1.0}$ between the AIA and ATA groups. Statistical evaluation using Fisher's exact probability test revealed that there was no significant difference in the number of severe asthmatic subjects between the ATA and AIA groups, suggesting that there was no significant difference in the prevalence of severe asthma between these groups. The dose of inhaled corticosteroids tended to be slightly higher in the AIA group than in the ATA group, but the difference was not statistically significant ($P = 0.06$).

Urinary mediator concentrations in AIA, ATA and healthy control groups

Table 2 shows a summary of urinary LXA_4, 15-epi-LXA_4 and LTE_4 concentrations. The urinary LTE_4 concentration was significantly higher in the AIA group than in the ATA ($P < 0.05$) and healthy control ($P < 0.01$) groups. The urinary 15-epi-LXA_4 concentrations were significantly higher than urinary LXA_4 concentration in the ATA ($P < 0.01$) and AIA subjects ($P < 0.05$). The urinary 15-epi-LXA_4 concentration was significantly higher in the ATA subjects than in the AIA ($P < 0.01$) and healthy control ($P < 0.01$) subjects. There was a significant correlation between urinary LXA_4 and 15-epi-LXA_4 concentrations in the ATA subjects ($r_s = 0.48$, $P = 0.05$), but not in the AIA or healthy control subjects. There was no significant correlation of urinary LTE_4 concentration with urinary LXA_4 or 15-epi-LXA_4 concentration in the AIA, ATA or healthy control subjects. A significantly negative correlation was found between urinary 15-epi-LXA_4 concentration and the number of peripheral blood eosinophils in the ATA subjects ($r_s = -0.71$, $P < 0.05$), but not in the AIA subjects. No significant correlation was observed between cumulative dose of aspirin during the provocation test and LTE_4 concentration or 15-epi-LXA_4 concentration in AIA subjects. There was no correlation between urinary LXA_4 or 15-epi-LXA_4 concentration and any of the other clinical parameters such as $FEV_{1.0}$.

When 15-epi-LXA_4 concentration in the same urine samples was measured twice, 2 months apart, there was no significant difference between the measured concentrations (mean ± SD, 14.73 ± 5.63 vs. 15.88 ± 8.68, $n = 8$), and the ratio of 15-epi-LXA_4 concentration in the first measurement to that in the second measurement was 1.02 ± 0.37, suggesting that 15-epi-LXA_4 remained stable during storage at −40°C for at least 2 months, and 15-epi-LXA_4 concentration can be measured with a good reproducibility.

Urinary LTE_4 and 15-epi-LXA_4 concentrations and their ratio depending on asthma severity

In the severe asthma subgroup, the AIA subjects showed significantly lower urinary 15-epi-LXA_4 and

Table 2. Urinary mediator concentrations and concentration ratios of urinary LTE_4 to 15-epi-LXA_4 in healthy control, ATA and AIA subjects

		ATA		AIA	
	Control	Non-severe	Severe	Non-severe	Severe
Urinary LTE_4 concentration, pg/mg-creatinine	53.4 (51.5–135.5)	144.3 (73.6–251.5)		345.1* (188.9–1231.0)	
		143.9 (74.2–184.6)	155.4 (74.1–310.0)	345.1 (162.3–431.7)	346.4† (222.9–1541.8)
Urinary LXA_4 concentration, pg/mg-creatinine	9.8 (8.0–15.1)	18.6 (11.4–25.1)		11.3 (6.0–16.3)	
		13.6 (11.3–19.2)	22.5 (18.8–34.2)	14.4 (11.3–16.6)	7.8 (3.1–15.7)
Urinary 15-epi-LXA_4 concentration, pg/mg-creatinine	22.5 (10.0–58.4)	95.3‡ (64.6–142.0)		21.5 (13.7–52.4)	
		88.8 (65.3–128.7)	139.2 (60.1–142.3)	21.5§ (13.5–51.7)	21.2¶ (14.3–47.3)
Concentration ratio of urinary LTE_4 to 15-epi-LXA_4	4.3 (1.3–9.4)	1.9 (1.0–2.9)		14.5‖ (6.2–37.0)	
		1.7 (0.8–3.2)	2.2 (1.4–2.8)	6.7** (4.2–15.6)	21.7†† (9.7–38.1)

Values are medians (interquartile range). The results were analysed using non-parametric Mann–Whitney *U*-test.

*Significantly higher in AIA subjects than in ATA ($P < 0.05$) and control subjects ($P < 0.01$).

†$P < 0.01$ when compared with severe ATA subjects.

‡$P < 0.01$ when compared with control subjects and AIA subjects.

§$P < 0.05$ when compared with non-severe ATA subjects.

¶$P < 0.01$ when compared with severe ATA subjects.

‖Significantly higher in AIA subjects than in ATA subjects ($P < 0.01$) and control subjects ($P < 0.05$).

**$P < 0.01$ when compared with non-severe ATA subjects.

††$P < 0.01$ when compared with severe ATA subjects.

AIA, aspirin-intolerant asthma; ATA, aspirin-tolerant asthma; LXA_4, lipoxin A_4; 15-epi-LXA_4, 15-epimer of LXA_4; LTE_4, leukotriene E_4.

higher urinary LTE_4 concentrations than the ATA subjects [median (interquartile range) AIA vs. ATA: 21.2 (14.3–47.3) vs. 139.2 (60.1–142.3) pg/mg-creatinine ($P < 0.01$) for 15-epi-LXA_4, 346.4 (222.9–1541.8) vs. 155.4 (74.1–310.0) pg/mg-creatinine ($P = 0.05$) for LTE_4]. In addition, even in the non-severe subgroup, the AIA subjects showed a significantly lower urinary 15-epi-LXA_4 concentration than the ATA subjects [21.5 (13.5–51.7) vs. 88.8 (65.3–128.7) pg/mg-creatinine ($P < 0.05$)] (Figs 3 and 4). The concentration ratio of urinary LTE_4 to 15-epi-LXA_4 was significantly higher in the AIA subjects than in the ATA subjects in both the severe and non-severe asthma subgroups [Fig. 5, 21.7 (9.7–38.1) vs. 2.2 (1.4–2.8) ($P < 0.01$) for severe asthma, 6.7 (4.2–15.6) vs. 1.7 (0.8–3.2) ($P < 0.01$) for non-severe asthma].

Receiver-operating characteristic curve analysis suggested that the concentration ratio of LTE_4 to 15-epi-LXA_4 was superior to 15-epi-LXA_4 concentration and LTE_4 concentration as a biomarker for diagnosis of aspirin sensitivity (Fig. 6). The area under curve values were as follows: 15-epi-LXA_4 concentration, 0.908 (95% CI, 0.743–0.971); LXA_4 concentration, 0.696 (0.477–0.852); LTE_4 concentration, 0.788 (0.576–0.910); the concentration ratio of LTE_4 to 15-epi-LXA_4, 0.977 (0.885–0.996); the concentration ratio of LTE_4 to LXA_4, 0.825 (0.620–0.932). As described above, the urinary LXA_4 concentration was significantly lower than the urinary 15-epi-LXA_4 concentrations in both ATA and AIA subjects, and the concentration ratio of LTE_4 to LXA_4 has a lower diagnostic value for AIA.

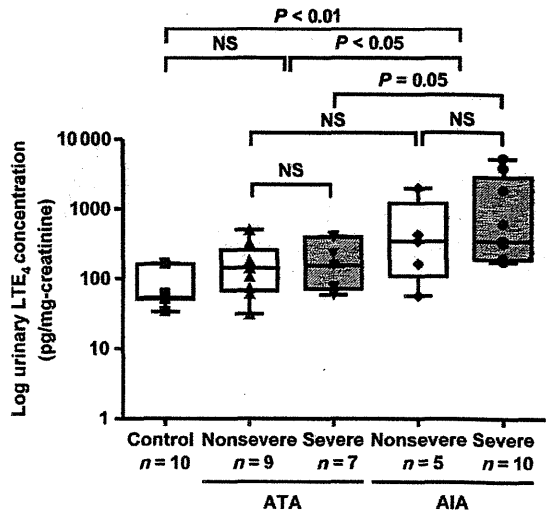

Fig. 3. Urinary leukotriene E_4 (LTE_4) concentrations in healthy control, aspirin-tolerant asthma (ATA) and aspirin-intolerant asthma (AIA) subjects. Data are presented as box plots showing medians and interquartile ranges. In the box plots, the lower boundary indicates the 25th percentile. The line within the box indicates the 50th percentile (median) and the upper boundary of the box indicates the 75th percentile. Error bars above and below the box indicate the maximum and minimum values, respectively. The results were analysed using non-parametric Mann–Whitney *U*-test.

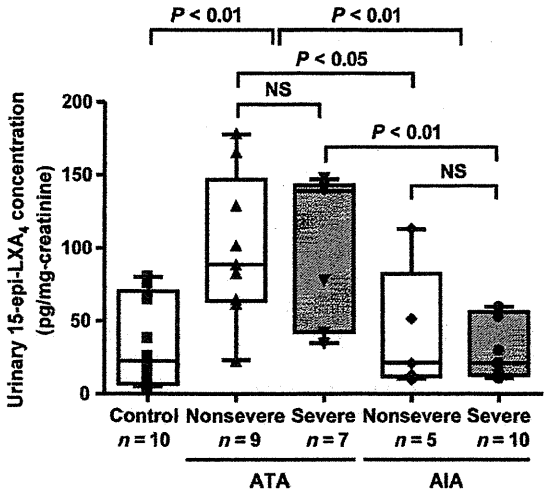

Fig. 4. Concentrations of urinary 15-epimer of LXA_4 (15-epi-LXA_4) in healthy control and aspirin-tolerant asthma (ATA) and aspirin-intolerant asthma (AIA) subjects. Data are presented similar to those in Fig. 3. The results were analysed using non-parametric Mann–Whitney *U*-test.

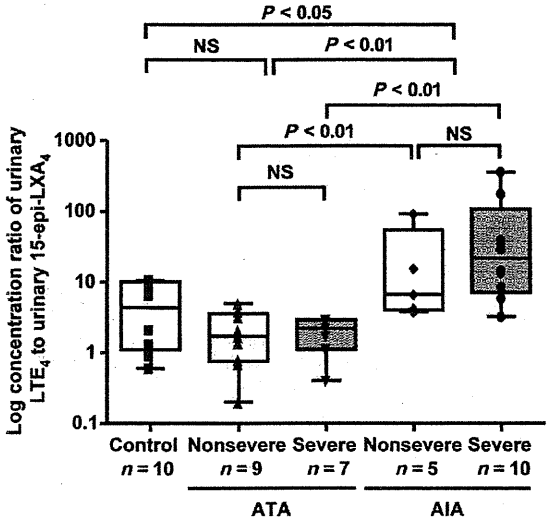

Fig. 5. Concentration ratio of urinary leukotriene E_4 (LTE_4) to 15-epimer of LXA_4 (15-epi-LXA_4) in healthy control, aspirin-tolerant asthma (ATA) and aspirin-intolerant asthma (AIA) subjects. Data are presented similar to those in Fig. 3. The results were analysed using non-parametric Mann–Whitney *U*-test.

Discussion

We have established a method for determining urinary LXA_4 and 15-epi-LXA_4 concentrations using the corresponding EIA kits after separating these compounds using HPLC to overcome the problem of significant cross-reactivity between the anti-LXA_4 antibody and 15-epi-LXA_4, which led us to an unexpected novel finding that the urinary 15-epi-LXA_4 concentration is significantly higher than the urinary LXA_4 concentration.

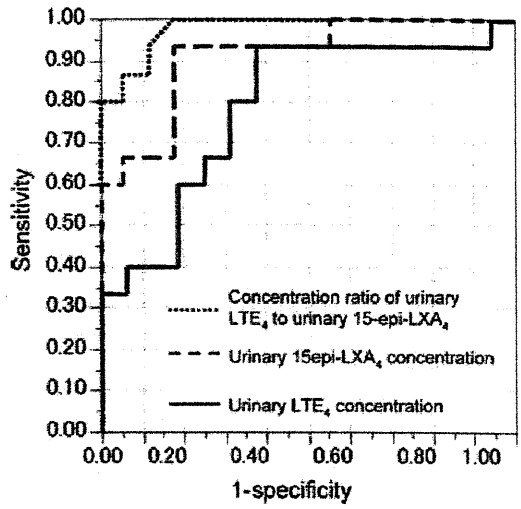

Fig. 6. Receiver operating characteristic curves for evaluation of urinary 15-epimer of LXA_4 (15-epi-LXA_4) concentration, urinary leukotriene E_4 (LTE_4) concentration and the concentration ratio of LTE_4 to 15-epi-LXA_4 for diagnosing aspirin sensitivity. The area under curve values for urinary 15-epi-LXA_4 concentration, urinary LTE_4 concentration and the concentration ratio of LTE_4 to 15-epi-LXA_4 were 0.908 (95% CI, 0.743–0.971), 0.788 (95% CI, 0.576–0.910) and 0.977 (95% CI, 0.885–0.996), respectively.

Both LXA_4 and 15-epi-LXA_4 are metabolized by 15-hydroxyprostaglandin dehydrogenase, which converts 15-epi-LXA_4 into 15-oxo-LXA_4 [22]. The rate of conversion of LXA_4 is twofold higher than that of 15-epi-LXA_4, which may be attributed to the higher 15-epi-LXA_4 concentration than LXA_4 concentration in biological samples. If this explanation is plausible for the concentration of 15-epi-LXA_4 being higher than that of LXA_4, this method might be applied to other samples of biological fluid. When we preliminarily quantified LXA4 and 15-epi-LXA_4 concentrations in BALF from subjects with interstitial lung disease (n = 6), it is surprising that the LXA_4 concentration in BALF was below the detection limit, whereas the median concentration of 15-epi-LXA_4 was 6.7 pg/mL in BALF (unpublished observations). This finding provides us further evidence that the 15-epi-LXA_4 concentration is significantly higher than the LXA_4 concentration in biological fluid. The results of our study indicated that LXA_4 concentration may be incorrectly determined in most previous studies in which 15-epi-LXA_4 and LXA_4 were quantified without separating them and have highlighted the significance of 15-epi-LXA_4 concentration as a biomarker of LX production.

Quantification of urinary LTE_4 provides a reliable means to assess the formation of total endogenous cysteinyl-leukotrienes (CysLTs) [23]. Urinary metabolites of

LXs have not been identified yet. Thus, it is still not clarified whether urinary LXs predominantly reflect local production in the kidney similar to urinary unmetabolized COX-derived prostaglandins [24, 25] or systemic production, which results from filtration of LXs from plasma. Although 15-epi-LXA_4 is also named aspirin-triggered 15-epi-LXA_4, urinary 15-epi-LXA_4 concentration was detectable even in the absence of aspirin administration.

Although no significant difference was observed in 15-epi-LXA_4 concentration between the severe asthmatic and non-severe asthmatic subjects in both the AIA and ATA groups, the 15-epi-LXA_4 concentration in the severe asthmatic subjects of the AIA group was significantly lower than that in severe asthmatic subjects of the ATA group. A similar difference was observed in the non-severe asthmatic subjects between the AIA and ATA groups, suggesting that the decreased concentration of 15-epi-LXA_4 may be related to aspirin intolerance. This finding is consistent with the findings of a lower LXA_4 concentration in the sino-nasal tissue in AIA subjects [26]. Aspirin/NSAID intolerance itself, but not severe asthma, may be associated with diminished 15-epi-LXA_4 production, and an imbalance of arachidonic acid metabolism including 15-epi-LXA_4 may be involved in AIA pathogenesis. 15(R)-hydroxyeicosatetraenoic acid, which is enzymatically formed by acetylated COX-2 or CYP2C9 in the cytochrome P-450 pathway [16, 27], is a precursor of 15-epi-LXA_4. Thus, the findings of this study suggest the possibility of a diminished expression of COX-2, acetylated COX-2 or cytochrome P-450 in AIA subjects. Further studies will be required to clarify the mechanism responsible for the decreased concentration of 15-epi-LXA_4 in AIA subjects.

It has been considered that baseline urinary LTE_4 concentration is a strong predictor of AIA [28]. When evaluated from ROC curves, the concentration ratio of urinary LTE_4 to urinary 15-epi-LXA_4 was a stronger predictive factor than the concentration of LTE_4 or 15-epi-LXA_4 for the diagnosis of aspirin sensitivity in asthmatic subjects.

In contrast, the ATA subjects exhibited a significantly higher urinary 15-epi-LXA_4 concentration than the healthy control subjects ($P < 0.01$), and a significantly negative correlation between urinary 15-epi-LXA_4 concentration and the number of peripheral blood eosinophils. These findings support the idea that 15-epi-LXA_4 plays a protective role against allergic inflammation in animal models of asthma [29, 30]. In allergic airway inflammation, LXA_4 and 15-epi-LXA_4 block both bronchial hyperresponsiveness and pulmonary inflammation induced by eosinophils via the LXA_4 receptor, leading to decreases in the numbers of eosinophils and T lymphocytes and decreases in the concentrations of interleukin-5, interleukin-13, eotaxin, immunoglobulin E, prostanoids and CysLTs [31].

Inhibition of LXA_4 production by dexamethasone in alveolar macrophages has recently been reported [32]. As there was no significant difference between the AIA and ATA subjects in the dose of an inhaled corticosteroid, a significant difference in 15-epi-LXA_4 concentration between AIA and ATA subjects ($P < 0.01$) may not have resulted from the regulation of 15-epi-LXA_4 production by a corticosteroid.

In conclusion, our findings indicate that an imbalance between locally produced proinflammatory CysLTs and anti-inflammatory 15-epi-LXA_4 at baseline concentrations may play an important role in the pathogenesis of AIA.

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ORIGINAL ARTICLE

Increase in Salivary Cysteinyl-Leukotriene Concentration in Patients with Aspirin-Intolerant Asthma

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ABSTRACT

Background: Cysteinyl-leukotrienes (CysLTs; LTC4, LTD4, and LTE4) play a considerable role in the pathophysiology of aspirin-intolerant asthma (AIA). Saliva has recently been validated as novel, simple, and noninvasive method for investigating inflammation in patients with asthma. The aim of this study is to clarify the molecular species of CysLT in saliva and to evaluate the CysLT and LTB4 concentrations in saliva in AIA patients. We also examined how the CysLT concentration in saliva reflects that of their corresponding urinary metabolite.

Methods: We preformed an analytical cross-sectional study. CysLT and LTB4 concentrations in saliva were quantified by enzyme immunoassay (EIA) following purification by high-performance liquid chromatography (HPLC).

Results:

1. When analyzed by EIA in combination with HPLC, saliva was found to consist of LTC4, LTD4 and LTE4 in similar amounts.
2. In saliva analysis among the three groups (AIA patients, aspirin-tolerant asthma [ATA] patients, and healthy subjects), both the concentrations of CysLTs and LTB4 were significantly higher in AIA patients than in ATA patients and healthy subjects.
3. We found significant correlations between CysLT concentration and LTB4 concentration in saliva in each group.
4. No significant correlation was found between the concentration of LTE4 in urine and that of CysLTs in saliva.

Conclusions: In this study, we found higher concentrations of CysLTs and LTB4 in saliva from AIA patients than in saliva from ATA patients, suggesting that the quantification of CysLT and LTB4 concentrations in saliva may be another diagnostic strategy for AIA.

KEY WORDS

aspirin-intolerant asthma (AIA), cysteinyl-leukotrienes (CysLTs), inflammatory mediator, leukotriene B4 (LTB4), saliva

ABBREVIATIONS

AIA, aspirin-intolerant asthma; CysLTs, cysteinyl-leukotrienes; LTB4, leukotriene B4; ENO, exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second; BAL, bronchoalveolar lavage; EBC, exhaled breath condensate; HPLC, high-performance liquid chromatography; EIA, enzyme immunoassay.

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INTRODUCTION

Aspirin-intolerant asthma (AIA) is characterized by a distinct clinical syndrome associated with aspirin intolerance, bronchial asthma, and chronic rhinosinusitis with nasal polyposis.[1] Cysteinyl-leukotrienes (CysLTs; LTC4, LTD4, and LTE4) play a considerable role in the pathophysiology of AIA.[2,3] AIA patients show an increased basal production of CysLTs even under a clinically stable condition.[4,5] In AIA patients, the concentrations of inflammatory mediators such as CysLTs and leukotriene B4 (LTB4) have been examined in various biological samples such as bronchoalveolar lavage (BAL) fluid,[6,7] induced sputum,[8,9] exhaled breath condensate (EBC),[10] and urine.[11-13] BAL has been typically performed to assess airway inflammation; however, the invasiveness of this technique limits its clinical application. Collection of induced sputum is relatively invasive because the technique involves inhalation of hypertonic saline, which induces an inflammatory response. Because the mediator concentration in EBC frequently falls below the detection limit when quantified by enzyme immunoassay (EIA) without condensation, the usefulness of mediator concentration in EBC for understanding the clinical conditions remains to be elucidated. Although urinary LTE4 has been regarded as an indicator of systemic CysLT biosynthesis, the precise origin of LTC4 production has not been established. On the other hand, saliva induction has recently been validated as simple and noninvasive research tool for investigating the inflammation in patients with asthma, and it has recently been reported that CysLTs can be measured in saliva.[14] Gaber *et al.*[15] showed that a 5-lipoxygenase inhibitor, zileuton, effectively inhibits salivary leukotriene levels, suggesting that saliva may be used for monitoring the effect of drugs that affect the leukotriene pathway. However, there are only a few studies that examined CysLT concentration in saliva in AIA patients. In addition, none of the studies have revealed the molecular species of CysLT in saliva, because the CysLT concentration has been measured in saliva without purification by high-performance liquid chromatography (HPLC). The aims of this study are to clarify the molecular species of CysLT in saliva and to evaluate the CysLT and LTB4 concentrations in saliva in AIA patients. We also examined how the CysLT concentration in saliva reflects that of their corresponding urinary metabolite.

METHODS

SUBJECTS AND DESIGN

Non-smoking asthmatic patients were recruited from the Sagamihara National Hospital. An analytical cross-sectional study design was used. The diagnosis of asthma was based on the American Thoracic Society criteria[16] and asthma severity was classified on the basis of daily medication regimen and response to treatment according to the Global Initiative for Asthma guidelines.[17] All the AIA patients had a positive reaction and all the patients with aspirin-tolerant asthma (ATA) have had a negative reaction to aspirin systemic challenge.[18] All the patients were in clinically stable condition. Ten healthy volunteers without subjective symptoms or objective findings of diseases were also enrolled in this study as healthy control subjects. The subjects gave written informed consent and underwent clinical assessment, spirometry and collection of saliva and urine. All samples were collected between 9:00 and 11:00 a.m. The Ethics Committee of Sagamihara National Hospital approved this study.

SALIVA COLLECTION

Saliva was collected by instructing participants to chew a cotton roll (Salivette, Becton Dickinson, North Ryde, Australia) for 3 minutes, after which the saliva was collected by centrifugation and the supernatant was subsequently analyzed.

URINE COLLECTION

Urine samples were collected and stored at -30℃ until analysis.

QUANTIFICATION OF CysLTs

An aliquot of saliva (1 ml) was used for the quantification of CysLTs by EIA following saliva purification by HPLC.[19] Briefly, after extraction with an Empore C18 disk cartridge (3M, St. Paul, MN, USA), the extract was purified by HPLC. The column effluents corresponding to the retention times of authentic LTC4, LTD4, LTE4 and LTB4 were collected and the concentrations were subsequently determined using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA). The retention times of LTC4, LTD4, LTE4 and LTB4 were approximately 6.9, 10.7, 13.1 and 10.1 min under the HPLC conditions used, respectively. The CysLT concentration in saliva was expressed as the total amounts of LTC4, LTD4 and LTE4. The EIA for peptidyl-LTs used in this study is reported to have the following cross-reactivities: LTC4, 100%; LTD4, 100%; LTE4, 70%; N-acetyl LTE_4, 10.5%; other eicosanoids, less than 0.01%. The lower detection limit of the kit was 13 pg/ml.

QUANTIFICATION OF LTB4

The antiserum is reported to have the following cross-reactivities: LTB_4, 100%; 20-hydroxy LTB4, 15%; 6-trans LTB4, 3% and its epimer, 0.03%; 20-carboxy LTB4, 0.14%; other eicosanoids, less than 0.01%. The lower detection limit of the kit was 13 pg/ml.

STATISTICAL ANALYSIS

Data were analyzed using SPSS for MS Windows, version 12.0 (SPSS, Inc., Chicago, IL, USA). Data are ex-

Table 1 Clinical characteristics

	AIA (n = 15)	ATA (n = 11)	P-value
Age (y)	51 (42-65)	55 (38-68)	
Male/female	6/9	4/7	
Atopy/non-atopy	6/9	5/6	
Duration of asthma (y)	20 (3-32)	17 (2-25)	
Blood eosinophils ($\times 10^6$/mm^3)	579 (8-4528)	652 (25-4891)	
Total serum IgE (IU/ml)	176 (17-1258)	321 (22-2556)	
Inhaled steroid dose (μg/day)	1600 (400-2000)	800 (200-2000)	
Histamine PC_{20} (mg/ml)	2.6 (0.1-7.5)	3.8 (0.2-5.8)	
Exhaled NO (ppb)	45.7 (12.1-96.5)	28.6 (10.8-66.3)	P = 0.037
FEV_1 (%predicted)	71.6 (65.5-96.0)	88.5 (61.2-98.2)	

Definitions of abbreviations: PC_{20}, provocation concentration causing a 20% fall in FEV_1 from baseline; Exhaled NO, exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second. Data are expressed as median (range).

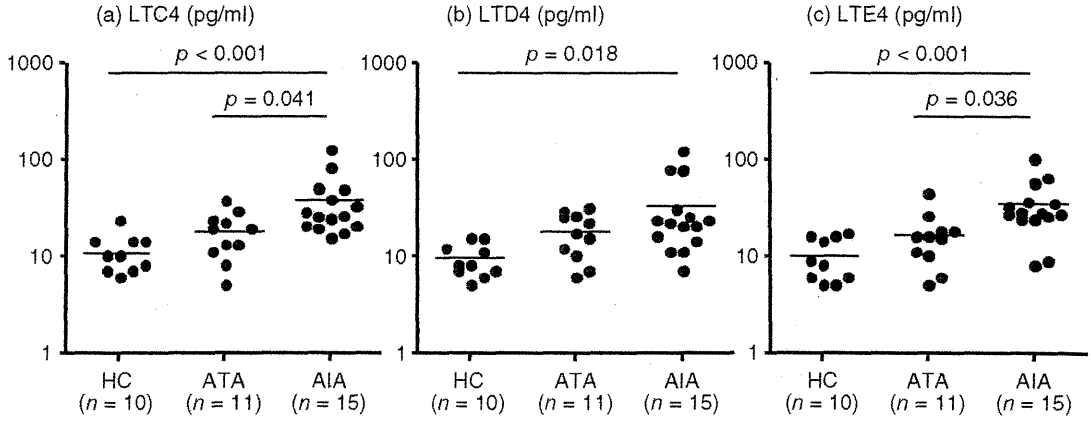

Fig. 1 Concentrations of LTC4, LTD4, and LTE4 in saliva among AIA patients, ATA patients and healthy control subjects. The concentrations of LTC4, LTD4 and LTE4 in saliva among the AIA patients (n = 15), ATA patients (n = 11) and healthy control subjects (n = 10) are shown. The concentrations of LTC4 and LTE4 were significantly higher in AIA patients than in ATA patients. The concentration of LTD4 was significantly higher in AIA patients than in healthy subjects.

pressed as median with range or mean ± SD. Paired data were compared using the Wilcoxon signed rank test for calculation of significance of differences. In all other calculations, an unpaired t-test or the Mann-Whitney test was used. When two groups were compared, Kruskal-Wallis ANOVA followed by the Mann-Whitney test with Bonferroni correction or the Friedman repeated measures of ANOVA followed by the Wilcoxon t-test with Bonferroni correction was carried out. Correlation was evaluated by the Spearman rank test. Differences were considered significant at a P value of less than 0.05.

RESULTS

CLINICAL CHARACTERISTICS OF THE SUBJECTS

The analysis consisted of 15 AIA patients, 11 ATA patients and 10 healthy control subjects. The clinical characteristics of the subjects are summarized in Table 1.

CysLT AND LTB4 CONCENTRATIONS IN SALIVA AMONG AIA PATIENTS, ATA PATIENTS AND HEALTHY SUBJECTS

Saliva collection resulted in a median (range) of 2.2 (0.8-3.8) ml. CysLT concentration was detectable in all saliva samples, although 3 samples were of insufficient volume to have the LTB4 concentration assessed. When analyzed by EIA in combination with HPLC, saliva was found to consist of LTC4, LTD4 and LTE4 in similar amounts. The mean concentrations of LTC4, LTD4 and LTE4 in all the subjects were 38.8 ± 36.2, 44.0 ± 46.9, and 51.1 ± 43.9 fmole/ml, respectively. The results suggest that saliva CysLT consists of LTC4 (29.5 ± 7.2%), LTD4 (32.1 ± 7.3%) and LTE4 (38.4 ± 7.7%) in asthmatic patients. These findings represent the first characterization of the molecular species of CysLTs present in saliva. As depicted in

Figure 1, the concentrations of LTC4, LTD4 and LTE4 were calculated as fmole/ml to express the composition of LTC4, LTD4 and LTE4 in saliva. Subsequently, we chose to express the total concentration of CysLTs as pg/ml.

We first compared the concentrations of LTC4, LTD4 and LTE4 in saliva among the AIA patients, ATA patients and healthy control subjects. The concentrations of LTC4 and LTE4 were significantly higher in AIA patients than in ATA patients (LTC4: $p = 0.041$, LTE4: $p = 0.036$) and healthy subjects (LTC4: $p < 0.001$, LTE4: $p < 0.001$) (Fig. 1a, 1c). The LTD4 concentration was significantly higher in AIA patients than in the healthy subjects ($p = 0.018$; Fig. 1b). In contrast, there was no significant difference in the concentrations of LTC4, LTD4 and LTE4 between ATA patients and healthy subjects. In the saliva analysis among the three groups, the CysLT concentration was significantly higher in AIA patients than in ATA patients ($p = 0.038$) and healthy subjects ($p < 0.001$; Fig. 2).

Subsequently, we also examined the LTB4 concen-

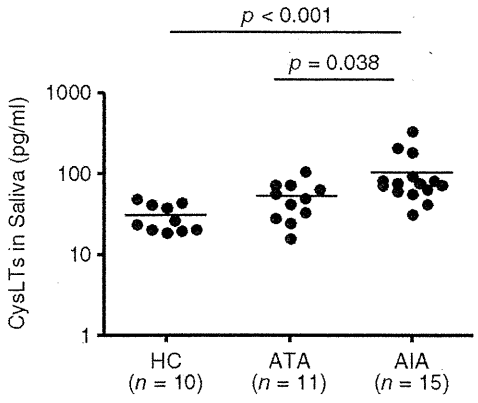

Fig. 2 Concentrations of CysLTs in saliva among AIA patients, ATA patients and healthy control subjects. Data from all the subjects included in this study are presented ($n = 36$). In the saliva the analysis among the three groups, the concentration of CysLTs was significantly higher in AIA patients than in ATA patients and healthy subjects.

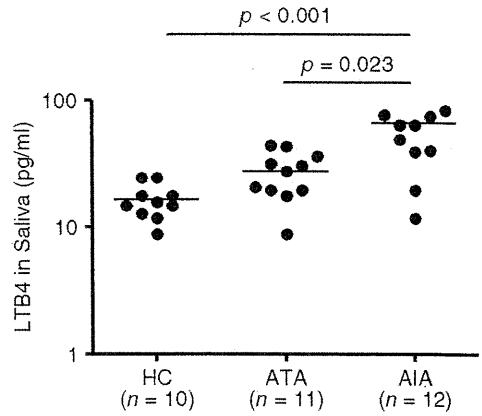

Fig. 3 Concentrations of LTB4 in saliva among AIA patients, ATA patients and healthy control subjects. LTB4 concentration in saliva in 23 patients with asthma (AIA: 12, ATA: 11) and 10 healthy subjects ($n = 10$) are shown. The results of LTB4 concentration in saliva among the three groups were similar to those of LTC4 or LTE4 concentration.

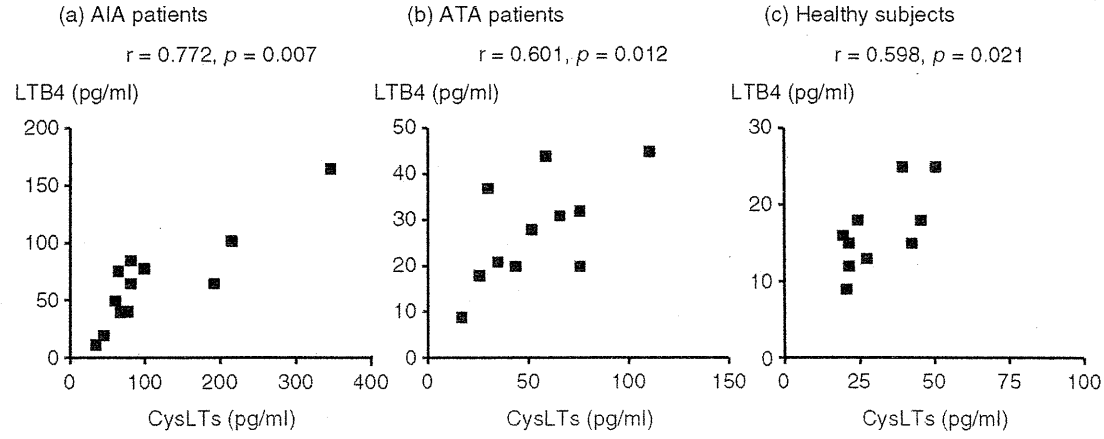

Fig. 4 Correlations between CysLT concentration and LTB4 concentration in saliva in each group. The concentrations of CysLTs and LTB4 in saliva among the AIA patients ($n = 12$), ATA patients ($n = 11$) and healthy control subjects ($n = 10$) are shown. There were significant correlations between CysLT concentration and LTB4 concentration in saliva in AIA patients, ATA patients and healthy subjects.

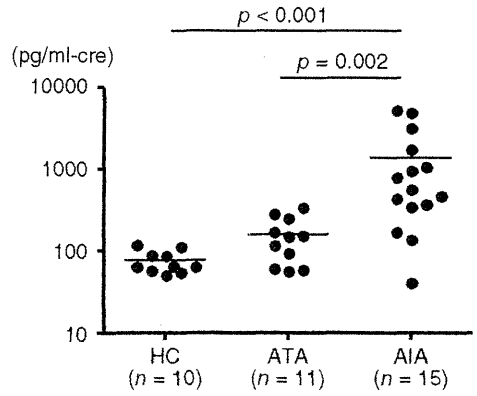

Fig. 5 Concentration of LTE4 in urine among AIA patients, ATA patients and healthy subjects. Data from all the subjects included in this study are presented (n = 36). In the urine analysis among the three groups, the concentration of LTE4 in urine was significantly higher in AIA patients than in ATA patients and healthy subjects.

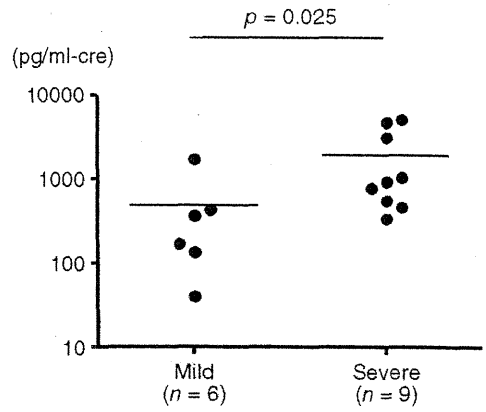

Fig. 6 Relationship between the LTE4 concentration in urine and asthma severity. CysLT concentrations in urine obtained from all the AIA patients (n = 15) are shown. When we classified AIA patients into two groups (mild [6] and severe [9]) on the basis of asthma severity, the concentration of urinary LTE4 significantly correlated with asthma severity.

tration in saliva in 23 patients with asthma (AIA: 12, ATA: 11) and 10 healthy subjects. The results were similar to those of LTC4 or LTE4 concentration among the three groups (Fig. 3). In addition, we found significant correlations between CysLT concentration and LTB4 concentration in saliva in AIA patients (r = 0.772, p = 0.007) as well as in ATA patients (r = 0.601, p = 0.012) and healthy subjects (r = 0.598, p = 0.021) (Fig. 4).

LTE4 CONCENTRATIONS IN URINE AMONG AIA PATIENTS, ATA PATIENTS AND HEALTHY SUBJECTS

In urine analysis among the three groups, the concentration of urinary LTE4 was significantly higher in AIA patients than that in ATA patients (p = 0.002) and healthy subjects (p < 0.001; Fig. 5). There was no significant difference in the concentration of urinary LTE4 between ATA patients and healthy subjects. In this study, we found no significant correlation between the concentration of LTE4 in urine and that of CysLTs in saliva or between the concentration of LTE4 in urine and that of LTB4 in saliva.

CLINICAL UTILITY OF URINARY LTE4 CONCENTRATION COMPARED WITH CysLT CONCENTRATION IN SALIVA IN AIA PATIENTS

Next, we examined the relationships between the clinical parameters and the CysLT concentration in saliva, that of LTB4 in saliva and that of LTE4 in urine, to investigate the significance of these mediators in relation to pathological condition of asthma. When we classified AIA patients into two groups (mild [6] and severe [9]) based on asthma severity, the concentration of urinary LTE4 significantly correlated with asthma severity (p = 0.025; Fig. 6). The concentration of urinary LTE4 was not associated with other clinical parameters such as age, gender, disease duration, percent predicted of FEV_1, exhaled NO concentration, and eosinophil count in peripheral blood. On the other hand, no clinical parameters correlated with the concentration of CysLTs or LTB4 in saliva.

DISCUSSION

This is the first study on the characterization of the molecular species of CysLT in saliva, which was found to consist of LTC4, LTD4 and LTE4 in similar amounts, when analyzed by EIA in combination with HPLC. In addition, we showed that both the concentrations of CysLTs and LTB4 in saliva were significantly higher in AIA patients than in ATA patients and healthy subjects. These results suggest that saliva may provide a new alternative method to distinguish AIA from ATA. Although the CysLT concentration in saliva has been reported,[14,15] the results of our study differ with respect to the CysLT concentration, which was approximately twofold lower than that measured in saliva without purification by HPLC. Because we preliminarily found the significant difference in CysLT concentration between saliva samples purified by HPLC and those assayed without purification by HPLC, we consider that purification by HPLC is required for obtaining reliable data on CysLT concentration in saliva.

With regard to the origin of CysLTs, it is known that LTE4 is a predominant molecular species of CysLT in urine, although a small amount of N-acetyl-LTE4 is also excreted into urine.[2] Thus, urinary LTE4 has been regarded as an indicator of systemic CysLT biosynthesis. In contrast, saliva may reflect the local

CysLT production because we could detect LTC4 and LTD4 in saliva, despite the fact that LTC4 and LTD4 are transitorily present in biological fluids during the course of metabolism and are easily metabolized to LTE4. Because it is known that the concentrations of arachidonic acid metabolites, such as prostaglandin E2, prostaglandin F2α and hydroxyeicosatetraenoic acids exhibit a circadian variation,[20] we collected saliva and urine samples between 9:00 and 11:00 a.m. Cells taken from the oral cavity have been found to produce leukotrienes.[21] In addition, another study has suggested that patients with asthma have local inflammation in salivary glands that seems to mirror airway inflammation.[22] It remains to be elucidated whether the leukotrienes are secreted only from the salivary glands or if further processes such as exchange over ductal epithelium and synthesis by cells in the oral cavity contribute to leukotriene levels in saliva.

To date, only one study has evaluated the relationship between CysLT concentration in saliva and LTE4 concentration in urine.[14] After an intravenous administration of ^{3}H-LTC4 to humans or monkeys, ^{3}H-LTE4 was found to be a predominant metabolite in their urine, and approximately 5% of the total radioactivity was recovered as LTE4; a substantial radioactivity was associated with more polar compounds.[23,24] This supports the fact that the metabolism of endogenously produced LTB4 is the same as that of exogenously administered LTB4, namely, approximately 0.2% of endogenous LTB4 is excreted into the urine as LTB4 glucuronide (LTBG).[25] Thus, the concentration of urinary leukotriene is associated with different metabolic rates in the liver and/or different kinetics of renal elimination of leukotriene between individuals. Metabolic degradation of leukotriene may also occur in the airways. This may contribute to the lack of correlation between CysLT concentration in saliva and LTE4 concentration in urine. Importantly, we showed that the LTE4 concentration in urine significantly correlated with asthma severity in AIA patients, although the CysLT concentration in saliva was not associated with any clinical parameters. A recent study has also shown the increase in the urinary excretion of LTE4 following aspirin-induced and allergen-induced bronchoconstriction, but there was no increase in salivary CysLT concentration after either challenge.[14] These results suggest that the CysLT concentration in saliva might be relatively insensitive to reflect the minor difference in the CysLT concentrations in the airway and might not necessarily reflect systemic CysLT biosynthesis. The source of the enhanced CysLT production in AIA patients may not be organ-specific such as the local bronchus, but systemic including the total pulmonary area. Taken together, the measurement of LTE4 concentration in urine may be superior to that in saliva as parameters in monitoring the pathophysiology of AIA.

In conclusion, we found in this study that the concentrations of CysLTs and LTB4 were higher in saliva from AIA patients than in saliva from ATA patients, suggesting that the quantification of CysLT and LTB4 concentrations in saliva may be another diagnostic strategy for AIA. Further studies are necessary to elucidate the mechanism underlying the increased concentrations of CysLT and LTB4 in the saliva of AIA patients.

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Bacteriologic Evaluation of Sinus Aspirates Taken by Balloon Catheter Devices in Chronic Rhinosinusitis: Preliminary Study

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Key Words
Bacteria · Balloon catheter · Chronic rhinosinusitis

Abstract
Purpose: Chronic rhinosinusitis (CRS) is known to be a polymicrobial infection involving both aerobes and Gram-positive and Gram-negative anaerobes. Accurate bacterial evaluation by adequate culture methods can justify subsequent antimicrobial strategies. ***Methods:*** Two specimens were obtained from each of 10 patients undergoing catheter-based Balloon Sinuplasty™, one from the middle meatus (endoscopic approach) and the other from the sinus (catheter-based approach). ***Results:*** The bacterial culture from the middle meatus was positive in 9 of 10 patients, including 6 different aerobes without anaerobes. The bacterial culture of aspirates from the sinuses were positive in 8 out of 10 patients, with 4 different aerobic bacteria and 4 different anaerobic bacteria. Anaerobes were isolated in 0% of middle meatus samples, which was significantly lower than the 62.5% (5/8) detected in the sinus samples. ***Conclusions:*** Bacterial culture of sinus aspirates using a catheter-based technique improves the recovery of bacterial pathogens from CRS patients.

Introduction

Although chronic rhinosinusitis (CRS) without nasal polyps is multifactorial, many published studies have revealed that bacteria play a major role in the etiology and pathogenesis. Numerous studies have reported the recovery of bacterial pathogens from patients with CRS, and concluded that the predominant isolates are anaerobic [1–4]. Brook [5] summarized 17 CRS studies (n = 1,758) that attempted to recover anaerobes. Anaerobes were recovered in 12–93% of these patients. These data indicate that anaerobic bacteria can be isolated in more than half of CRS patients when adequate methods are utilized [1, 3, 4, 6, 7]. Variability in the isolation rates of anaerobic bacteria is probably due to the differing methods of collection, transportation and cultivation; contamination; and previous antimicrobial treatment.

The clinical application of endoscopic techniques to collect sinus cultures provides reductions in discomfort, pain, and complications as compared with an invasive procedure or a Caldwell-Luc operation [3]. However, it has been suggested that specimens obtained by maxillary endoscopy or endoscopic sinus surgery contain aerobic organisms derived from the nasal mucosa [3, 8, 9].

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Recently developed balloon catheter technology (Balloon Sinuplasty™) appears to be safe and effective for relieving ostial obstructions [10, 11]. This system has also been designed to insert special catheters for sinus lavage, drainage, and antibiotic irrigation. The catheter-based approach may enable the practitioner to selectively collect the sinus effusion with less contamination, as compared with the swab method when utilizing an endoscopic approach.

In the present study, we conducted a bacterial examination of the maxillary sinus aspirates collected using this catheter-based approach. Then the results were compared with those collected using the endoscopic approach in order to gain an accurate picture of the microbiologic environment of the maxillary sinus.

Patients and Methods

Ten patients (3 females and 7 males, mean age 51.2 years, range 38–79 years) with unilateral chronic maxillary sinusitis undergoing balloon catheter sinuplasty at the outpatient clinic of Department of Otorhinolaryngology of Juntendo University Faculty of Medicine between September 2008 and July 2009 were enrolled after giving informed consent. The study was approved by the ethics committee of Juntendo University Faculty of Medicine.

CRS without nasal polyps was diagnosed based on the criteria of the European position paper [12]: they had two or more symptoms one of which should be either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip), ± facial pain/pressure, ± reduction or loss of smell; and endoscopic signs of mucopurulent discharge primarily from middle meatus, and edema/mucosal obstruction primarily in the middle meatus, and computed tomographic changes within the ostiomeatal complex and unilateral maxillary sinus.

The duration of the sinus-related symptoms ranged from 7 months to 2 years. No visible polyps in the middle meatus were observed in any patients. The patients took no antimicrobial therapy for at least 14 days prior to surgery.

Balloon Sinuplasty

Balloon Sinuplasty was performed under general anesthesia according to the previous reports [10, 11]. Before surgery, the nasal vestibule was sterilized with 0.02% chlorhexidine gluconate swabs. Two specimens were obtained from the patients undergoing a balloon catheter sinusotomy. The first specimen was endoscopically obtained from the middle meatus by a microtip cotton applicator before surgery. The second specimen was collected by aspiration during the catheter-based surgery. In the catheter-based approach, a C-arm fluoroscope with minimal time of exposure to radiation was utilized in addition to the standard endoscopic sinus surgery equipment. The catheter devices used here included sinus-guiding catheters, sinus guidewires, sinus balloon catheters, and sinus lavage catheters (Acclarent Inc., Menlo Park, Calif., USA). The guide catheter was introduced into the nasal cavity under endoscopic guidance and placed adjacent to the maxillary ostium. Thereafter, the sinus was catheterized and dilated under fluoroscopic control with a balloon catheter. Finally, the content inside the maxillary sinus was aspirated with the use of a sinus lavage catheter.

Bacterial Culture

The specimens for all bacterial culture were transported immediately to the laboratory (<30 min) in culturette tubes and kept moist with Stuart's bacterial transport medium. These were kept at room temperature until processing. All aerobic specimens were inoculated onto sheep blood/chocolate biplate agar medium (Nissui Pharmaceutical, Tokyo, Japan) in an ambient atmosphere of 35°C for 48 h, then Sabouraud dextrose agar (Eiken Chemical, Tokyo, Japan) and CHROMagar Candida (CHROMagar Paris, France) in ambient atmosphere of 20–25°C for 7 days. All anaerobic specimens were inoculated onto Anaero Columbia with rabbit blood agar (Nippon Becton Dickinson, Tokyo, Japan) in an anaerobic atmosphere at 35°C for 48 h; Brucella HK semisolid medium (Kyokuto Pharmaceutical, Tokyo, Japan) was also used as an enrichment culture.

The identification of isolates from sinus specimens was performed by routine methods in use at the laboratory. The colony morphology and Gram stain reaction were used for identification prior to using the automated identification system. Staphylococci, enterococci, *Enterobacteriaceae*, and glucose non-fermentative Gram-negative bacilli were identified by the MicroScan WalkAway 96SI (Siemens Healthcare Diagnostics, West Sacramento, Calif., USA) automated identification system following the manufacturer's recommendations. For *Staphylococcus aureus*, methicillin resistance was determined using minimal inhibitory concentrations of both oxacillin and cefoxitin by reference to the broth microdilution method using the automated system (as recommended by the Clinical and Laboratory Standards Institute). *Streptococcus pneumoniae* was differentiated by colony morphology and optochin (ethyl hydrocupreine) susceptibility. Major β-hemolytic streptococci (including groups A, B, C, and G) were differentiated using the Seroiden Strept Kit (Eiken Chemical, Tokyo, Japan) as the latex agglutination test.

For isolates that were not identified by the automated identification system, we used the following identification kits and or conventional methods. The ID test EB-20 ID, NF-18, and HN-20 rapid (Nissui Pharmaceutical) were used for *Enterobacteriaceae*, glucose non-fermentative Gram-negative bacilli, and *Haemophilus* species, respectively. API 20 STREP and API 20 A (bioMérieux, Marcy l'Etoile, France) were used for *Streptococcus* species (other than group A, B, C, and G β-hemolytic streptococci) and anaerobic Gram-negative bacilli, respectively. Differentiation of anaerobes was undertaken using Gram characteristics, cell morphology, and the aerotolerance test. Yeast isolates were identified by pigmentation on CHROMagar Candida as a choromogenic agar, and by ID32 C (bioMérieux) for confirmation if necessary. Molds were identified by morphology of colony and microculture. Coagulase-negative staphylococci and *Corynebacterium* species were identified by only colony morphology and Gram characteristics, because almost all isolates from sinuses are recognized as contaminants.

Statistics

The comparison of the culture was analyzed by a χ^2 test. Values of $p < 0.05$ were considered to be significant.

Table 1. Pathogens isolated from 10 patients with chronic maxillary sinusitis

Patient No.	Gender	Age years	Bacteria from the middle meatus (endoscope)	Bacteria from the sinus (catheter-based devices)
1	F	56	negative	negative
2	M	48	*Corynebacterium* sp., coagulase-negative staphylococci	*Prevotella/Porphyromonas*
3	M	79	microaerophilic *Streptococcus*	microaerophilic *Streptococcus, Parvimonas micra*
4	F	57	*Pseudomonas aeruginosa*	*Citrobacter freundii, Pseudomonas aeruginosa, Eikenella* sp.
5	M	44	microaerophilic *Streptococcus,* coagulase-negative staphylococci	microaerophilic *Streptococcus*
6	M	38	microaerophilic *Streptococcus,* coagulase-negative staphylococci	microaerophilic *Streptococcus, Parvimonas micra, Lactobacillus* sp.
7	M	40	coagulase-negative staphylococci	*Fusobacterium* sp., *Parvimonas micra, Prevotella/ Porphyromonas*
8	F	58	*Serratia marcescens,* methicillin-susceptible *Staphylococcus aureus*	negative
9	M	52	coagulase-negative staphylococci	*Prevotella/Porphyromonas*
10	M	40	coagulase-negative staphylococci	microaerophilic *Streptococcus*

Results

Bacteriology findings of the specimens taken from the middle meatus (endoscope) and sinus (balloon catheter) are listed in table 1. One specimen from the middle meatus showed no growth. Bacterial profiles including coagulase-negative staphylococci, *Corynebacterium*, microaerophilic *Streptococcus, Pseudomonas aeruginosa*, methicillin-susceptible *Staphylococcus aureus*, and *Serratia marcescens* were recovered from the middle meatus. No anaerobes were recovered. The bacterial cultures of sinus aspirates were positive in 8 out of 10 patients, with 4 different aerobic bacteria (including 4 isolates of microaerophilic *Streptococcus*, 1 isolate of *Citrobacter freundii*, 1 isolate of *Eikenella* sp. and 1 isolate of *Pseudomonas aeruginosa*) and 4 different anaerobic bacteria (including 3 isolates of *Parvimonas micra*, 3 isolates of *Prevotella/Porphyromonas*, 1 isolate of *Lactobacillus* sp. and 1 isolate of *Fusobacterium* sp.). All organisms were bacterial pathogens. Anaerobic bacteria were found in 5 of 8 culture-positive specimens (62.5 %), which was significantly more than in the endoscopic approach ($p < 0.01$). No perioperative or postoperative complications during Balloon Sinuplasty were recognized.

Microaerophilic streptococcus (4 patients) and *Pseudomonas aeruginosa* (1 patient) were recovered using both procedures. However, coagulase-negative staphylococci, *Corynebacterium*, and *Serratia marcescens* may be contaminates from the nasal cavity, as anaerobes have never been detected by the middle meatus method. Patient No. 1 showed negative culture results from both methods. In cases 3–6, one organism was independently recovered from both methods. Cases 2, 7, and 8–10 showed complete discrepancies in findings between the two methods.

Discussion

In the present study, we utilized a catheter-based approach combined with endoscopy to collect maxillary sinus aspirates. Although a full set of balloon catheter devices is considerably expensive, this technique essentially requires a sinus guide catheter, guidewire, and sinus lavage catheter, which are reasonable expenses. Furthermore, as this equipment develops and becomes more widespread, we can expect reductions in prices.

The relative advantages and disadvantages of the antral puncture and endoscopic approaches are being de-

bated. Endoscopic approaches clearly offer several benefits over an antral puncture, such as being able to obtain cultures from any sinus, direct examination of the status of the sinonasal mucosa, minimal contamination from nasal flora, and minimal morbidity [14, 15]. However, although the use of an endoscope allows direct culture of mucopurulent secretions at their source in the sinus ostia, it does not permit this from the sinus itself [16]. Based on a comparison between specimens obtained by sinus endoscopy and surgical drainage, the usefulness of endoscopic aspiration from the chronic maxillary sinusitis has been recognized. However, the origin of the isolates in several cases was the nasal mucosa rather than the sinus cavity, indicating contamination with nasal flora was taking place [3]. Appropriate sampling and culture techniques have generally resulted in the isolation of significant fractions of anaerobes. Contamination of a sample with nasal flora increases the proportion of aerobic organisms, and samples should therefore be obtained by direct puncture of the sinus [4].

There were no bacteria derived from the nasal cavity in the catheter-based approach, whereas the endoscopic method included isolates of nasal flora. Furthermore, the fraction of anaerobes recovered by the catheter-based approach was significantly higher than that of the endoscopic one. Thus, the catheter-based approach is likely to directly reflect the microenvironmental conditions of the maxillary sinus itself, which is superior to the antral puncture and endoscopic approaches.

There is one potential limitation of this study. Since balloon catheter devices have not yet been approved as a medical tool in Japan, the number of patients employed here was limited and the data were preliminary. A more general problem is that balloon catheter devices are disposable and quite expensive (USD 2,000). This high price may limit their widespread use.

Rhinosinusitis is generally not life-threatening, but causes considerable long-term morbidity that often encourages the emergence of resistant organisms, particularly in conjunction with misuse of antimicrobials [4]. Treatment failures may result in serious secondary infections, such as infectious complications involving the orbit and central nervous system. Therefore, accurate microbial evaluation is required to create adequate antimicrobial strategies.

The catheter-based approach is nowadays assisted by fluoroscopy, which carries the potential risk of radiation-induced complications of the lens [17]. However, current technology using illumination enables a more convenient approach without fluoroscopy under local anesthesia [18]. In addition to its being a safe, effective and minimally invasive tool, sampling from each sinus can be performed.

In conclusion, this new technique using catheter-based devices is likely to provide promising outcomes in terms of accurate microbiologic identification from individual inflamed sinuses. Further studies are expected to provide more accurate and detailed findings.

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ORIGINAL ARTICLE

Comparison of bacterial examinations between eosinophilic and neutrophilic chronic rhinosinusitis with nasal polyps

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Abstract

Conclusion: We found no significant differences in the bacterial features of the maxillary sinuses between eosinophilic and neutrophilic chronic rhinosinusitis (CRS) with nasal polyps. *Objectives:* Since neutrophilic CRS is often influenced by a predisposition to bacterial infection, and eosinophilic CRS is likely to be developed by allergic antigens, differences in the microbiology between the two pathologies of CRS can be expected. The present study was designed to investigate the bacterial findings from the maxillary sinus in eosinophilic and neutrophilic CRS. *Methods:* Seventy patients with CRS with nasal polyps were divided into eosinophilic and neutrophilic types based on histopathological observations of the nasal polyps. The specimens for bacterial culture were obtained from the maxillary sinus during endoscopic sinus surgery. *Results:* In all, 29 and 41 patients were classified as having eosinophilic and neutrophilic CRS with nasal polyps, respectively. The isolation rate of bacteria showed no significant difference between eosinophilic (90%) and neutrophilic CRS (98%). Aerobic bacteria were found in 25 patients (86%) with eosinophilic CRS, which was not significantly different from that in neutrophilic CRS (40 patients, 98%). The isolation rate for aerobic and anaerobic bacteria showed no significant differences.

Keywords: *Maxillary sinus, bacteria, infection*

Introduction

Although chronic rhinosinusitis (CRS) is a multifactorial disease in a heterogeneous group of diseases, with different underlying etiologies and pathophysiologies, European and US studies proposed differentiation between CRS without nasal polyps and CRS with nasal polyposis [1,2]. Histomorphological patterns of CRS with nasal polyps are characterized by Th2-driven immune responses including the predominance of eosinophils and mixed mononuclear cells with a relative paucity of neutrophils [3]. Mucosal infiltration with eosinophils in CRS with nasal polyps may be more refractory to surgical cure and is frequently associated with bronchial asthma [4]. Patients with CRS without nasal polyps are more likely to manifest signs of bacterial infection and have been reported to have a better response to medical treatment [5]. The phenotype of CRS without nasal polyps can be characterized by Th1-dominant responses that demonstrate predominantly mononuclear cells and interferon gamma-positive cells [6].

Differing from European and US patients, Japanese patients with CRS with nasal polyps are known to show two distinct phenotypes, (i) tissue eosinophilia characterized by Th2-polarization [7] and marked expression of eotaxins [8] and (ii) poorly expressed eosinophilia characterized by Th1-shifted immunity and prominent expression of IL-8 [7,9]. The former type can be designated as eosinophilic CRS with nasal polyps. The latter type displays neutrophilic inflammation where neutrophil recruitment into the sinus effusion is induced by both the up-regulation of adhesion molecules of the vascular endothelium induced

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