Table 1. Clinical characteristics of patients

	Aspirin-induced asthma ($n = 7$)	Aspirin-tolerant asthma ($n = 6$)	*P*-value
Age (years)	49.9 ± 19.4	45.5 ± 18.0	NS
Male/female	3/4	4/2	NS
Atopy/non-atopy	2/5	3/3	NS
Duration of asthma (years)	11.7 ± 15.9	6.8 ± 4.0	NS
Severity	NS		
Mild persistent asthma	2	3	
Moderate persistent asthma	3	3	
Severe persistent asthma	2	0	
FEV_1 (% predicted)	82.5 ± 14.3	99.2 ± 21.4	NS
Blood eosinophils (/mm^3)	956 ± 738	288 ± 240	NS
Serum IgE (IU/mL)*	312 (18–925)	199 (20–2470)	NS
Dose of inhalation steroids (μg/day)*	800 (0–1600)	400 (0–800)	NS
No. of patients receiving prednisolone	0	0	
No. of patients receiving leukotriene receptor antagonist	5	1	
No. of patients receiving salmeterol	0	0	

*Median (range).
NS, non-significant.

Permission to conduct the study was obtained from the National Sagamihara Hospital Ethics Committee and all subjects gave their informed consent. Urine samples were collected in polypropylene tubes containing 4-hydroxy-TEMPO and stored at −35 °C until analysis. Urine was analysed within a year.

Aspirin challenge test

The study group consisted of seven patients with AIA and six patients with aspirin-tolerant asthma (ATA). All patients were in a clinically stable condition. All medications were stopped for at least 12 h prior to the challenge test. At the time of the study, forced expiratory volume in 1 s (FEV_1) exceeded 70% of the predicted value. The characteristics of the patients receiving aspirin challenge test are summarized in Table 1. There were no significant differences between the two groups in any of the parameters.

The challenge test was performed at about 9:00 to 12:00 hours as previously reported [2, 11]. Briefly, urine samples were collected at the beginning of the study. After intravenous injection of 1 mL of saline, if FEV_1 did not change by more than 10% compared with the pre-challenge baseline, double doses of lysine aspirin (12.5, 25, 50, 100, and 200 mg equivalent of aspirin) were intravenously administered. FEV_1 was recorded every 10 min after aspirin administration and the time interval between administrations of increasing doses was 30 min. The challenge test was stopped when FEV_1 decreased by 20% or more from the baseline. ATA patients did not exhibit changes in respiratory function even after receiving the highest dose of aspirin. Urine samples were collected during the following periods: 0–3, 3–6, 6–9, and 9–24 h after the onset of bronchoconstriction.

Quantification of urinary LTE_4

Urinary LTE_4 was quantified by enzyme immunoassay after purification by HPLC as reported previously [17].

Statistical analyses

Data were expressed as the mean ± SD unless otherwise specified. Differences between groups were analysed by the Mann–Whitney *U*-test. A non-parametric statistical test was performed using Friedman's test for time-course experiments. When the test indicated significant differences, the Tukey-type test was used to compare the data. Correlation between parameters was analysed using Spearman's rank correlation test. A *P*-value of less than 0.05 was regarded as statistically significant.

Results

Verification of the presence of urinary LTB_4 after hydrolysis with β-glucuronidase

For LTB_4 identification by GC-MS-SIM, a large volume of urine was hydrolysed by β-glucuronidase as described above and the eluate from HPLC was derivatized to the LTB_4 pentafluorobenzyl ester. After the LTB_4 pentafluorobenzyl derivative was purified by HPLC using a normal-phase column [19], the derivative was converted to its corresponding di-trimethylsilyl ether derivative. When the ions at *m/z* 389 and *m/z* 479 were monitored by GC-MS-SIM, the peak area ratio of the ion at *m/z* 389 to that at *m/z* 479 was 0.329 ± 0.003 ($n = 4$) for the authentic LTB_4 derivative. When the samples extracted from urine were analysed, the peak having two fragment ions appeared at the same retention time as that of the authentic LTB_4 derivative. The peak area ratio of the ion at *m/z* 389 to that at *m/z* 479 was 0.336 ± 0.008 ($n = 3$), which agreed with that of authentic LTB_4. This observation suggests that the presence of LTB_4 can be confirmed based on mass spectral patterns in addition to retention times on gas chromatography.

Exogenously administered LTB_4 is not excreted into the urine [20]. However, measurable concentrations of urinary LTB_4 were obtained in this study. To ascertain whether LTB_4 is present in human urine or whether the observed concentrations resulted from the cross-reactivity of the antibody for other compounds, we measured the peak area ratio of the LTB_4 derivative in urine by GC-MS-SIM without enzymatic hydrolysis. The peak area ratio was 0.344, confirming the presence of LTB_4 in human urine (Fig. 1).

Quantification of urinary LTB_4 by enzyme immunoassay in combination with HPLC

Rates of LTB_4 recovery from urine The average recovery rate was 72.6 ± 3.2% ($n = 6$) after all the purification methods. LTB_4 was stable during incubation without β-glucuronidase and the recovery rate was 99.0 ± 1.3% after incubating overnight at 37 °C. However, LTB_4 concentration decreased to 80% of the control (80.6 ± 2.9%, $n = 4$) after incubation of LTB_4 in the presence of β-glucuronidase, thus

indicating that concentration of LTBG may be underestimated by approximately 20%.

Accuracy of the method The amount of LTB_4 was 13.7 pg when hydrolysing urine with β-glucuronidase. This amount shows endogenous LTB_4 and LTB_4 generated from LTBG by hydrolysis. Using three urine samples to which 42 pg of LTB_4 was added, and which were measured by the same method, we obtained the following results: 72.1, 52.2 and 61.3 pg. The rates of recovery from the urine sample with 55.7 pg (13.7+42 pg) of LTB_4 were 129%, 93.4%, and 110% (110.8 ± 17.8%). Similarly, the LTB_4 recovery was 116.1 ± 12.5% when 120 pg of LTB_4 was added to the pooled urine samples. The accuracy of this method was within acceptable limits.

The precision of the method was evaluated by analysing eight aliquots from the same urine specimen on the same occasion. Four aliquots were analysed without hydrolysis and the remaining were analysed with hydrolysis using β-glucuronidase. The endogenous LTB_4 concentration was 25.0 ± 2.9 pg/mL. After hydrolysis, the LTB_4 concentration was 41.7 ± 4.7 pg/mL. The coefficient of variation calculated as a measure of intra-assay variation was about 11% in the case of analysis with or without hydrolysis.

Urinary concentrations of LTB_4, LTBG and LTE_4 in asthmatic patients and healthy subjects

The bronchial asthmatic patients, which excluded AIA patients, showed significantly higher urinary LTBG concentrations than the healthy subjects (median, 3.32 pg/mg creatinine [range, 0.14–10.5] vs. 5.37 pg/mg creatinine [range, 1.2–13], $P = 0.0159$; Fig. 2), although there was substantial overlapping between the two groups. Significant difference in the concentration of urinary LTE_4, a confirmed marker for LTC_4 production *in vivo*, was also observed between the two groups (median, 35.7 pg/mg creatinine [range, 9.9–91.9] vs. 81.6 pg/mg creatinine [range, 19.2–255.3], $P = 0.014$; Fig. 2). There was no significant difference in urinary LTB_4 concentration between the two groups (median, 0.59 pg/mg creatinine [range, 0.3–4.3] vs. 0.99 pg/mg creatinine [range, 0.14–5.1], $P = 0.232$; Fig. 2). Moreover, no significant correlation between urinary LTE_4 and urinary LTBG concentrations was observed in the asthmatic patients when the data were analysed by Spearman's rank correlation test ($r = 0.040$, $P = 0.865$).

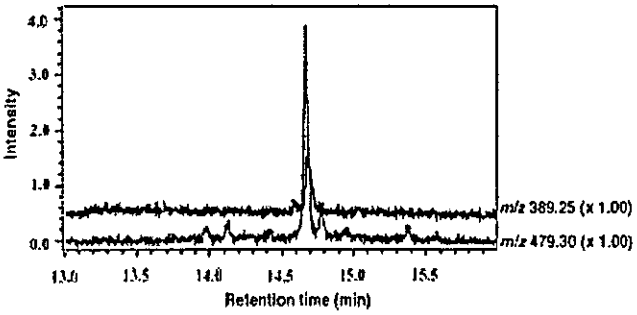

Fig. 1. Detection of leukotriene B_4 as pentafluorobenzyl ester di-trimethylsilyl ether derivative in human urine by gas chromatography-mass spectrometry-selected ion monitoring.

Baseline urinary LTBG and LTE_4 concentrations in AIA patients

As supported by previous reports, the AIA patients ($n = 7$) had a significantly higher urinary LTE_4 concentration than the ATA patients ($n = 6$) at the pre-challenge baseline (median, 331 pg/mg creatinine [range, 75–4946] vs. median, 54.5 pg/mg creatinine [range, 27.8–255.7], $P < 0.01$). In contrast, the two groups showed no significant difference in the concentrations of LTBG (median, 5.6 pg/mg creatinine [range, 3.6–18.9] for the AIA patients vs. median, 5.32 pg/mg creatinine [range, 0.46–8.7] for the ATA patients) and LTB_4 (median, 2 pg/mg creatinine [range, 0.6–6.4] for the AIA patients vs. median, 0.95 pg/mg creatinine [range, 0.42–2.1] for the ATA patients). There was no significant correlation between the basal concentrations of LTE_4 and LTBG in seven AIA patients ($r = 0.107$, $P = 0.819$).

Changes in urinary LTBG and LTE_4 concentrations after intravenous aspirin challenge in AIA and ATA patients

The seven AIA patients showed a greater than 20% decrease (21.1–30%, mean 26%) in FEV_1 after stimulation with a cumulative dose of 25–75 mg of aspirin. There was no significant correlation between a decrease in FEV_1 and the maximal concentrations of urinary eicosanoids. The number of patients may be too small to allow us to determine the correlation between a decrease in FEV_1 and urinary eicosanoid concentrations.

In all AIA patients, the intravenous injection of aspirin induced significant increases in both urinary LTBG and LTE_4 concentrations at the 0–3 and 3–6 h collection periods compared with the baseline concentrations (Fig. 3). The urinary

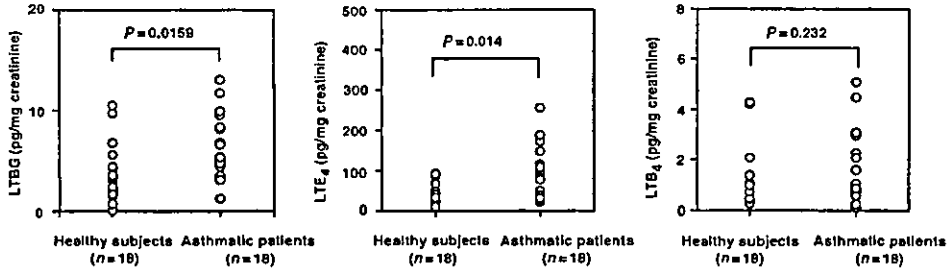

Fig. 2. Urinary concentrations of LTBG (left), LTE_4 (center) and LTB_4 (right) in asthmatic patients and healthy subjects. LTBG, LTB_4 glucuronide; LTE_4, leukotriene E_4; LTB_4, leukotriene B_4.

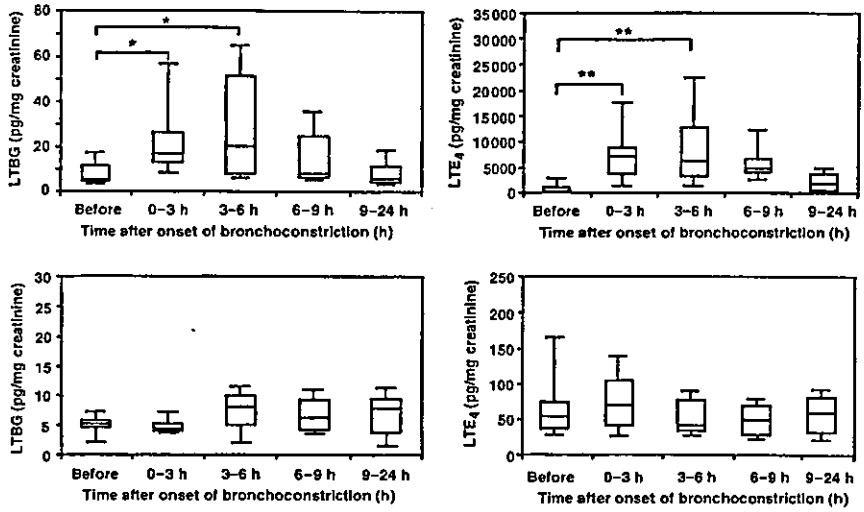

Fig. 3. Changes in urinary LTBG (left) and LTE_4 (right) concentrations in patients with aspirin-intolerant asthma (upper) and aspirin-tolerant asthma (lower) after intravenous aspirin challenge. Data are presented as box plots displaying medians and inter-quartile ranges. In the box plots, the lower 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 90th and 10th percentiles, respectively. The results were analysed using Friedman's test with a *post hoc* test. *Significantly different from the baseline concentration ($P<0.05$). **Significantly different from the baseline concentration ($P<0.01$). LTBG, LTB_4 glucuronide; LTE_4, leukotriene E_4; LTB_4, leukotriene B_4.

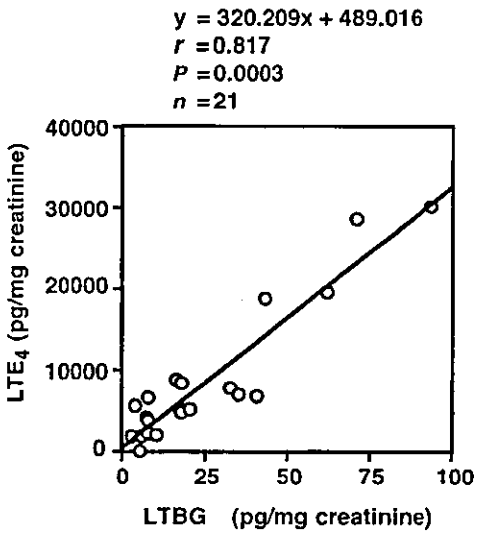

Fig. 4. Correlation between urinary LTBG and LTE_4 concentrations at the 0–3, 3–6 and 6–9 h collection periods after intravenous aspirin challenge in aspirin-intolerant asthma patients. LTBG, LTB_4 glucuronide; LTE_4, leukotriene E_4.

LTBG concentration increased from the baseline to 17.4 pg/mg creatinine (median [range, 5.7–93.7], $P<0.05$) at the 0–3 h collection period and to 20.7 pg/mg creatinine (median [range, 3.2–71.2], $P<0.05$) at the 3–6 h collection period. Similarly, the urinary LTE_4 concentration increased significantly from the baseline concentration at the 0–3 h collection period (median, 6932 pg/mg creatinine [range, 222–30176], $P<0.01$) and at the 3–6 h collection period (median, 6696 pg/mg creatinine [range, 1859–28560], $P<0.01$). Urinary LTB_4 concentration did not change significantly after aspirin challenge in the AIA patients. After aspirin challenge of six ATA patients, urinary LTBG and urinary LTE_4 concentrations did not change significantly in 24 h (Fig. 3).

Urinary LTE_4 concentration reached the peak in the first 3 h in four patients and during the collection period of 3–6 h in the remaining three patients. The periods in which the maximum LTE_4 and LTBG concentrations were reached coincided in six of seven AIA patients. Both urinary LTBG and LTE_4 concentrations almost returned to their baseline concentrations at the 9–24 h collection period. When the data from seven AIA patients during the 0–3, 3–6 and 6–9 h collection periods were taken together, a significant linear correlation was found between urinary LTBG and LTE_4 concentrations (Fig. 4, $r = 0.817$, $P = 0.0003$, $n = 21$).

Discussion

The LTBG concentration was approximately 3.8 pg/mg creatinine (median) in urine samples from the healthy subjects, suggesting that the urinary LTBG concentration is approximately 10-fold lower than the concentration of urinary LTE_4, which is an established *in vivo* marker of cys-LT production. Supposing that the metabolism of endogenously produced LTB_4 is the same as that of exogenously administered LTB_4 [15], namely, about 0.2% of endogenous LTB_4 is excreted into the urine as LTBG and the total amount of creatinine excreted is 1500 mg/day, the calculated whole-body LTB_4 production may be 2.85 μg/day. If metabolic clearance systems including liver function and UDP-glucuronosyl transferase activity were not altered in the disease state, urinary LTBG concentration provides a new and easily performed method to monitor whole-body LTB_4 production.

It has been inferred from chromatographic results that various glucuronide conjugates of LTB_4 exist in human urine [15]. We do not know whether each conjugate is sufficiently cleaved by hydrolysis although Karin et al. [15] reported that LTBG hydrolysis is completed in 20 h.

In spite of the findings that exogenous LTB_4 is not excreted into the urine [20], the analysis by GC-MS-SIM confirmed the presence of LTB_4 in human urine. The most likely source of urinary LTB_4 may be the kidney, where LTA_4 hydrolase is present [21].

Our data are the first to demonstrate that, similar to LTE_4 concentration, LTBG concentration increases in urine after aspirin challenge of AIA patients, suggesting that aspirin challenge results in the increased production of LTB_4 in AIA patients. On the other hand, urinary LTB_4 concentration did

not increase after aspirin challenge in AIA patients. Ferreri et al. [22] reported that following oral aspirin challenge of AIA patients, LTC_4 concentration in nasal lavage fluid increased but LTB_4 concentration in nasal lavage fluid remained unchanged. These results indicate that LTB_4 itself is not an appropriate marker of LTB_4 production, probably because it is rapidly degraded.

It was reported that aspirin administration to AIA patients increased urinary concentrations of not only LTE_4 but also 9α, 11β-PGF_2 and methylhistamine [2, 23]. Furthermore, an increased concentration of plasma 9α, 11β-PGF_2 following aspirin administration to AIA patients was also reported [10]. These findings support the possibility that LTC_4 may be produced by activated mast cells in AIA patients following aspirin administration, because PGD_2 is the predominantly produced PG by human mast cells and histamine is produced by mast cells and basophils. On the other hand, it is known that mast cells produce only small amounts of LTB_4 [24–26]. It is of interest to identify cells producing LTB_4 and to clarify the underlying production mechanism. It is known that eosinophils fail to produce LTB_4 [27, 28]. Taking also into consideration the absence of a significant increase in the concentration of 3-bromotyrosine produced by activated eosinophils, as demonstrated in our previous study [11], the possibility of LTC_4 production by activated eosinophils may as well be eliminated.

Some probable mechanisms underlying LTB_4 production are assumed. (1) Monocytes and macrophages or cells capable of producing both LTB_4 and LTC_4 are activated. Macrophages are also capable of producing PGD_2 [29, 30]. (2) In addition to mast cells, cells capable of LTB_4 production, such as monocytes, macrophages and neutrophils, are also activated simultaneously. However, our previous study showed that the urinary concentration of 3-chlorotyrosine known to be produced by activated neutrophils remains unchanged despite the increased urinary LTE_4 concentration following aspirin administration to AIA patients [11]. Based on these findings, there seems to be little possibility of neutrophil activation. (3) The possibility of LTB_4 and LTC_4 production by transcellular biosynthesis is also assumed [31–33]. That is, LTA_4 is released into the extracellular space along with LTB_4, and LTA_4 is converted to LTC_4 by cells that express LTC_4 synthase, such as mast cells, eosinophils, platelets [34–36] and endothelial cells [37–39]. Nasser et al. [40] reported that large numbers of eosinophils and mast cells are present in tissues obtained from AIA patients by bronchial biopsy compared with those in tissues obtained from ATA patients. Cowburn et al. [41] reported that counts of cells expressing LTC_4 synthase are significantly higher in bronchial biopsies from AIA patients than those from ATA patients. On the basis of these reports, the possibility of transcellular biosynthesis of LTB_4 and LTC_4 is also considered. On the other hand, it is also assumed that in relation to LTC_4 production by mast cells, LTA_4 released into the extracellular space may be transformed to LTB_4 by neighbouring cells such as epithelial cells expressing LTA_4 hydrolase [42].

In summary, further studies are necessary to elucidate the mechanism underlying LTC_4 and LTB_4 production following aspirin administration to AIA patients. The possibility that LTBG is produced by events starting from mast cell activation cannot be excluded. It was reported that an allergen challenge of patients with bronchial asthma leads to an increased urinary LTE_4 concentration [43–47]. It was suggested that this increased urinary LTE_4 concentration following the allergen challenge is due to mast cell activation by the IgE antibody. The determination of changes in urinary LTE_4 and LTBG concentrations after the allergen challenge of patients with bronchial asthma may contribute to the clarification of whether mast cells are the main cellular source of urinary LTBG. On the other hand, the increase in urinary LTE_4 concentration following the inhalation allergen challenge of atopic patients was lower than the increase in LTE_4 concentration following the intravenous aspirin challenge of AIA patients. These findings also suggest another possibility that LTBG concentration shows only minute changes that are undetectable. Investigation of this possibility is in progress in our laboratory.

Since we used the intravenous route for aspirin challenge, it is impossible to identify the tissue that produced eicosanoids. In this study, we present the whole-body production of eicosanoids in AIA patients. When we used the inhalation route for aspirin challenge, eicosanoids were produced only in the lungs; therefore, it is necessary to confirm in future studies whether LTBG is also produced only in the lungs. However, an increase in urinary LTE_4 concentration in AIA patients challenged via the inhalation route was not as large as that via the intravenous route, and the increase in LTBG concentration is not expected to be significant; therefore, it may be difficult to obtain clear differences.

No correlation between pre-challenge LTE_4 and LTBG concentrations was observed. When statistical analysis for correlation was repeated for 20 AIA patients, which included 13 AIA patients added to the original seven AIA patients, no correlation between the two was observed. It seemed that LTC_4 overproduction observed in AIA patients in the stable condition did not involve LTB_4 overproduction. Although it may be proved unreasonable to compare baseline concentrations of metabolites whose metabolic pathways and metabolic rates differ from each other, it is difficult to assume the sustained production of a large amount of LTC_4 alone if the same types of cell produce LTC_4 and LTB_4. Only cells that produce LTC_4 may be subject to continuous activation in the clinically stable state. On the other hand, as shown in Fig. 3, there was a significant correlation between increased urinary concentrations of LTC_4 and LTB_4 following aspirin administration. LTBG and LTE_4 concentrations peaked simultaneously, namely, LTGB and LTE_4 concentration changes followed the same time course. These results suggest that both LTs produced following aspirin administration are closely associated with each other.

In conclusion, the estimation of urinary LTBG concentration may be a very useful method of assessing the biosynthesis of LTB_4, whose role in bronchial asthma remains to be elucidated. The determination of LTBG concentration indicated that not only LTC_4 production but also LTB_4 production increased in AIA patients following aspirin challenge. These findings provide significant information regarding cells involved in the pathogenesis of AIA. The elucidation of a more important issue, that is, the mechanism underlying the production of both LTs, is required in further studies.

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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

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Summary

Background Eosinophil peroxidase and myeloperoxidase halogenate tyrosine residues in plasma proteins and generate 3-bromotyrosine (BY) and 3-chlorotyrosine (CY), respectively.

Objectives (1) To estimate urinary concentrations of BY and CY in asthmatic patients. (2) To investigate BY concentration in relation to urinary leukotriene E4 (LTE4) concentration in order to evaluate the activation of eosinophils in patients with aspirin-induced asthma (AIA).

Methods BY and CY were quantified with a gas chromatograph-mass spectrometer using ^{13}C-labelled compounds as internal standards.

Results (1) Activation of eosinophils and neutrophils by immobilized IgG1 induced preferential formation of BY and CY, respectively. (2) A significantly higher concentration of BY was observed in the urine from asthmatic patients than in that from healthy control subjects (45 ± 21.7 vs. 22.6 ± 10.8 ng/mg-creatinine, $P<0.01$). CY concentration was also elevated in the urine from asthmatic patients (4.4 ± 3.2 vs. 1.5 ± 1.0 ng/mg-creatinine, $P<0.01$). (3) After intravenous aspirin challenge of aspirin-induced asthmatic patients, the concentration of BY in urine did not significantly change. No significant change was also observed in the ratio of BY concentration to total tyrosine concentration in plasma proteins. In contrast, the concentration of urinary LTE4 significantly increased after the intravenous aspirin challenge.

Conclusion Determination of BY and CY concentrations may be useful for monitoring the activation of eosinophils and neutrophils in asthmatic patients, respectively. After aspirin challenge of AIA patients, the increased concentration of urinary LTE4 did not accompany changes in BY concentration in both urine and plasma proteins. These results may preclude the activation of eosinophils after aspirin challenge in patients with AIA.

Keywords aspirin-induced asthma, 3-bromotyrosine, 3-chlorotyrosine

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Introduction

Eosinophils play a role in the pathogenesis of various inflammatory diseases including asthma. Eosinophils possess a wide range of biological properties that may participate in the pathogenesis of a disease by releasing proteins, eicosanoids, platelet-activating factor and cytokines. Eosinophil peroxidase (EPO) resides in a matrix of cytoplasmic granules and is one of the most abundant proteins of eosinophils. Eosinophils activated by various stimuli generate large amounts of hydrogen peroxide (H_2O_2) and secrete EPO. Halogenated oxidants formed by the EPO–H_2O_2-halide system are potent cytotoxic oxidants possessing microbicidal and viricidal activities and also contribute to inflammatory injury in host tissues. Although chloride is found *in vivo* at concentrations at least 1000-fold higher than those of other halide ions, human EPO preferentially oxidizes bromide ions under physiological conditions, and the major product of EPO is hypobromous acid (HOBr) [1]. HOBr is too reactive to measure directly in biological fluids. However, HOBr easily reacts with primary amines to form bromamines, and the intermediates convert tyrosine into 3-bromotyrosine (BY) as a stable end product [2–5]. The presence of BY is considered as a chemical marker of eosinophil activation. On the other hand, myeloperoxidase (MPO), which is stored in neutrophils, monocytes and macrophages, converts H_2O_2 into hypochlorous acid (HOCl) at plasma chloride concentrations, and the reaction of tyrosine with HOCl yields 3-chlorotyrosine (CY) [3, 6, 7]. Thus, detection of CY may provide a means for studying oxidative damage promoted by MPO. BY concentration in proteins in bronchoalveolar lavage fluid (BALF) significantly increased after segmental allergen challenge of asthmatic patients [3]. A significant increase in BY, CY and 3-nitrotyrosine concentrations was also observed in BALF from severely asthmatic patients [8]. In addition, a

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recent study has shown that BY concentration in total protein was significantly elevated in sputa collected from stable asthmatic patients [9]. In addition, EPO and MPO also use nitrite to generate 3-nitrotyrosine [10–13].

Urine has been found to be a useful biological fluid in monitoring the endogenous release of chemical mediators such as arachidonic acid metabolites and histamine by measuring their stable urinary metabolites [14]. In addition to the ease in collecting urine, measurements of metabolites in urine are not confounded by problems involving artificial *ex vivo* formation of metabolites during sampling, which could be a major problem when arachidonic acid metabolites and histamine in plasma were measured. On the other hand, the major drawback of using urine is that a reliable marker of endogenous production of the mediator needs to be identified and urine analysis cannot provide any information on the cellular origin of mediators. It has been reported that leukotriene E4 (LTE4) concentration in the urine of patients with aspirin-induced asthma (AIA) is elevated even in a clinically stable condition and is further elevated after aspirin challenge [15–20]. Although the cellular source of leukotriene C4 (LTC4) release has not been identified, aspirin-induced reactions seem to involve activation of mast cells and/or eosinophils. However, previous studies do not provide information regarding which cells are the target of aspirin to initiate the adverse reaction. If LTC4 is released from eosinophils, it is possible to detect an increase in the concentration of urinary BY.

In this study, we evaluated whether the concentrations of BY and CY increase in urine from asthmatic patients. We also analysed the concentration of BY in relation to urinary LTE4 concentration to assess the contribution of eosinophils to the pathogenesis of AIA.

Materials and methods

Patients

The study group consisted of 12 patients with AIA, 12 patients with aspirin-tolerant asthma (ATA) and 18 healthy control subjects. All patients were in a clinically stable condition. All medications were stopped for at least 12 h prior to the challenge test. Permission to conduct the study was obtained from the National Sagamihara Hospital Ethics Committee and all the subjects gave their informed consent. Urine was collected in the morning (9:00–11:00 hours).

Aspirin challenge test

The 12 AIA patients received intravenous aspirin challenge. These patients were divided into two groups. The challenge test was performed at about 9:00 hours to noon as previously reported [20]. Briefly, urine or blood samples were collected at the beginning of the study. After intravenous injection of 1 mL of saline, if $FEV_{1.0}$ did not change by more than 10% from the prechallenge baseline, doubled doses of lysine aspirin (12.5, 25, 50, 100 and 200-mg equivalent of aspirin) were intravenously administered. $FEV_{1.0}$ was recorded every 10 min after the administration and the time interval between administrations of increasing doses was 30 min. The challenge test was stopped when a positive reaction occurred, which was defined as a decrease in $FEV_{1.0}$ by 20% or more from the baseline. ATA patients did not show a changed respiratory function even after receiving the highest dose of aspirin. Urine samples were collected for the measurement of LTE4, BY and CY during the following periods: 0–3, 3–6, 6–9 and 9–24 h after the onset of bronchoconstriction. Urine samples were collected in polypropylene tubes containing 4-hydroxy-TEMPO and were stored at −35 °C until analysis. The second subgroup of six AIA patients also underwent aspirin challenge, and blood samples were collected 1 and 3 h after the aspirin challenge.

Preparation of internal standards

$^{13}C_6$-labelled BY was prepared by reacting $^{13}C_6$-tyrosine (L-tyrosine-ring $^{13}C_6$, ^{13}C-99%, Cambridge Isotope Laboratories, Inc., Andover, MA, USA) with bromine water in 50 mM phosphate buffer (pH 7.3). $^{13}C_6$-BY was isolated by high-performance liquid chromatography (HPLC) using a NOVA-PAK C18 column (Waters, Milford, MA, USA) with a solvent mixture of 10 mM ammonium formate containing 5% methanol (adjusted to pH 3.2 with formic acid). Similarly, $^{13}C_6$-CY was prepared by reacting $^{13}C_6$-tyrosine with NaOCl in 1 N HCl. The retention times of BY and CY were about 6 and 5 min under the HPLC conditions used, respectively.

Analysis of BY and tyrosine in biological fluids

Urine: $^{13}C_6$-BY (50 ng) and $^{13}C_6$-CY (30 ng) were added to 1 mL of urine as internal standards and the urine was passed through a Bond Elut C18 column (Varian, Harbor City, CA, USA) to remove polar compounds. After addition of 1 mL of 0.2% trifluoroacetic acid, the solution was loaded on an LC18 column (Supelco, Bellefonte, PA, USA) and tyrosine and its derivatives were eluted with 1.6 mL of 25% methanol from the column. Heptafluorobutyryl derivatives were prepared according to the method of Frost et al. [21]. After derivatization, the reaction mixture was concentrated under a nitrogen stream, to which 0.2 mL of 0.1 N HCl and 0.6 mL of ethyl acetate were added. Heptafluorobutyryl derivatives were extracted into the organic phase and then converted into the corresponding tert-butyldimethylsilyl derivatives [21, 22]. The concentrations of BY and CY were determined 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, Bellefonte, PA, USA) in the negative ion chemical ionization mode with methane as the reagent gas. The ion source and interface temperatures were set at 250 °C. The initial column temperature was maintained at 50 °C for 2 min and then increased to 250 °C at 15 °C/min. BY and CY concentrations were determined by measuring the fragment ions at *m/z* 489.10 for endogenous compounds and *m/z* 495.15 for the internal standards.

Plasma protein Proteins were precipitated from 0.02 mL of plasma using a mixture of water:methanol:water-washed diethyl ether (1:3:7, v/v/v) [23]. After addition of $^{13}C_6$-tyrosine (25 μg) and $^{13}C_6$-BY (50 ng) as internal standards, proteins were hydrolysed in 0.5 mL of 4 N methanesulfonic acid containing 1% phenol at 110 °C for 24 h. Tyrosine and BY in the hydrolysate were quantified as described earlier. An aliquot of the eluate from the LC18 column (0.05 mL) was

separated for quantitation of tyrosine. Tyrosine was quantified using the fragment ions at *m/z* 407.15 and *m/z* 413.15, which is the corresponding fragment ion derived from $^{13}C_6$-tyrosine. The ratio of BY concentration to total tyrosine concentration in plasma proteins has been calculated and used as a measure of bromination of plasma proteins.

Activation of eosinophils and neutrophils by immobilized human IgG1

Eosinophils and neutrophils were isolated from peripheral blood as reported previously [24]. The procedure yielded >99% pure eosinophils. On the other hand, the neutrophil preparation contained a small percentage of eosinophils in four of five cases (0.3–5.2%). Eosinophils and neutrophils were suspended in RPMI-1640 containing fetal calf serum (5%), penicillin, streptomycin and 100 μM NaBr at densities of 2.5×10^6/mL and 7.5×10^6/mL, respectively. The suspension (0.2 mL) was placed in a tube (MaxiSoap, Nalge Nunc Int. Rochester, NY, USA), which had been coated with human IgG1λ (Sigma, St Louis, MO, USA), and cultured in humidified 95% air and 5% CO_2 at 37 °C overnight. As a control, the cells were cultured in tubes coated with human albumin instead of human IgG1λ. After proteins in the supernatant were hydrolysed, tyrosine and its derivatives were quantified as described above.

Quantitation of LTE4 and EDN in urine

LTE4 was quantified by enzyme immunoassay after purification by HPLC as reported previously [25]. EDN was measured using an EDN enzyme immunoassay kit (MBL, Nagoya, Japan) after diluting the urine from most of our patients 50 times with a dilution buffer. The concentrations were expressed as ng per mg of creatinine.

Statistical analyses

Data were expressed as the mean ± SD unless otherwise specified. A non-parametric statistical test was performed using Friedman's test for time-course experiments. When the test indicated significant differences, the Tukey-type test was used to compare the data. The Mann–Whitney *U*-test was used to examine differences between asthmatic patients and healthy controls. Spearman's rank correlation test was used to evaluate correlated data. Differences were considered significant for *P*-values less than 0.05.

Results

Quantification of tyrosine and its derivatives

When a calibration graph was constructed by plotting the peak area against the concentration, a linear standard graph was obtained in the concentration range of 1.2–36 ng/mL. The peak area ratio of *m/z* 489.10 and *m/z* 495.15 was almost the same as the mixing molar ratio of BY and $^{13}C_6$-BY.

To confirm the formation of BY and CY during sample preparation, urine to which $^{13}C_6$-tyrosine and NaBr (100 μM) were added in the absence of internal standards was analysed. $^{13}C_6$-BY and $^{13}C_6$-CY were not generated, suggesting that BY and CY are not formed from tyrosine during sample preparation.

Effect of activation of eosinophils and neutrophils by immobilized human IgG1 on BY and CY formation

Immobilized IgG stimulates degranulation and superoxide production by eosinophils and neutrophils through an Fcγ receptor II (CD32)-mediated mechanism [26–28]. Thus, we examined BY and CY formation by eosinophils and neutrophils after stimulation with immobilized IgG1. As shown in Fig. 1, activation of eosinophils through Fcγ

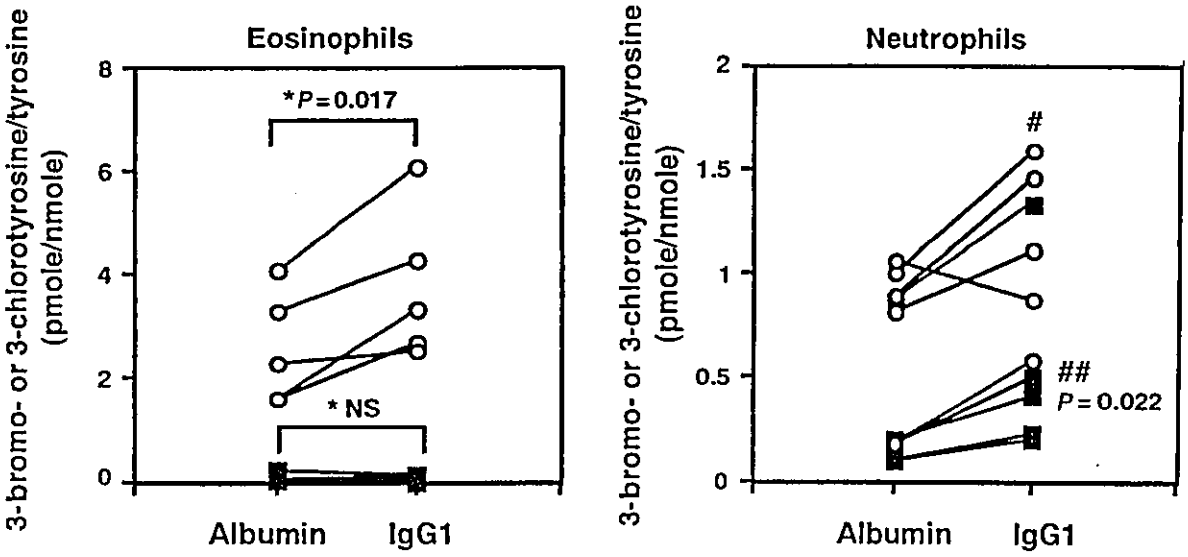

Fig. 1. Generation of 3-bromotyrosine (BY) and 3-chlorotyrosine (CY) by eosinophils (left) and neutrophils (right) through Fcγ receptor activation. Open circle and closed square indicate BY and CY, respectively. *Generation of BY, but not CY, was significantly increased by immobilized IgG ($P = 0.017$). #No statistical significance was found in BY generation by neutrophils. ##CY generation by neutrophils in an IgG1-coated tube was significantly higher than that in an albumin-coated tube ($P = 0.022$).

receptors resulted in a significant increase in the concentration of BY (2.57 ± 1.07 vs. 3.78 ± 1.47 pmol/nmol tyrosine, $P = 0.017$ by a paired two-tailed Student's t test). No significant increase in CY concentration was observed in the supernatant proteins. In contrast, a significant increase in CY concentration was observed in the supernatant proteins when neutrophils were stimulated with immobilized IgG1 (0.29 ± 0.32 vs. 0.53 ± 0.46 pmol/nmol tyrosine, $P = 0.022$). BY concentration also increased in the proteins in four of five patients, albeit to a lesser extent. In one of these patients, whose cell suspensions were not contaminated with eosinophils, no increase in BY concentration was observed. We could not determine whether contaminating eosinophils in a small percentage were responsible for BY generation in neutrophils or whether neutrophils can generate low concentrations of HOBr. Nevertheless, the difference failed to reach statistical significance ($P = 0.08$). These results indicate that BY and CY were preferentially produced by activated eosinophils and neutrophils, respectively.

Characteristics of patients participating in the study

The characteristics of the patients are summarized in Table 1. There were no significant differences in terms of age, gender, duration of asthma, severity and dose of inhaled steroids between AIA and ATA patients. Although IgE concentration was not significantly different between these groups, most of the ATA patients were atopic.

Baseline concentrations of BY, CY and LTE4 in urine

The concentrations of BY in urine from asthmatic patients and control subjects are shown in Fig. 2. Asthmatic patients showed a significantly higher BY concentration than control subjects (45 ± 21.7 vs. 22.6 ± 10.8, $P<0.01$). A significant difference was also observed between the two groups even when data from six AIA patients were excluded from analysis. A significant correlation between BY and EDN concentrations in urine could not be observed in patients with asthma. Neither EDN concentration nor BY concentration showed a significant correlation with the number of peripheral blood eosinophils.

Table 1. Clinical characteristics of patients

	AIA ($n = 12$)	ATA ($n = 12$)	P-value
Age (years)	53.7 ± 13	53.8 ± 16.7	NS
Male/female	4/8	7/5	NS
Atopy/non-atopy	4/8	10/2	$P<0.05$
Duration of asthma (years)	18.7 ± 13.4	19.1 ± 17.6	NS
Severity			NS
Mild persistent asthma	0	2	
Moderate persistent asthma	4	4	
Severe persistent asthma	7	4	
$FEV_{1.0}$ (% predicted)	73.8 ± 13.1	86.0 ± 27.6	NS
Blood eosinophils (/mm^3)	759 ± 636	625 ± 365	NS
Serum IgE (IU/mL)*	110 (18–2490)	300 (19–20 200)	NS
Inhaled steroid dose (mg/day)*	1600 (0–1600)	800 (400–2400)	NS
Patients (n) receiving predonisolone	1	1	
Dose (mg/day)	4	5	
Patients (n) receiving leukotriene receptor antagonist	6	2	
Patients (n) receiving salmeterol	0	2	
Dose (μg/day)		100	

AIA, aspirin-induced asthma; ATA, aspirin-tolerant asthma; $FEV_{1.0}$, forced expiratory volume in 1 s; NS, non-significant.
*Median (range).

The concentration of CY was about 10-fold lower than that of BY, which was below the sensitivity of the assay, in two patients and one healthy subject. Urinary CY concentration was also significantly different between asthmatic patients and control subjects (4.4 ± 3.2 vs. 1.5 ± 1.0 ng/mg-creatinine, $P<0.01$). There was no correlation between CY and BY concentrations in urine of patients with asthma.

Urinary LTE4 concentration was significantly higher in asthmatic patients (median, 107 pg/mg-creatinine; range, 26–5816 pg/mg-creatinine) than in control subjects (median, 50.9 pg/mg-creatinine; range, 18.8–77.6 pg/mg-creatinine). However, when the data of six AIA patients were excluded, this difference was not statistically significant, suggesting that the higher concentration of LTE4 in asthmatic patients is not related to their asthmatic status, but rather to the presence of aspirin sensitivity. When examined in AIA patients showing increased urinary LTE4 concentration (>200 pg/mg-creatine), a significant correlation could not be observed between the concentrations of urinary BY and LTE4.

No significant difference was observed in the concentration of urinary EDN between AIA patients (1588 ± 1464 ng/mg-creatinine) and ATA patients (865 ± 864 ng/mg-creatinine), although the possibility that the number of AIA patients may have been too small to detect the difference cannot be ruled out.

Changes in urinary BY and LTE4 concentrations after intravenous aspirin challenge

After the aspirin challenge of the first subgroup of six AIA patients, urinary BY concentration did not change significantly in 24 h (Fig. 3). There were considerable interindividual differences in the extent and time course of the increase in urinary LTE4 concentration in the AIA patients. However, a significant increase has been observed in urinary LTE4 concentration during the 3–6 h period in these patients after the onset of bronchoconstriction ($P<0.01$). The urinary LTE4 concentration increased from 210 pg/mg-creatinine (median; range, 75–5819 pg/mg-creatinine) as the baseline concentration to 6485 pg/mg-creatinine (median; range, 408–33 600 pg/mg-creatinine) in the 3–6 h period, that is, the concentration increased by 12-fold (median; range, 3.4- to 448-fold) of the baseline concentration. There were also no significant changes in CY concentration in AIA patients after the onset of bronchoconstriction (data not shown). Urinary LTE4 and BY concentrations did not increase significantly in ATA patients after aspirin challenge (Fig. 4).

The second subgroup of six AIA patients also received aspirin challenge. The ratio of BY concentration to total tyrosine concentration in plasma proteins did not show any significant change at 1 h and at 3 h after aspirin challenge (Fig. 5). On the other hand, urinary LTE4 concentration increased significantly from the baseline concentration (median, 182 pg/mg-creatinine; range, 31.5–979 pg/mg-creatinine) at the 0–3 h period (median, 957 pg/mg-creatinine; range, 476–8486 pg/mg-creatinine, $P<0.05$). Overall, we

failed to obtain evidence for enhanced formation of BY in urine and plasma proteins after aspirin challenge of AIA patients. BY generation did not occur in AIA patients who demonstrated increased urinary LTE4 concentration, which indicates that cells other than eosinophils may be responsible for LTC4 production in AIA patients after aspirin challenge.

Discussion

BY and CY concentrations in bronchial lavage fluid [3, 8], sputa [9] and tracheal aspirate [29] were measured, and the results clarified that patients with bronchial asthma or respiratory diseases have high concentrations of BY and

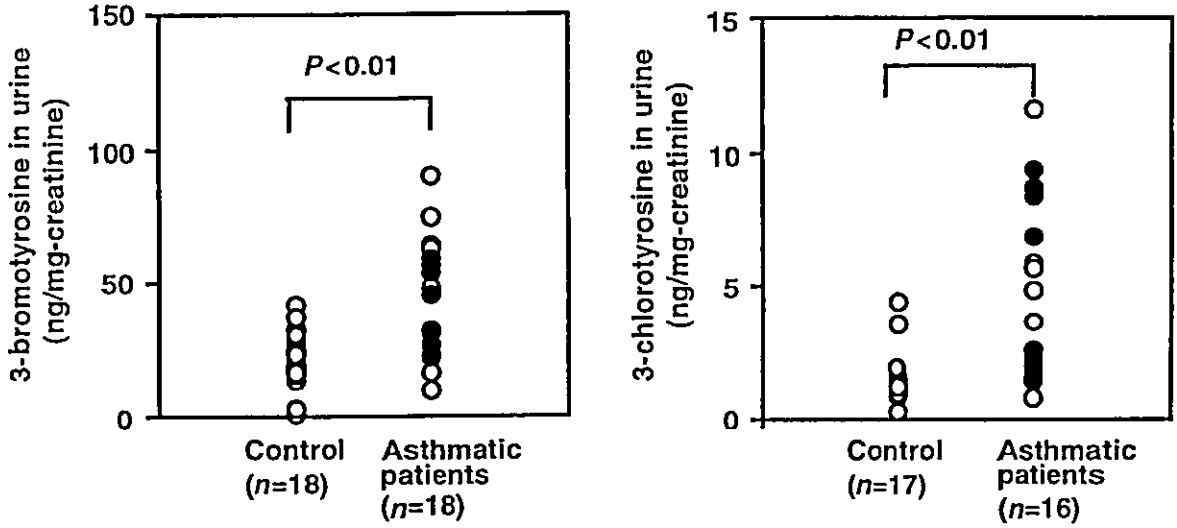

Fig. 2. Concentrations of urinary 3-bromotyrosine (left) and urinary 3-chlorotyrosine (right) in patients with asthma and healthy subjects. Closed symbols indicate the patients with aspirin-induced asthma.

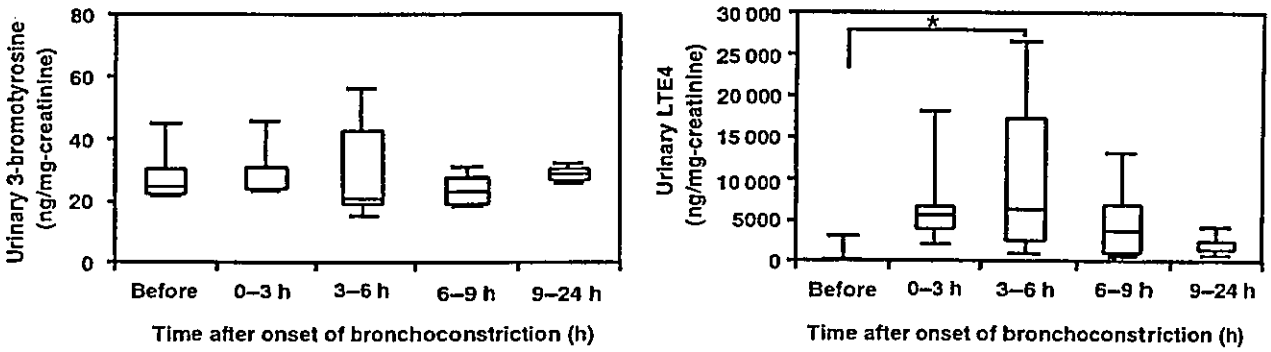

Fig. 3. Changes in urinary 3-bromotyrosine (left) and leukotriene E4 (LTE4) (right) concentrations in patients with aspirin-induced asthma after intravenous aspirin challenge. Data are presented as box plots displaying medians and interquartile ranges. In the box plots, the lower boundary indicates the 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 90th and 10th percentiles, respectively. The results were analysed using Friedman's test with a *post hoc* test. *Significantly different from the baseline concentration ($P<0.01$).

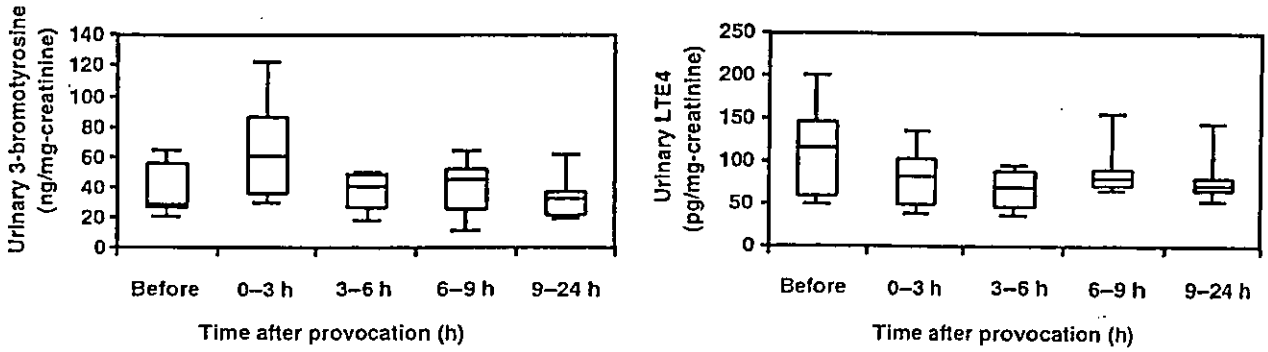

Fig. 4. Changes in urinary 3-bromotyrosine (left) and leukotriene E4 (LTE4) (right) concentrations in patients with aspirin-tolerant asthma after intravenous aspirin challenge.

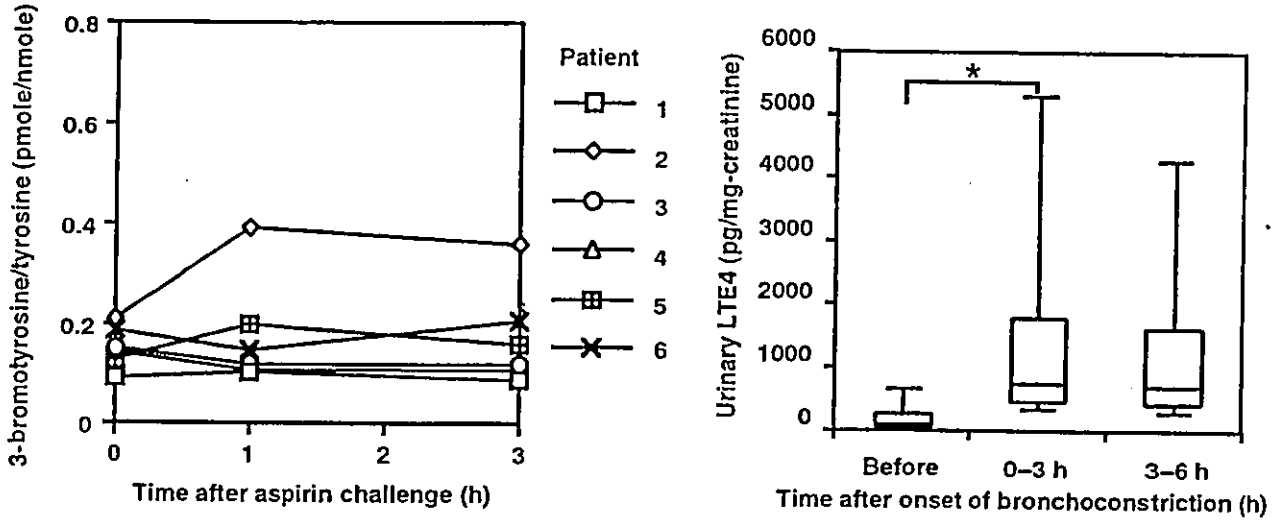

Fig. 5. Changes in the ratio of 3-bromotyrosine concentration to total tyrosine concentration in plasma proteins (left) and urinary leukotriene E4 (LTE4) concentration (right) after aspirin challenge in patients with aspirin-induced asthma. *$P<0.05$ compared with the baseline concentration.

CY. To investigate the clinical significance of BY concentration as a marker of eosinophil activation, we first measured urinary BY concentration in asthmatic patients and compared it with that in healthy control subjects. To date, there have been no studies regarding the measurement of urinary BY or CY concentration. Urinary BY concentration in the asthmatic group was significantly higher than that in the control group (Fig. 2). Since urine samples were used in this experiment, it is impossible to identify the cellular origin of BY. In addition, there has been no information about urinary metabolite of BY. However, our result provides compelling evidence that a large amount of HOBr is produced in asthmatic patients, thereby, tissue damage and oxidative injury associated with eosinophil activation may occur. On the other hand, CY is considered to be a marker of the activation of cells including MPO. According to previous reports, CY concentration in bronchial lavage fluid increased following an allergen challenge test [3], and a high concentration of CY was also observed in bronchial lavage fluid obtained from severely asthmatic patients [8]. In contrast, no significant difference in the concentration of CY in sputa between asthmatic patients and controls was observed [9]. In our current study, urinary CY concentration in the asthmatic group was significantly higher than that in the control group (Fig. 2). This result may suggest the involvement of neutrophils and monocytes/macrophages in the pathogenesis of bronchial asthma. There have been many reports on the involvement of neutrophils in bronchial asthma. Neutrophil influx and activation and a high concentration of MPO were observed in the sputa of asthmatic patients [30–34]. Neutrophils obtained from asthmatic patients showed an increased release of MPO when stimulated with a bacterial peptide, and the concentration of MPO released was associated with lung function [35, 36]. In addition, neutrophils from asthmatic patients have been shown to generate a larger amount of superoxide anions than those from control subjects [37]. These results support the idea that neutrophils are involved in airway injury in asthma. However, it is unclear to what extent the activation of neutrophils affects the deterioration of the pulmonary function of asthmatic patients.

A significant correlation between BY concentration and EPO concentration in sputa was observed [9]; however, in the current study, no significant correlation was observed between urinary BY concentration and urinary EDN concentration, which is another marker of eosinophil degranulation. EDN is released from eosinophil granules together with eosinophil cationic proteins and EPO. The molecular weight of EDN is 18–19 kDa, which means that EDN is excreted in the urine more easily than EPO, which has a molecular weight of 66 kDa. Namely, EDN easily permeates through the glomerulus. Therefore, EDN is used as an eosinophil degranulation marker in urine [38]. Concentrations of mediators released at local sites in airways are easily detected in sputa. This is why BY concentration correlates with EPO concentration. Our data showed that the concentration of BY might be regulated in a manner different from that of EDN in urine. EDN is released from eosinophils not only by their degranulation but also by their degradation. However, BY is generated by the interaction between hydrogen peroxide and EPO; therefore, the activation of eosinophils is definitely necessary. Such a difference in the production mechanism may account for the low correlation between EDN concentration and BY concentration in urine. Alternatively, BY and EDN are eliminated from the systemic circulation and enter the urine at various rates.

The overproduction of cysteinyl-LT is considered to be an indication of AIA. However, cells that produce a large amount of LTC4 have not yet been clarified. 5-Lipoxygenase, the enzyme required for the initiation of LT production, is expressed predominantly by cells of myeloid origin. In contrast, enzymes involved in the second step of LT biosynthesis, LTC4 synthase and LTA4 hydrolase, are more broadly expressed in various cell types. LTC4 is considered to be released mostly from mast cells, basophils and eosinophils, as well as from monocytes/macrophages at a lesser concentration. In our previous study, the concentration of 9α, 11β prostaglandin F2 (9α, 11β-PGF2), which is a metabolite of prostaglandin D2 (PGD2), increased with increasing LTE4 concentration, and methylhistamine concentration slightly increased in some of the AIA patients [20]. These results

suggest the activation of mast cells following the administration of aspirin [39]. PGD2 was reported to be produced not only by mast cells but also by macrophages/monocytes [14], T cells [40] and fibroblasts [41]. In addition, recently, a divergent pathway from isoprostane, which is produced non-enzymatically, to PGD2 via epimerization has been discovered [42]. PGD2 and its metabolites have been used as markers of mast cell activation [43]. Recently, an increase in blood tryptase concentration following the aspirin challenge in AIA patients has been reported [44]. This result also strongly suggests the activation of mast cells. On the other hand, eosinophils are known to produce a large amount of cysteinyl-LT, and an increased number of infiltrating eosinophils in the lungs of AIA patients was also reported [45]. Therefore, we cannot exclude the possibility of the involvement of eosinophils in the reaction following aspirin administration in AIA patients. How eosinophils participate in the induction of AIA remains to be elucidated. If indeed eosinophils participate in aspirin-induced bronchoconstriction and cysteinyl-LT is, at least in part, released from eosinophils, it is possible to detect an increase in BY concentration in biological fluids. We, therefore, measured BY concentration before and after aspirin administration in the first AIA subgroup and the results were compared with those observed in ATA patients after aspirin administration. After aspirin was administered to AIA patients, urinary LTE4 concentration increased by 3- to 448-fold of the baseline concentration; in contrast, urinary BY concentration did not show any significant changes (Fig. 3).

In our laboratory, aspirin was intravenously administered to subjects in order to test their aspirin sensitivity. We then investigated whether aspirin activates eosinophils in peripheral blood and whether tyrosine residues in plasma proteins are brominated in the second subgroup of AIA patients. As shown in Fig. 5, even at the 1-h time-point after aspirin challenge, the ratio of brominated tyrosine concentration to total tyrosine concentration in plasma proteins did not show any significant changes compared with the ratio obtained before the test. Namely, although AIA patients have demonstrated an increased concentration of LTE4 in urine, this increase has not been associated with a change in the concentrations of both urinary free BY and brominated tyrosine in plasma proteins. The ratio of brominated tyrosine residue concentration to total tyrosine concentration was 0.152 ± 0.04 pmol/nmol in plasma proteins from the patients with AIA. This ratio is similar to that in BALF that was collected from mildly asthmatic patients [3].

We may have to consider the possibility that urinary BY concentration does not show changes that are sufficiently large to reflect the activation of eosinophils. However, in our preliminary study, the urinary BY concentration in patients with Churg Strauss syndrome in the active phase was 197.1 ng/mg-creatinine (median), and the LTE4 concentration was 506.4 pg/mg-creatinine (median). Thus, both the LTE4 and BY concentrations were higher than those in healthy controls. As described above, when aspirin was administered to AIA patients, LTE4 was excreted in the urine at a concentration 10-fold that in urine from patients with Churg Strauss syndrome; nevertheless, BY concentration in the AIA patients did not show any significant increase. Based on these results, it is unlikely that changes in the concentration of urinary BY were not sufficiently large to detect the activation of eosinophils.

Collectively, our results demonstrated that (1) there were significantly high urinary BY and CY concentrations in patients with bronchial asthma, and (2) although urinary LTE4 concentration in AIA patients after administration of aspirin significantly increased, increases in BY concentrations in urine and plasma proteins were not confirmed. These results suggest that eosinophils are not directly activated by aspirin administration in AIA patients. Further studies need to focus on identifying the specific mechanism underlying the increased generation of cysteinyl-LT in patients with AIA.

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Expression of membrane-bound CD23 in nasal mucosal B cells from patients with perennial allergic rhinitis

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Keywords: allergic rhinitis, nasal mucosa, CD23, Th2, CxCR3, CCR4

Abstract

Background: CD23 is the low-affinity receptor for IgE on B cells, and is thought to play an important role in regulation of IgE production.

Method: To clarify the role of CD23 in allergic rhinitis, we examined its expression in nasal mucosal B cells of patients with perennial allergic rhinitis, using flow cytometric analysis with double, direct immunofluorescence staining of the mucosal-infiltrating lymphocytes. The expression of CD23 in nasal B cells of patients with non-atopic rhinosinusitis served as a control.

Result: The ratio of $CD23^+$ B cells to total B cells in patients with perennial allergic rhinitis was significantly higher than in non-atopic controls, while that of B cells to total lymphocytes was unchanged. The ratio of $CCR4^+$ CD4 cells to total CD4 cells in allergic patients was significantly higher than in non-atopic controls, while the ratio of $CXCR3^+$ CD4 cells to total CD4 cells was unchanged. There was no significant correlation between the percentages of $CD23^+$ B cells and $CCR4^+$ CD4 cells. In addition, the percentage of $CD23^+$ B cells did not correlate with the total IgE level, or with the specific IgE level.

Conclusions: Our results indicate that nasal mucosal CD23-bearing B cells, as well as Th2 cells, increase in patients with perennial allergic rhinitis. However, the expression of CD23 did not directly correlate with the number of Th2 cells in the nasal mucosa.

Keywords: allergic rhinitis, nasal mucosa, CD23, CxCR3, CCR4

INTRODUCTION

Allergic rhinitis occurs through fundamental mechanisms that involve induction of allergen-specific IgE antibodies. Allergen-specific T cell-B cell interactions are indispensable for the induction of human IgE synthesis, and it has recently been reported that interleukin (IL)-4 and other cytokines released from CD4 helper cells (Th2 cells) affect T cell - B cell interactions and play a role in the induction of IgE synthesis in B cells [1,2].

Human CD23 exists in two isoforms (CD23a and CD23b), which differ only in 6 or 7 amino acids at the N terminus. CD23 has the potential to associate with HLA-DR at the surface of B cells, and in doing so may help to stabilize T cell-B cell interactions, which in turn contribute to T cell activation [3]. The membrane-bound CD23 on B cells is thought to enhance IgE-dependent antigen presentation to T cells and also to influence IgE synthesis in the B cells. However, CD23 expression on B cells in the nasal mucosa and its possible correlation with relevant Th2 cells in patients with allergic diseases has yet to be clarified.

In the present study, we measured the expression of membrane-bound CD23 in nasal B cells, and examined its correlation with CD4 subtypes or serum IgE levels in patients with perennial allergic rhinitis.

MATERIALS AND METHODS

PATIENTS

Eleven Japanese patients (23-69 years old, mean 41.1 ± 18.7, 5 males and 6 females) with serious perennial allergic rhinitis due to *Dermatophagoides pteronyssinus* were enrolled in this study. The diagnosis of allergic rhinitis was made based on Okuda's criteria [4], including a positive CAP-RAST test (greater than class 2; SRL, Tokyo, Japan) against *Dermatophagoides pteronyssinus*. None of the patients received immunotherapy or immunosuppressive drugs (including steroids) during the study. Eleven Japanese patients (24-71 years old, mean 50.4±14.3, 7 males and 4 females) with non-atopic rhinosinusitis were enrolled as controls. Informed consent for participation in the study was obtained from each subject.

TISSUE SAMPLES

Inferior turbinate mucosa or paranasal mucosa was obtained by endonasal sinus surgery. After the mucosa was cut into small pieces (approx. 2mm), tissue-infiltrating lymphocytes (TILs) were collected with a cell strainer (Falcon, Becton Dickinson Labware, NJ, USA), using the Ficoll-Hypaque separation technique (Lymphocyte Separation Solution, Nacalai Tesque Inc., Tokyo) was used. TILs were washed twice with PBS and re-suspended in a freezing solution (Cell Banker, Nihon Zenyaku, Fukushima, Japan). The cells were stored at -80°C until examination.

ANTIBODIES

Anti-human CD4, CD19, CD23, and CXCR3 mAbs were purchased from DAKO (Tokyo, Japan). Anti-human CCR4 mAb was obtained from Genzyme (Boston, MA USA)

FLOW CYTOMETRIC ANALYSIS

The frozen cells were rapidly thawed and diluted 10 times with PBS containing 1% BSA. After two washes with PBS in 1% BSA, the cells were stained with an FITC-conjugated monoclonal anti-CD19 antibody combined with an RPE-conjugated anti-CD23 antibody, or with a FITC- or RPE-conjugated negative control antibody, according to the manufacturer's protocol. The cells were also stained with an FITC-conjugated anti-CD4 antibody combined with RPE-conjugated anti-CXCR3 or anti-CCR4 antibodies.

Cells were subjected to flow cytometric analysis using a FACScan flow cytometer (Becton and Dickinson, USA). A lymphocyte gate was set, based on the pattern of forward and side scatter. A minimum of $5x10^4$ cells in the gate was analyzed on the same day. B-lymphocytes were identified as $CD19^+$ lymphocytes, and T helper (Th) cells were identified as $CD4^+$ lymphocytes. Cell viability was demonstrated by negative staining with 7-aminoactinomycin D (Sigma,

USA), which showed that at least 98% of the cells were viable.

STATISTICAL ANALYSIS

Statistical analysis was performed using a Wilcoxon rank-sum test or a Wilcoxon signed rank test for paired and unpaired data. Statistical analysis was also performed using a Spearman rank correlation test for correlation between the data. A p value of <0.05 was considered statistically significant.

RESULTS

Dot plots for CD19 FITC and CD23 RPE

Typical dot plots for CD19 FITC and CD23 RPE staining are shown for the control group and the allergic rhinitis (AR) group in Fig. 1a and Fig. 1b, respectively. Only $CD19^+$ cells expressed CD23 on mucosal lymphocytes, and CD23 expression on B cells from AR mucosa was higher than that of controls. The dot plot pattern of CD23 expression on nasal B cells suggested that this was not an all or nothing effect for a given cell, but rather that B cells expressed various levels of CD23. Therefore, we measured the % positive and mean fluorescence intensity (MFI) of CD23 on B cells, where the % positive value indicates the relative amount of $CD23^+$ B cells to total B cells, and the MFI indicates the mean level of CD23 expression per B cell.

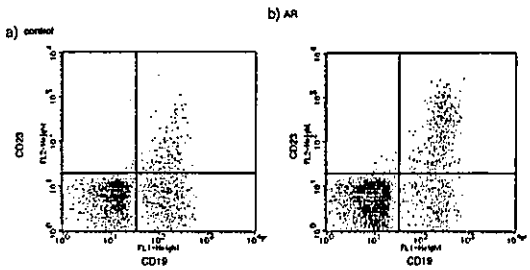

Figure 1

Expression of CD23 on mucosal B cells

The % positive value for CD23 on mucosal B cells in the AR group (43.9 ± 5.8%, mean ± SD) was significantly higher than in the control group (19.9 ± 9.0%, $p<0.01$) (Fig. 2a), while that of B cells to total lymphocytes was unchanged (data not shown). The MFI of CD23 on mucosal B cells in the AR group (40.76 ± 20.62, mean ± SD) was also significantly higher than in the control group (16.9 ± 4.68, $p<0.01$) (Fig. 2b).

The % positive value and the MFI for CD23 were significantly correlated, with the correlation coefficients for control subjects and AR subjects being 0.71 ($p<0.05$) and 0.99 ($p<0.01$), respectively (Fig. 2c).

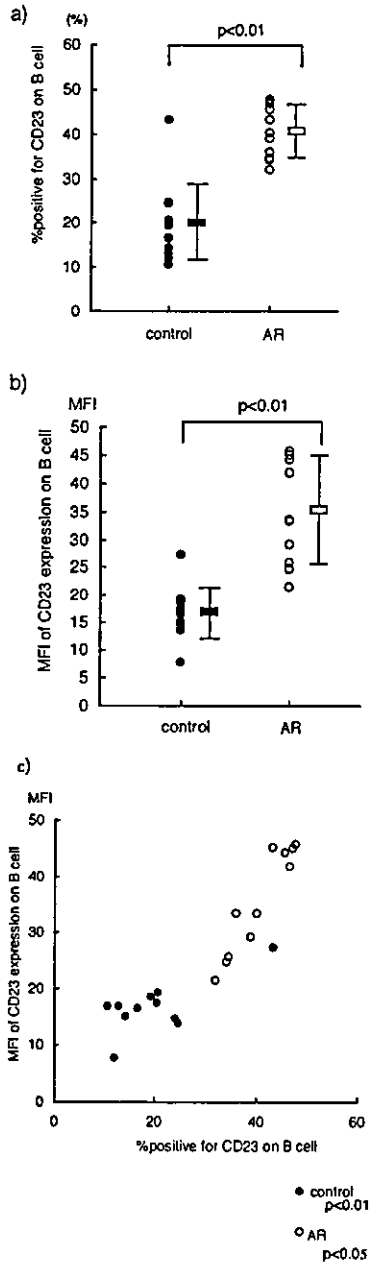

Figure 2

Expression of chemokine receptors on mucosal CD4 cells

CXCR3 and CCR4 were used as Th1 and Th2 markers, respectively [5]. There was no difference between the % positive value for CXCR3 on mucosal CD4 cells in the AR group (16.3 ± 8.1%, mean ± SD) and in the control group (18.8 ± 13.8%, mean ± SD) (Fig. 3a), while the % positive value for CCR4 on mucosal CD4 cells in the AR group (7.5 ±5.7%, mean ± SD) was significantly higher than in the control group (1.3 ± 2.5%, mean ± SD $p<0.05$) (Fig. 3b).

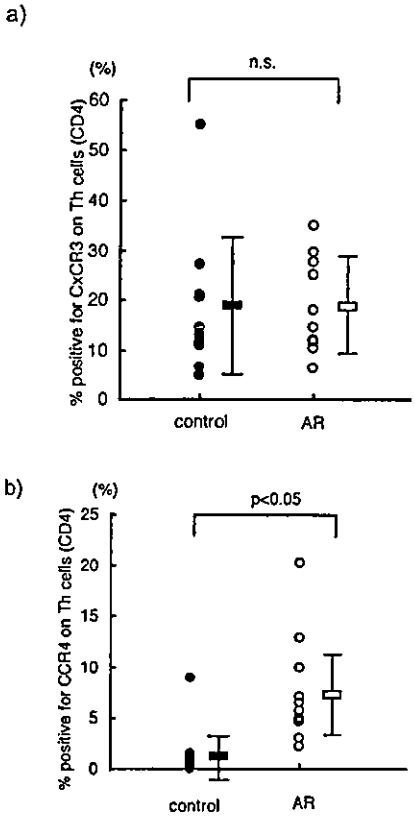

Figure 3

Correlation between % positive values for CD23 on B cells and CCR4 on CD4 cells

No significant correlation was observed between the % positive values for CD23 on mucosal B cells and CCR4 on CD4 cells from the same mucosa. The correlation coefficients for control subjects and AR subjects were 0.21 (n.s.) and 0.26 (n.s), respectively (Fig. 4), and hence there was no significant correlation in either group.

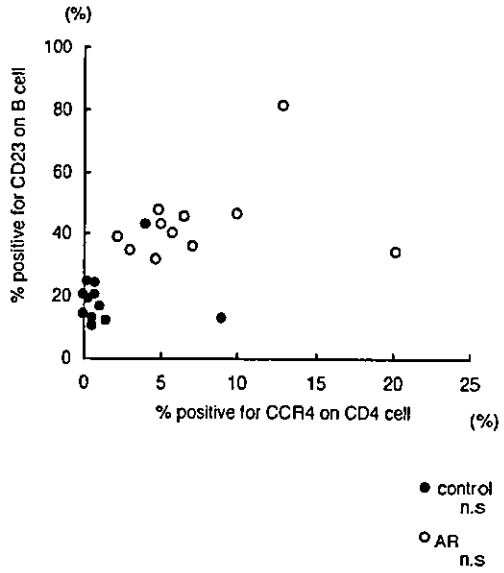

Figure 4

Correlation between the % positive value for CD23 on B cells and the serum IgE level

The correlation between the % positive values for CD23 on mucosal B cells and total serum IgE levels is shown in Fig. 5. No significant correlation was observed between these values, or between the % positive values for CD23 on B cells and specific IgE levels (data not shown).

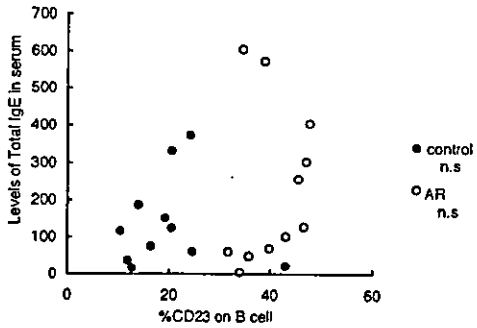

Figure 5

DISCUSSION

The role of CD23 in IgE synthesis is still controversial and remains to be elucidated. The binding of the antigen-IgE complex to CD23-bearing B cells has been shown to augment IgE-mediated responses [6], and also CD23 is the enhancement of IgE-dependent antigen presentation to T cells [3,7,8]. In clinical studies, the cell surface expression in peripheral

blood B-lymphocytes have shown increased CD23 expression in allergic children and adults, including patients with allergic rhinitis compared with non-allergic controls [9,10], and have further shown that CD23 expression decreased after successful hyposensitization [11,12]. Moreover, since IgE levels in serum were assessed as atopy marker, a significant correlations were reported between the levels of soluble form CD23 and the levels of IgE in serum in patients with atopy. [13]

In the present study, we examined the expression of CD23 on mucosal B cells and found higher percentages of CD23-bearing B cells in patients with perennial allergic rhinitis, compared to those in non-allergic patients. In addition, we investigated Th1 and Th2 cells in the nasal mucosa by staining for expression of CXCR3 and CCR4 chemokine receptors, respectively. The results showed that the Th2/CD4 ratio in patients with perennial allergic rhinitis was indeed higher than in non-allergic controls, while the Th1/CD4 was unchanged. However, no significant correlation was found between the Th2/CD4 ratio and the CD23/Bcell ratio. Furthermore, no significant correlation was found between the CD23/B cell ratio in the nasal mucosa and the total IgE level or specific IgE level in serum (data not shown).

The T cell-B cell interaction must play an important roll in allergic inflammation. IL-4 and IL-13 are known to promote the switching of B cells from IgM to IgE production and expression of CD23 [14], while interferon-gamma (IFN-γ) , IL-10 and IL-12 inhibit this effect [15-17]. Other than Th2 cells, various kinds of cells in the nasal mucosa, such as mast cells, basophils, and CD8 cells, have been shown to produce IL-4 and IL-13 [18,19]. The lack of a significant correlation between the ratios of Th2/CD4 T cells and CD23/B cells may suggest that the level of CD23 may be correlated with the amount of IL-4/IL-13 produced, and that not only from Th2 cells, influences CD23 expression in nasal mucosal B cells. In this study, Th2 cells were shown to make up about 7.15% of the infiltrating CD4 T cells in the nasal mucosa of patients with allergic rhinitis. However, only a small portion of these Th2 cells could recognize the house dust mite allergen. An ELISPOT study has shown a very low frequency population of allergen-specific IL-4 or IL-13 producing Th cells, which represented about 1 spot per 10,000-100,000 peripheral CD4 T cells [20,21]. Th1/Th2 cytokine dysregulation is thought to be a fundamental pathogenesis of allergic rhinitis, but only a few T and B cells are allergen-specific. The major source of IL-4 and IL-13 production in the effector phase in the nasal mucosa of patients with allergic rhinitis may be mast cells or basophils, and not Th2 cells [22]. The role of Th2 cytokines from mast cells remains to be clarified, but recent study showed Th2 cytokines from mast cells induced by antigen stimulation [23] and influence not only the differentiation of naïve T cells toward Th2 cells [24] but also B cell activation [25]. In addition, the lack of correlation between the number of nasal B cells and the serum IgE level observed in this study may suggest that the nasal mucosa could synthesis IgE independently from peripheral blood [26].

Overall, the results in this study suggest that enhanced expression of CD23 on nasal mucosal B cells occurs in patients with allergic rhinitis. However, further analysis is required regarding the significant of CD23 in nasal mucosa at the site of the allergic reaction.

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