

Cellular Allergen Stimulation Test with Acetylsalicylic Acid-Lysine Is Not a Useful Test to Discriminate between Asthmatic Patients with and without Acetylsalicylic Acid Sensitivity

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Key Words

Aspirin sensitivity • Asthma • Nasal polyp • Cysteinyl leukotrienes • Cellular allergen stimulation test assay

Abstract

Background: The aspirin provocation test, considered to be the gold standard in the diagnosis of aspirin sensitivity, may be associated with severe adverse reactions; thus, alternative procedures with a higher safety profile are highly desirable. Although the cellular antigen stimulation test (CAST) has been proposed to be such an alternative, there is limited information about its clinical usefulness. **Objective:** It was the aim of our study to evaluate the clinical usefulness of CAST in the diagnosis of aspirin sensitivity. **Material and Methods:** Patients with aspirin-sensitive asthma and/or nasal polyps ($n = 40$), patients with aspirin-tolerant asthma and/or nasal polyps ($n = 13$) and healthy volunteers ($n = 26$) were included. A 2-day, single-blind placebo-controlled oral aspirin provocation test was performed. In vitro release of cysteinyl leukotrienes (Cys-LTs) by peripheral blood leukocytes was measured after stimulation with both stimulation buffer and lysine aspirin (2.5 mg/ml) by CAST. **Results:** Baseline Cys-LT levels were similar among the 3 groups. After lysine aspirin stimulation, the net increase in Cys-LTs was sig-

nificantly higher in patients with aspirin sensitivity (median 91 pg/ml, interquartile range 22–206) compared with aspirin-tolerant patients (20 pg/ml, range 0–46) and healthy controls (23 pg/ml, range 0–71; $p = 0.004$). The assay had a sensitivity of 25%, a specificity of 92.3%, and positive and negative predictive values of 28.7 and 90.7%, respectively.

Conclusion: Although the leukocytes of patients with aspirin sensitivity produce higher amounts of Cys-LTs as measured by CAST, the low sensitivity and predictive values limit the clinical usefulness of this test in the diagnosis of aspirin sensitivity.

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Introduction

Acetylsalicylic acid (ASA) and other nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most frequently used drugs because of their antipyretic and anti-inflammatory actions. High frequency of ASA sen-

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sitivity has been reported in a group of patients with asthma, nasal polyposis and chronic urticaria [1].

It is commonly accepted that sensitivity to ASA results from inhibition of cyclooxygenase. This inhibition diminishes the production of prostaglandin E_2 which normally acts as a 'brake' on the production of cysteinyl leukotrienes (Cys-LTs: LTC_4 , LTD_4 , LTE_4), which are not only major mediators for the clinical symptoms observed in ASA sensitivity, but also induce the release of other mediators by the mast cell [2, 3]. There is clear evidence that most patients with ASA intolerance synthesize excessive amounts of Cyt-LTs after oral aspirin challenges [4].

Despite extensive data on the mechanism of the disease, the diagnosis of ASA intolerance is currently based on history and oral or inhalation provocation tests with ASA. Provocation tests, especially oral provocation tests, are considered to be the golden standard in the diagnosis of ASA intolerance. However, they are time-consuming and potentially harmful procedures and should be performed under rigorous surveillance [5, 6]. Therefore, safe and easy in vitro diagnostic tests would be extremely useful both for patients with ASA intolerance and for the allergists who are dealing with these patients. However, currently, there is no established in vitro method for the diagnosis of ASA sensitivity.

The cellular antigen stimulation test (CAST) is based on IL-3 priming and stimulation of isolated human leukocytes with the allergen and measuring the concentration of the generated Cys-LTs in the supernatant [7, 8]. The test is a fast, simple and commercially available tool and has been applied as an in vitro diagnostic procedure in allergic reactions to inhalant allergens, insects and foods [9]. Only a few studies have investigated the use of CAST assay in the diagnosis of ASA sensitivity [9–11]. At present, the role of CAST for diagnosis of ASA sensitivity is a contradictory issue, and as outlined in a recent review, these contradictions are mainly associated with technical problems and methodological issues related to patient selection [9]. In general, these studies included a relatively small number of patients, and ASA challenges have been performed in only a subset of these individuals. More importantly, as a direct consequence of these methodological issues, the sensitivity and specificity of CAST in the diagnosis of ASA sensitivity remain for the most part unknown.

Therefore, we aimed to evaluate the clinical utility of ASA-induced Cyt-LT production in the diagnosis of ASA sensitivity in a group of patients with asthma and/or nasal polyposis using CAST (CAST™ 2000 ELISA, Bülh-

mann Laboratories, Allschwil, Switzerland). The sensitivity and specificity of the assay were calculated against the results of the oral provocation testing.

Material and Methods

Patient Selection

Patients who presented to the Department of Allergy at Ankara University between March 2006 and April 2007 with a suspected history of ASA sensitivity, histories of naso-ocular symptoms, mild to severe bronchospasm and/or anaphylactoid reaction within 2 h after ingesting a prescribed ASA/NSAID, paracetamol or metamizol, and asthma and/or nasal polyposis were included in the study. Asthma was diagnosed by the presence of recurrent symptoms of wheezing, shortness of breath, cough and demonstration of an objective sign of reversible airway obstruction as stated by the American Thoracic Society [12]. The diagnosis of nasal polyposis was based on visualization of bilateral polyps in the nasal cavities by an ENT specialist on endoscopic examination or by the CT scan of the paranasal sinuses.

Patients underwent an oral ASA provocation as outlined below. In order to be eligible for ASA provocation, asthma patients were required to be stable for at least 2 weeks to have a forced expiratory volume in 1 s (FEV_1) >70% of the predicted value for the patient and not to have a respiratory infection for at least 6 weeks prior to the study.

They were allowed to continue their maintenance therapy with inhaled corticosteroids and inhaled salbutamol as needed, but the drug was not allowed at least 6 h before the challenge procedure. Additionally, aminophylline and long-acting β_2 -agonists were withheld for at least 24 h before the challenge. Patients taking systemic antihistamines, systemic corticosteroids, leukotriene modifiers, cromolyn, oral sympathomimetics or β -blocker drugs within the last week were excluded.

According to the results of ASA provocation tests and the history of a reaction to ASA/NSAIDs, the patients were divided into 2 groups: patients with asthma and/or nasal polyps with ASA sensitivity (ASA sensitive, $n = 40$) and patients without ASA sensitivity (ASA tolerant, $n = 13$). Healthy controls ($n = 26$) were recruited from among the hospital staff who denied any acute or chronic illnesses and had normal physical examination findings. These individuals have confirmed using ASA without any apparent reactions.

All study procedures were performed in accordance with a protocol previously approved by the Ethics Committee of Ankara University. All patients provided written informed consent for the study procedures.

Study Measurements

In vitro release of Cys-LTs by isolated peripheral blood leukocytes, at baseline and after Lys-ASA stimulation, was measured by CAST according to the manufacturer's instructions [9]. To determine the most suitable dose for the stimulation, we tried several doses such as 0.0625, 0.125, 1, 2.5 and 5 mg/ml. The concentration of Lys-ASA (2.5 mg/ml) was selected according to both our results and the results of the preliminary experiments [13]. Briefly, patients' leukocytes were incubated with Lys-ASA (BAG2-C51, Bülhmann Laboratories) at a final concentration of 2.5 mg/ml.

Stimulation buffer was used to obtain background values, and monoclonal anti-IgE anti-receptor antibody (Bühlmann Laboratories) was used as positive control. Cys-LT concentration was measured by ELISA.

A stimulation index (test value/background value) >2 and a net Cys-LT value (test value – background value) >100 pg/ml were considered positive [9].

Aspirin Provocation

The oral ASA provocation test was performed according to Nizankowska et al. [14], and the responses were classified as negative, upper airway reaction, classical reaction and systemic reaction. Upper airway reaction was defined as the presence of rhinorrhea and nasal congestion, classical reaction as a >20% decrease in FEV₁ associated with upper airway reaction, and systemic reaction as extra bronchial symptoms such as hoarseness, redness of the face and the upper chest, ocular injection and/or periorbital swelling, nausea, vomiting and stomach cramps, and hypotension with or without cutaneous/bronchial symptoms. Each additional bronchial symptom was scored as follows: 0 = no symptom; 1 = small intensity; 2 = moderate intensity; 3 = strong intensity; 4 = very strong intensity. The symptoms were considered strong when a patient attained a total score of ≥12 points. The reaction was considered to be systemic in the presence of strong extra bronchial symptoms with or without cutaneous/bronchial symptoms [14].

On the first day of ASA provocation, 3 capsules of placebo were administered every 2 h. FEV₁ was measured every 30 min, and if FEV₁ dropped more than 15% during the placebo challenge, further oral ASA provocation was not performed. On the second day, oral provocation with consecutive doses of ASA was carried out according to the protocol. The starting dose of 10 mg was followed by 17, 44, 117 and 312 mg of ASA every 1.5–2 h until a cumulative dose of 500 mg was reached. FEV₁ was measured before each consecutive dose of ASA and subsequently at 30, 60, 90 and 120 min. The provocation test was considered positive if the FEV₁ value decreased by at least 20% from a post-saline baseline value and/or if strong extra bronchial symptoms occurred. The provocative dose of ASA causing a 20% fall in FEV₁ (PD₂₀) was calculated as the PD₂₀ ASA oral. The lowest FEV₁ out of 3 measurements (at 30, 60 and 120 min) obtained after each dose was plotted against the log₁₀ cumulated dose of ASA in order to obtain a dose-response curve. The PD₂₀ was derived by linear interpolation from this dose-response curve [14].

Evaluation of Atopy

Atopy was defined as a positive skin prick test to at least 1 of the aeroallergens. Glycerinated extracts (Stallergenes, Antony, France) of the following allergenic sources were used in skin prick tests: *Dermatophagoides pteronyssinus*, *D. farinae*, cockroach, grass, tree, weed pollens, cat, dog, *Alternaria* and *Cladosporium* antigens). Positive (histamine 10 mg/ml) and negative (saline solution) controls were included. The puncture method with a 1-mm-tip disposable lancet was used, and a mean diameter of 3 mm greater than the negative control was considered positive.

Statistical Analyses

Statistical analyses were performed by Statistical Package for Social Sciences (SPSS) version 11.0 for Windows. Nominal values were expressed numerically or as percentages, and results were

expressed as the median (interquartile ranges), unless stated otherwise. The χ^2 test was used to compare nominal data. Nonparametric tests were used due to the heterogeneity of the groups. Differences between study groups were examined by the Kruskal-Wallis test. For pairwise comparisons of the non-normally distributed data, the Mann-Whitney U test with Bonferroni correction was used. All directional p values were 2-tailed, and the significance was assigned to values <0.05.

Sensitivity, specificity, as well as the positive predictive value (PPV) and the negative predictive value (NPV) of CAST were calculated using cross-tables, as defined previously. Briefly, sensitivity was defined as the proportion of CAST-positive subjects with a positive oral ASA provocation and specificity as the proportion of CAST-negative subjects with a negative oral ASA provocation. The PPV was defined as the number of true positives/the number of true positives + the number of false positives. The NPV was defined as the number of true negatives/the number of true negatives + the number of false negatives. The PPV and NPV likelihood ratio (LHR) was calculated considering the prevalence of ASA-sensitive asthma which was reported to be 11% in Turkey [15]. The positive LHR was defined as sensitivity/1 – specificity. The LHR was classified as important (LHR >10), moderately important (5 < LHR < 10), sometimes important (2 < LHR < 5) and unimportant (1 < LHR < 2).

Results

Forty patients with ASA sensitivity, 13 patients with ASA-tolerant asthma and/or nasal polyps and 26 healthy controls constituted the study population. Characteristics of the study population are shown in table 1. Males were only predominant in the ASA-sensitive group. The atopy rate and the presence of asthma were statistically higher in the ASA-sensitive group than in the ASA-tolerant group. Two of 40 ASA-sensitive patients (5%) had no previous drug reaction. The median duration of drug reaction in the remaining ASA-sensitive patients was 5 years [2, 10]. The culprit drug was ASA in 29, other NSAIDs (etodolac, naproxen, fluorbiprofen, ibuprofen, diclofenac) in 22, metamizol in 14 and paracetamol in 2 patients.

Oral ASA Provocation Results

A total of 42 patients were challenged with oral ASA. Thirteen of them completed the oral ASA provocation without any reactions and were considered ASA tolerant. The remaining 29 patients reacted to ASA challenge and were considered ASA sensitive. Of these 29 patients, 20 had a classical response to oral ASA with a median PD₂₀ ASA of 140 mg (range 58–312); 4 patients had systemic reactions with a median extrapulmonary score of 12 [10, 13]; 4 patients had upper airway symptoms and 1 had cutaneous symptoms. Due to a clear history of life-threat-

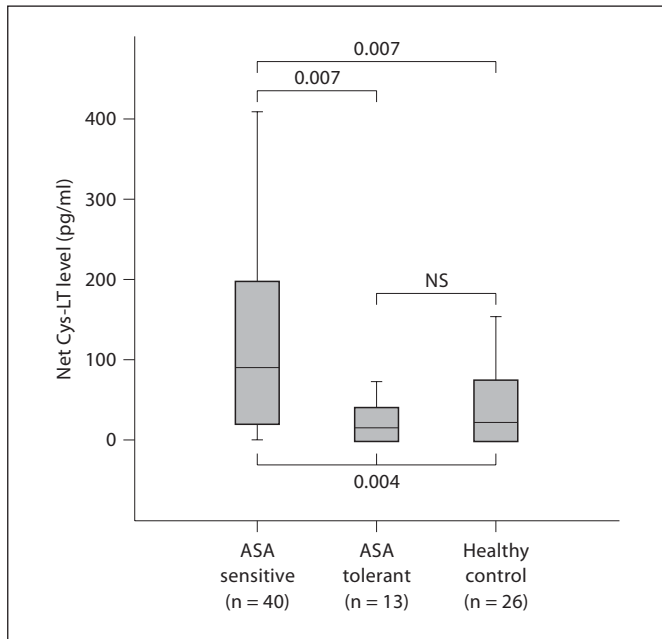


Fig. 1. Cys-LT levels according to the drug reaction. NS = Not significant.

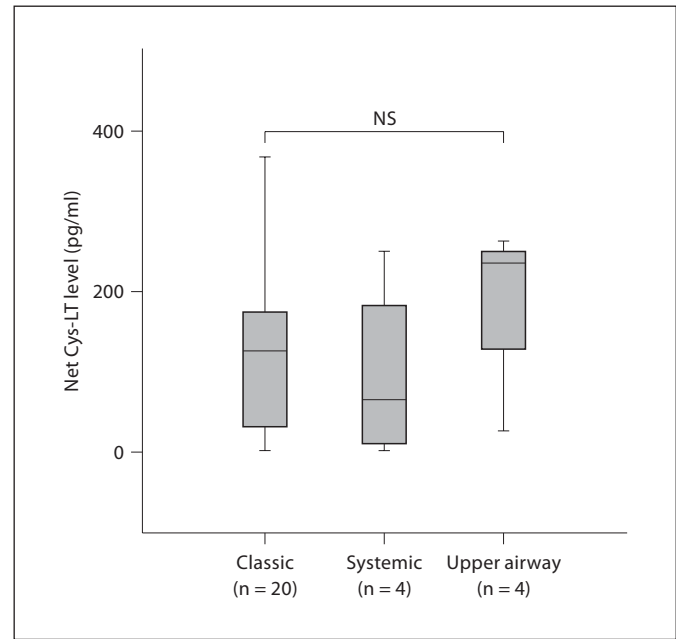


Fig. 2. Net Cys-LT levels according to the response type to oral ASA provocation test (1 patient not included in the graph had a cutaneous reaction).

Table 1. Characteristics of the study population

	ASA sensitive (n = 40)		ASA tolerant (n = 13)	Healthy control (n = 26)	p
	APT- NA (n = 11)	APT+ (n = 29)			
Gender					
Female	9 (82)	20 (69)	4 (30.8)	16 (61.5)	0.01
Male	2 (18)	9 (31)	9 (69.2)	10 (38.5)	
Age, years	39.19 ± 1.2	38.2 ± 0.9	42.9 ± 2.1	34.4 ± 1.5	>0.05
Skin test to common aeroallergens					
Positive	3 (27)	13 (44)	2 (15.4)	NA	0.04
Negative	8 (73)	16 (56)	11 (84.6)		
Nasal polyp only	2 (18)	6 (21)	10 (77)	–	0.01
Nasal polyp with asthma	9 (82)	20 (69)	3 (23)	–	0.01

Figures in parentheses are percentages. APT = Oral ASA provocation test; NA = not applicable.

ening reactions to ASA/NSAIDs, oral ASA provocation was not performed in 11 patients, but these patients were added to the ASA-sensitive group. Therefore, the ASA-sensitive group consisted of 40 patients (29 + 11). All healthy controls have used ASA and other NSAIDs without any adverse reactions.

Cys-LT Results

Baseline Cys-LT levels were similar among the 3 groups ($p > 0.05$). After Lys-ASA stimulation, the net increase in Cys-LTs was significantly higher in patients with ASA sensitivity (91 pg/ml, interquartile range 22–206) compared with ASA-tolerant patients (20 pg/ml, range 0–46) and healthy controls (23 pg/ml, range 0–71;

p = 0.004) (fig. 1). The type of response to oral the ASA provocation test had no effect on the amount of Cys-LTs released by the blood leukocytes (fig. 2).

Stimulation indices were calculated as 1.48 (interquartile range 1.08–2.18) in ASA-sensitive, 1.15 (range 0.87–1.3) in ASA-tolerant patients and 1.18 (range 0.83–1.57) in healthy controls. The details of stimulation indices are shown in table 2.

Median duration between the time of oral ASA provocation and the time of in vitro stimulation of peripheral blood leukocytes with Lys-ASA was 7 months [3–12]. In 12 patients with positive CAST, oral provocation dated back longer than 6 months before CAST was performed, and in 17 patients, the reaction took place within the past 6 months. Net Cys-LT release was higher in patients whose reaction took place more than 6 months ago (170 pg/ml, range 31–316) compared with those with a reaction within the past 6 months (54 pg/ml, range 0–122; p = 0.048). This difference disappeared when the comparison was done for 12 months.

According to our positivity criteria, 3 of the 39 ASA-tolerant patients and healthy controls and 10 of the 40 ASA-sensitive patients were identified as CAST positive. The sensitivity and specificity of the CAST assay was 25 and 92.3%, respectively. PPVs and NPVs were calculated to be 28.7 and 90.9%, respectively. The positive LHR was calculated to be 3.3 and indicated that the test was important in some cases.

There was no difference in the cumulative dose of oral ASA which induced objective symptoms between CAST-positive and CAST-negative patients (median 188 mg, range 84–500, vs. 500 mg, range 188–500; p = 0.062).

Discussion

Our study shows that peripheral blood leukocytes from patients with ASA sensitivity produce increased amounts of Cyt-LTs upon in vitro stimulation with ASA. Our results also suggest that even though the test has a high specificity (92.3%) for ASA sensitivity, relatively lower values obtained for sensitivity (25%), PPV (28.7%) and NPV (90.9%) may limit its usefulness in the diagnosis of ASA sensitivity.

CAST has been introduced as the first in vitro test for the diagnosis of ASA/NSAID intolerance in the early 1990s [16]. Since then, there have been several attempts evaluating the use of the CAST assay in the diagnosis of ASA sensitivity, but there are very limited data about its clinical usefulness [9].

Table 2. Stimulation indices of the study groups

	ASA sensitive ¹	ASA tolerant ²	Healthy control ³
Stimulation index	1.48 (1.08–2.18)	1.15 (0.87–1.3)	1.18 (0.83–1.57)

Figures in parentheses are interquartile ranges.
¹ ASA sensitive versus ASA tolerant, p = 0.038.
² ASA sensitive versus healthy control, p = 0.036.
³ ASA tolerant versus healthy control, p = 0.826.

There are conflicting data on the utility of CAST for the diagnosis of ASA sensitivity (table 3) [10, 11, 13, 16–26]. Pierzchalska et al. [11], in a well-documented trial, found no difference between Cys-LT release to ASA stimulation from peripheral blood leukocytes of patients with and without ASA sensitivity. Similarly, Lebel et al. [17] have concluded that the CAST assay is not useful due to its low sensitivity of 21% and a specificity of 88%. On the other hand, Czech et al. [18] demonstrated that Cys-LT release from human leukocytes after stimulation with C5a (100 µg/ml) and ASA (10 µg/ml) in patients with ASA sensitivity was significantly higher when compared with normal controls. One year later, Mewes et al. [10] evaluated Cys-LT release from blood leukocytes after ASA challenges in patients with asthma, nasal polyps and ASA sensitivity, in ASA-tolerant patients with nasal polyps and in controls. Blood leukocytes were prestimulated with IL-3 and incubated with ASA (10 and 100 µg/ml) together with C5a (10⁻⁸ M) for 18 h. Baseline Cys-LT levels were similar, and an increase in Cys-LT levels after in vitro ASA has been observed only in patients with asthma and nasal polyps and ASA sensitivity. The study group concluded that the Cys-LT release assay could be of potential use in the in vitro diagnosis of ASA sensitivity. Three years later, May et al. [19] showed that ASA itself induced a slight but significant increase in Cys-LT release, whereas ASA plus C5a resulted in a higher significant increase in Cys-LT release in asthma or urticaria patients with ASA sensitivity compared with patients with allergic rhinitis and healthy controls. This study also clearly showed that the use of several NSAIDs, in particular diclofenac, in addition to ASA markedly improved sensitivity (from 50 to 72%) while not impairing a high specificity (>99%). As a nonspecific activator of basophils, C5a has been used by some in vitro studies evaluating ASA sensitivity and has been thought to augment the stimulation induced by ASA [10, 18, 19]. Contrarily, more recently, it was reported that Cys-LT release in ASA-sensitive asthmatics after basophil stimulation with C5a was not different from those of ASA-tolerant asthmatics (ta-

Table 3. CAST publications in ASA/NSAID hypersensitivity

Reference	AIA	AIU	ATA	AC	HC	Prov+	Stimulant	Sensitivity, %	Specificity, %	Concentrations µg/ml
De Weck et al. [16], 1993		10			10	?	ASA	60	100	NR
Czech et al. [18], 1995	3	5		7	8	8	ASA, C5a	100 (C5a)	100 (C5a)	10, 100
Wolanczyk-Medrała et al. [24], 1995	15		10			15	ASA	66	100	2, 20, 200
Brunner et al. [22], 1996		9		8	10	9	ASA	sLT >in AIU		NR
Mewes et al. [10], 1996	8		8		6	8	ASA, C5a	sLT >in AIA		10, 100, 10 ⁻⁷ 8 M C5a
Kubota et al. [21], 1997		25		3	5	?	ASA	62.5–80	70–100	0.5, 5, 50, 500
May et al. [19], 1999	22	16			20	30	ASA, diclofenac, others	50–72	96	20, 200
Wedi and Kapp [23], 2000		18			66	18	C5a	83	94	100, 10 ⁻⁷ M C5a
Busse et al. [25], 2000		29			15	8	ASA, C5a	72	100	100, 10 ⁻⁷ M C5a
Pierzchalska et al. [11], 2000	26		32	20	30	26	ASA	0	0	18
Lebel et al. [17], 2001	19				17	19	ASA	21	88	0.2, 2, 20, 200
Celik et al. [3], 2001	13		12		13	ND	ASA	sLT >in AIA		50
Abrahamsen et al. [20], 2001	10		12		9	10	C5a	sLT, all intrinsic asthmatics		10 ⁻⁷ M C5a
Sanz et al. [13], 2005	20	40		21	9	16	ASA, diclofenac, others	21–31	83–96	300, 1,250
Current study, 2008	40		13		26	29	ASA	25	92.3	2,500

AIA = Aspirin induced asthma; AIU = aspirin induced urticaria; ATA = aspirin tolerant asthma; AC = atopic control; HC = healthy control; Prov+ = provocation test; NR = not reported; sLT = cysteinil leukotriene; ND = not defined.

ble 3) [20]. At present, the general recommendation is not to use C5a as an adjuvant [9]. In keeping with that and according to the manufacturer's instructions, we did not use C5a in our assay. Recently, in the largest series of in vitro diagnostic tests, in which flow cytometric basophil activation and CAST has been combined, Sanz et al. [13] reported that CAST has a lower sensitivity (21.6%) with ASA stimulation; however, the addition of CAST increased the overall sensitivity of the corresponding flow cytometric basophil activation assay.

A variety of factors may account for the observed discrepancy among the results regarding the clinical suitability of CAST for the diagnosis of ASA sensitivity. These factors include random effects observed in the relatively small number of subjects, heterogeneity of the study group (asthma and urticaria) and the dose of drug used, difference in the CAST-positivity criteria and a number of technical factors [9, 13, 19, 26]. So far, the largest study of the CAST assay included 60 patients. However, in this study, ASA sensitivity was diagnosed mainly by clinical diagnosis without provocation, and only 20 of the patients with ASA sensitivity had asthma [13]. In our study, we included 40 patients with only asthma/nasal polyp phenotype with ASA sensitivity and it seems that it included the highest number of patients with the same phenotype. Moreover, in our study, ASA sensitization has been confirmed by oral provocation test in all patients except for those with a his-

tory of life-threatening reactions. Increased Cyt-LT production has also been demonstrated in chronic urticaria patients with ASA-induced flares [23, 27]. In keeping with that, rather high sensitivity and specificity of the CAST assay have been reported in these patients [16, 21, 23, 25, 26]. However, the mechanism of ASA intolerance resulting in acute urticaria/angioedema may be different than that of patients with chronic urticaria, and this needs further investigation [5]. The optimal dose of drug concentration for the CAST assay has not yet been clearly defined [9]. ASA has been used most commonly at a dosage ranging between 10 and 100 µg/ml [10, 18]. However, the dose of ASA in a completely negative study was almost 20–50 times lower than those used in these studies (0.018 mg/ml) [11]. We used 50 µl of ASA at a final concentration of 2.5 mg/ml, according to the manufacturer's instructions. In addition, it seems important to test more than 1 NSAID to increase the sensitivity of the CAST assay [13, 19, 26].

In support of a previously published report [19], we also failed to show any difference in the cumulative dose of ASA causing a clinical response in the oral provocation test between the CAST-positive (n = 10) and -negative patients (n = 30; p > 0.05). Even though these results suggest that the amount of the leukotrienes released by the leukocytes is not a direct function of the dose of ASA, a definitive conclusion will require more individuals than the number we have included in our study.

The optimal time period to perform a CAST assay has been reported to be within 6–12 months following clinical exposure or provocation testing to ASA/NSAIDs [13]. However, in contrast, we found that the net Cys-LT levels were significantly higher in patients in whom the CAST assay was performed 6 months after ASA provocation. The underlying physiology for this observation remains unclear.

To conclude, our results suggest that CAST may be a potentially important test for the diagnosis of ASA sen-

sitivity as it allows the detection of significant increases in the levels of Cyt-LTs after stimulation of peripheral blood leukocytes with ASA. However, in its present form, its clinical use is greatly limited by the low sensitivity.

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