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Upregulation of CD63 or CD203c Alone or in Combination Is Not Sensitive in the Diagnosis of Nonsteroidal Anti-Inflammatory Drug Intolerance

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Key Words
Aspirin sensitivity · Asthma · Nasal polyp · Basophil activation · CD63 · CD203c · Flow cytometric cellular allergen stimulation test · Cellular antigen stimulation tests · Allergenicity

Abstract
Background: The oral aspirin (ASA) provocation test is considered to be the gold standard in the diagnosis of ASA sensitivity. However, since it may be associated with severe adverse reactions, safer alternatives would be highly desirable. The basophil activation test has been proposed as such an alternative, but there is limited information about its usefulness. Our aim was to evaluate the clinical usefulness of flow cytometric basophil activation in the diagnosis of ASA sensitivity. ***Methods:*** Patients with ASA sensitivity (n = 18), patients with ASA tolerance (n = 12) and healthy volunteers (n = 12) were included in the study. A 2-day single-blind placebo-controlled oral ASA provocation test was performed on all patients. Basophil activation after lysine-ASA and diclofenac stimulation was measured by Flow-CAST™ (Bühlmann Laboratories) for CD63 and an allergenicity kit (Beckman Coulter) for CD203c. The results of CD63 and CD203c were compared within groups, and sensitivity and specificity of the assay were measured against oral ASA provocation. ***Results:*** The highest sensitivity and specificity of CD63 were 33.3 and 79.2%, respectively, and of CD203c were 16.7 and 100%, respectively, for ASA. The highest sensitivity and specificity of CD63 were 16.7 and 91.7%, respectively, and of CD203c were 22.2 and 100%, respectively, for diclofenac. Neither the addition of CD203c to CD63 nor the addition of diclofenac improves the overall sensitivity and specificity of CD63 to ASA. ***Conclusion:*** At present, basophil activation using CD63 and CD203c does not seem to be optimally sensitive for the diagnosis of ASA sensitivity.

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Introduction

Acetyl salicylic acid (ASA) and other nonsteroidal anti-inflammatory drugs (NSAID) are among the most frequently used drugs because of their antipyretic and anti-inflammatory actions. The high frequency of ASA intolerance has been reported in a group of patients with asthma, nasal polyposis and chronic urticaria [1].

It is commonly accepted that intolerance reactions to ASA result from inhibition of cyclooxygenase by ASA and other NSAID [2–4]. Despite extensive data on the mechanism of the disease, the diagnosis of ASA hypersensitivity is currently based on history and provocation tests with ASA. Provocation tests, especially oral provocation tests, are considered to be the gold standard in the

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diagnosis of ASA hypersensitivity. However, they are both time-consuming and potentially harmful procedures which 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 hypersensitivity and for the allergists who are dealing with these patients. However, at present, there is no established in vitro method for the diagnosis of ASA hypersensitivity.

Flow cytometry is a useful tool for the analysis of different cellular types, and can be used to identify specific cell populations. Basophils are considered as primary effector cells of allergic reactions. The flow cytometric analysis of in-vitro-activated peripheral blood basophils relies upon quantification of phenotypic alterations, i.e. changes in the expression of basophilic activation markers following a challenge from specific allergens [7]. So far, the most commonly applied markers in flow cytometric studies for basophilic activation have been CD63 and CD203c. CD63 is a membrane protein and is expressed on basophils, tissue mast cells, macrophages and platelets. CD63 is expressed on the membrane of basophil granules, and is therefore not detectable on the surface of resting basophils. In contrast, CD203c is constitutively and exclusively expressed on the surface of basophils, mast cells and their progenitors. Following allergen challenge, CD203c is rapidly upregulated in sensitized subjects, and thus a promising target molecule for flow-cytometry-based evaluations. Determination of basophil activation through the use of just CD63 may lead to false-negative results, while the use of only CD203c may lead to false-positive results. For this reason, a combination of CD63 and CD203c is recommended for the analysis of allergen stimulation [8, 9]. Flow cytometric analysis of in-vitro-activated basophils using CD63 and CD203c has been proved to be reliable for the diagnosis of different IgE-mediated allergies, including inhalant allergens, food allergies, natural rubber latex and venom [7–10].

Diagnosis of a drug allergy is a major problem, because a wide range of drugs or their metabolites can induce clinical manifestations with immune or non-immune, or even unknown, mechanisms. There are several trials indicating the contribution of flow cytometric analysis of basophil activation, mainly via CD63, to the diagnosis of hypersensitivity reactions to neuromuscular-blocking agents and β-lactam antibiotics [11–13]. However, only a few studies have investigated basophil activation using CD63 in the diagnosis of hypersensitivity to ASA/NSAID, with the clinical usefulness of CD63 measurement for the diagnosis of ASA hypersensitivity still being a controversial issue [7, 8, 11]. Moreover, there is no study evaluating the value of CD203c expression in response to in vitro stimulation of ASA for ASA hypersensitivity.

Therefore, in this study, we aimed to evaluate the application of basophil activation using CD63 and CD203c molecules following in vitro ASA challenge in the diagnosis of ASA hypersensitivity. The sensitivity and specificity of the assay were measured against the results of the oral provocation testing.

Material and Methods

Patient Selection

One hundred and twenty patients who presented to the Department of Allergy at Ankara University between March 2005 and December 2006 with a suspected history of ASA hypersensitivity, histories of naso-ocular symptoms, mild to severe bronchospasm and/or anaphylactoid reaction within 2 h of ingesting a prescribed ASA/NSAID, paracetamol or metamizole, and asthma and/or nasal polyposis, were screened and tested with ASA provocation for the study. Asthma was diagnosed by the presence of recurrent symptoms of wheezing, shortness of breath, a cough and the demonstration of an objective sign of reversible airway obstruction as stated by the Global Initiative for Asthma guidelines [14]. The diagnosis of nasal polyposis was based on visualization of bilateral polyps in the nasal cavities by either an ear-nose-throat specialist on endoscopic examination or by a CT scan of the paranasal sinuses. All patients, except those with life-threatening reactions, underwent oral ASA provocation (as outlined in 'Oral ASA provocation test results'). In order to be eligible for ASA provocation, asthma patients were required to have been stable for at least 2 weeks prior to the study, to have a forced expiratory volume in 1 s (FEV_1) >70%, and not to have had a respiratory infection for at least 6 weeks prior to the study.

Patients were permitted to continue their maintenance therapy with inhaled corticosteroids and inhaled salbutamol as needed, with the stipulation that the drug was not to be used for at least 6 h before the challenge procedure. In addition, aminophylline and long-acting β_2-agonists were withheld for at least 48 h before the challenge. Patients who had taken systemic antihistamines, systemic corticosteroids, cromolyn, oral sympathomimetics or β-blocker drugs within the last week were excluded.

From among the 120 patients reviewed, 30 patients who agreed to take part in the study were included. According to the results of ASA provocation tests, the patients were divided into 2 groups: patients with asthma and/or nasal polyps with ASA hypersensitivity (ASA sensitive, n = 18), and those without ASA sensitivity (ASA tolerant, n = 12). Healthy controls (n = 12) were recruited from hospital staff who denied any acute or chronic illnesses, and all of whom had normal physical examination findings. They reported using ASA/NSAID without any apparent reactions, and therefore were not challenged with ASA.

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.

Aspirin Provocation

The oral ASA provocation test was performed according to Nizankowska et al. [15] with responses classified as negative, upper airway reaction, classical reaction or systemic reaction. Upper airway reaction was defined as the presence of rhinorrhea and nasal congestion; classical reaction as more than a 20% decrease in FEV_1 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 extra-bronchial symptom was scored as follows: 0 = no symptom; 1 = mild symptoms; 2 = moderate symptoms; 3 = severe symptoms; 4 = very severe symptoms. These 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 [15].

On the first day of ASA provocation, 3 capsules of placebo were administered every 2 h. FEV_1 was measured every 30 min and if FEV_1 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. FEV_1 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 FEV_1 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 FEV_1 (PD_{20}), was calculated as the PD_{20} ASA oral. The lowest FEV_1 of the 3 measurements (at 30, 60 and 120 min) obtained after each dose was plotted against the log 10 cumulated dose of ASA in order to obtain a dose-response curve. The PD_{20} was derived by linear interpolation from this dose-response curve [15].

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 and *Alternaria* and *Cladosporium* antigens. Positive (histamine: 10 mg/ml) and negative (saline solution) controls were also included. The puncture method with a 1-mm-tipped disposable lancet was used, and a wheal reaction with a mean diameter of 3 mm greater than the negative control was considered a positive response.

In-vitro Basophil Activation

Basophil Isolation. To begin, 6 ml of venous blood samples were drawn into ACD (acid citrate dextrose) tubes (Vacutainer, Becton Dickinson, Plymouth, UK) containing 10 mM EDTA to prevent coagulation. Basophils were obtained either from unmanipulated whole blood in the allergenicity kit or leukocytes isolated by density gradient sedimentation using Ficoll separating solution (Biocoll-Biochrom, Berlin, Germany) in the Flow-CAST™ study.

Flow-Cytometry-Based Basophil Activation Test. All the tests were carried out within 2 h of blood sampling. CD63-induced expression was evaluated using the Flow-CAST Basophil Activation Test (Bühlmann Laboratories, Schönenbuch, Switzerland) according to the manufacturer's instructions [10]. Briefly, after cell separation, 50 μl of the patient's cell suspensions were incubated with allergens [50 μl ASA (lysine acetylate, Bühlmann Laboratories) at final concentrations of 2.5 and 0.5 mg/ml, and diclofenac (Bühlmann Laboratories) at 0.025 and 0.005 mg/ml] and negative and positive controls in corresponding pyrogen-free polypropylene tubes. For basal values (negative control), 50 μl of stimulation buffer (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, $CaCI_2$ 7 mM, $MgCI_2$ 3.5 mM, bovine serum albumin 1 mg/ml, pH 7.4) containing IL-3 (2 ng/ml) and 10 μl heparin (5,000 IU/ml: ROVI, Madrid, Spain) was used. As positive control, 50 μl of a monoclonal anti-IgE receptor (FcεRI) antibody (Bühlmann Laboratories) at a final concentration of 1 μg/ml was used. Tubes were then vortexed gently, and incubated at 37°C in a water bath for 40 min. The reaction was halted by adding 50 μl of pre-cooled blocking buffer [HEPES buffer pH 7.3 exclusive of calcium and magnesium, but containing EDTA (HEPES 20 mM, NACl 133 mM, KCl 5 mM, EDTA 0.27 mM)] to each tube. The basophils from the pellet were then double labeled by adding 20 μl of anti-CD63 PE-labeled antibody (Caltag Laboratories, Burlingame, Calif., USA) and anti-IgE FITC monoclonal antibodies (Caltag Laboratories), and then incubated for 30 min at 4°C with protection from light exposure. Tubes were then vortexed gently and incubated at room temperature for another 5 min with 3.5 ml of pre-warmed lysing reagent, after which the reaction was blocked by 1 ml blocking buffer. After centrifuging for a further 5 min at 1,000 *g*, the supernatant was decanted and 500 μl of blocking buffer was added to each tube and vortexed gently. All tubes were processed by flow cytometric analysis within 2 h. Dual color staining (CD63 PE and anti-IgE FITC) of surface markers was performed at 488 nm on a Cytomics FC 500 Flowcytometer (Beckman Coulter, Marseille, France) equipped with an argon laser, and analyzed by CXP 2.2 software. On the histogram defined by forward scatter and side scatter, the initial cell gate was mapped around lymphocytes. The second gate was defined around cells showing high-density anti-IgE, identifying them as basophils. At least 500 basophils were assessed in each assay.

CD203-induced expression was evaluated using an allergenicity kit (Cellular Analysis of Allergy, Beckman Coulter) according to the manufacturer's instructions. So, 100 μl of the patient's venous blood was incubated with 20 μl of NSAID drugs, such as ASA (lysine-ASA, Bühlmann Laboratories; final concentrations of 2.5 and 0.5 mg/ml) and diclofenac (Bühlmann Laboratories; final concentrations of 0.025 and 0.005 mg/ml), in polypropylene tubes. In order to evaluate the background basal values without stimulation (negative control), we added 20 μl of PBS (Sigma Diagnostics, St. Louis, Mo., USA) to whole blood in another tube. As positive control, we used 20 μl of a monoclonal anti-IgE receptor (FcεRI) antibody; 20 μl of CRTH-2 (clone: BM16)-FITC/CD203c (clone: 97)-PE/CD3 (clone: UCHT-1)-PC7 monoclonal antibodies, and 100 μl of activation solution containing calcium was added and incubated at 37°C in a water bath for 15 min. The reaction was halted by adding 100 μl of stop solution. Tubes were then incubated for another 10 min for lyses at room temperature. Three-color staining CRTH-2-FITC/CD203c-PE/CD3-PC7 (Beckman Coulter) of surface markers was performed at 488 nm on a FC 500 Flowcytometer (Beckman Coulter) equipped with an argon laser and analyzed by CXP 2.2 software. On the histogram defined by

Table 1. Characteristics of the study population

	ASA sensitive (n = 18)	ASA tolerant (n = 12)	Healthy control (n = 12)	p
Gender				
Female	14 (78)	7 (58)	8 (67)	>0.05
Male	4 (22)	5 (42)	4 (33)	
Age, years	36.3 ± 13.4	41.8 ± 5.2	32.3 ± 7.8	0.03*
Skin prick test for aeroallergens				
Positive	6 (33)	7 (58)		
Negative	12 (67)	5 (42)	12 (100)	0.009*
Nasal polyp only	1 (6)	4 (33)	–	n.s.
Nasal polyp + asthma	17 (94)	6 (50)	–	n.s.
Drug reaction		2 (17)	–	n.s.

Figures in parentheses are percentages. Ages are given as means ± SD. * Healthy controls were significantly different from ASA-sensitive and ASA-tolerant patients.

forward scatter and side scatter, the initial gate was mapped around all the peripheral blood cells. The second gate was defined as cells that did not express CD3. Cells expressing both CRTH-2-FITC and CD203c-PE were identified as basophils. At least 500 basophils were assessed in each assay.

Evaluation of the Results

Results were considered as positive for both assays when the absolute percentage of basophils expressing CD63 or CD203c after contact with lysine-ASA/diclofenac was equal or higher than 5%, and the stimulation index (calculated as the ratio between percentage of basophils expressing CD63 or CD203c to lysine-ASA/diclofenac stimulation and percentage of basophils expressing CD63 or CD203c to negative) was higher than 2 [7, 16].

Allergen Preparation

Allergens [lysine-ASA (BAG2-C51) and diclofenac (BAG2-C52); Bühlmann Laboratories] were selected for the trial through the recommendations of a recent study by Sanz et al. [16], since the combination of ASA and diclofenac yielded the highest sensitivity and specificity among the 4 NSAID tested. Two concentrations of each drug, lysine-ASA (A: 2.5 mg/ml and B: 0.5 mg/ml) and diclofenac (A: 0.025 mg/ml and B: 0.005 mg/ml) were prepared daily according to the instructions of the producer company via resuspension of the lyophilized drug with 250 μl stimulation buffer (concentration A), and dilution of concentration A (50 μl) + 200 μl stimulation buffer (concentration B).

Statistical Analysis

Statistical analysis was performed using SPSS 15.0 for Windows (SPSS, Chicago, Ill., USA). Differences among ASA-sensitive patients, ASA-tolerant patients and healthy controls were evaluated by Kruskal-Wallis analysis of variance. When the p value from the Kruskal-Wallis test statistics was statistically significant, multiple comparison tests were used to discern which groups differed from which others [17]. Nominal values were expressed as numerals and percentages, and numerical variables were expressed as medians and interquartiles, with the exception of age, which was expressed as the mean ± SD. Non-parametric tests were used due to the heterogeneity of the groups. Comparisons between 2 groups were evaluated by the Mann-Whitney U test for non-normally distributed continuous variables and Student's t test for normally distributed continuous variables. A p value of less than 0.05 was considered significant.

Sensitivity was defined as the proportion of CD63- and CD203c-positive subjects whose provocation tests were positive with oral ASA provocation. Specificity was defined as the proportion of CD63- and CD203c-negative subjects whose provocation tests were negative with oral ASA provocation. The positive predictive value was defined as the number of true positives/number of true positives + number of false positives. The negative predictive value was defined as the number of true negatives/number of true negatives + number of false negatives. The positive and negative predictive values were calculated considering the prevalence of ASA sensitive asthma, reported to be 11% in Turkey [18]. The positive likelihood ratio (LHR) was also defined as sensitivity/1 – specificity. LHR was classified as LHR > 10: important; 5 ≤ LHR ≤ 10: moderately important, 2 ≤ LHR ≤ 5: sometimes important and 1 ≤ LHR ≤ 2: unimportant.

Results

The characteristics of the study population are shown in table 1. During comparison of the groups, there were no difference with regards to demographic data, with the exception of the healthy controls who were significantly younger and nonatopic ($p < 0.03$ and $p < 0.009$, respectively).

The mean duration of drug reaction was 5 years [2, 10] among ASA-sensitive patients, except for 1 patient who had no previous history of sensitivity to ASA, but whose oral ASA provocation test result was positive with a classical reaction. The culprit drug was ASA alone in 12, other NSAID in 9, and metamizole along with ASA in 10 patients.

Oral ASA Provocation Test Results

Due to a clear-cut history with a life-threatening reaction to ASA/NSAID, oral ASA provocation was not performed in 2 of the patients. Of the remaining 16 patients, 12 had a classic response to oral ASA with a median PD_{20} ASA of 189 (108, 257) mg, and 3 patients had systemic reactions (extrapulmonary scores were 12, 13 and 13). Only 1 patient had an upper airway response. One patient, who initially appeared to be only intolerant to ASA without asthma or nasal polyp, later responded to oral ASA provocation with a classical reaction. During follow-up, she turned out to be asthmatic. Twelve patients

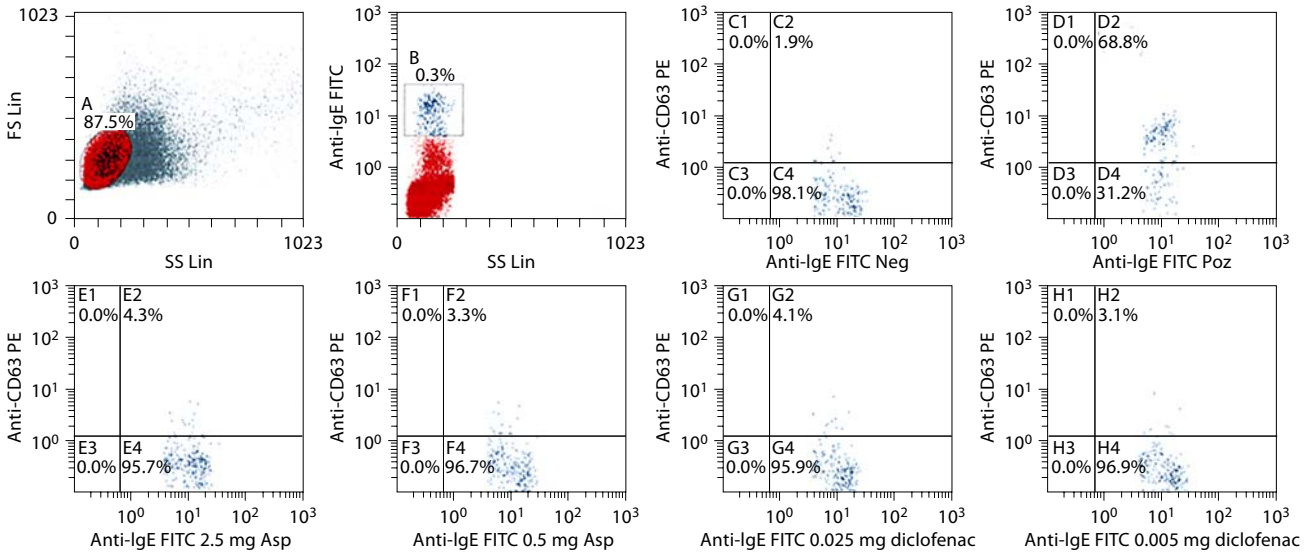

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Fig. 1. Expression of CD63 on basophils in a healthy control. Basophils were stimulated with a stimulation buffer containing IL-3 as negative control, with monoclonal anti-IgE receptor antibody as positive control, and lysine-ASA at final concentrations of 2.5 and 0.5 mg/ml, and diclofenac at final concentrations of 0.025 and 0.005 mg/ml as allergen stimulaters. FS Lin = Forward scatter (linear); SS Lin = side scatter (linear); Neg = negative; Poz = positive; Asp = aspirin.

completed the oral ASA provocation without any reactions, and were considered as ASA tolerant. Among them, 2 had a history of sensitivity to antibiotics and ASA/NSAID, and paracetamol without asthma and/or nasal polyp. None of the patients reacted to the oral placebo. The median duration between the time of oral ASA provocation and the time of flow cytometry was 11 months (interquartile range: 4–19 months, with a minimum of 4 months and a maximum 26 months between the time of oral ASA provocation and the time of flow cytometry, respectively). There was no statistically significant effect of the time interval on flow cytometry results.

Flow Cytometry

The median numbers of basophils recovered were 913 (range: 521–1,941), 603 (range: 539–1,640) and 687 (range: 504–2,960) in patients with ASA sensitivity, ASA tolerance and healthy controls, respectively. A significantly higher number of basophils were recovered by the allergenicity method as compared to Flow-CAST (1,076.9 vs. 896.6; p = 0.026).

The percentages of basophils expressing CD63 and CD203c to negative control stimulation varied between 0.1 and 3.5% (patients) and 0.2 and 2.7% (healthy controls), with the exception of 1 healthy control who had 9.5% expressing CD63. For patients and healthy controls, the percentages of basophils expressing CD63 and CD203c to positive control stimulation varied between 7.4 and 85.4% (patients), and 7.0 and 93.3% (controls) (fig. 1, 2). There were 3 subjects (7% of the group) with CD63 expression to the positive control ranging from 1.7 to 8.6%, and all demonstrated >15% of CD203c expression to positive control stimulation. Similarly, there were 2 subjects (4% of the group) with a CD203c expression to the positive control ranging from 7.0 to 9.6% and exhibiting >15% of CD63 expression to positive control stimulation. They therefore were not considered as true non-responders. CD63 expression to negative and positive control stimulation was comparable among patients and healthy controls. This was also the case for CD203c expression to positive control; however, there was a significant difference between ASA-sensitive and ASA-tolerant patients (p = 0.002), and ASA-sensitive patients and healthy controls (p = 0.002) for CD203c expression to the negative control (p = 0.002). Furthermore, all patients and healthy controls demonstrated a comparable per-

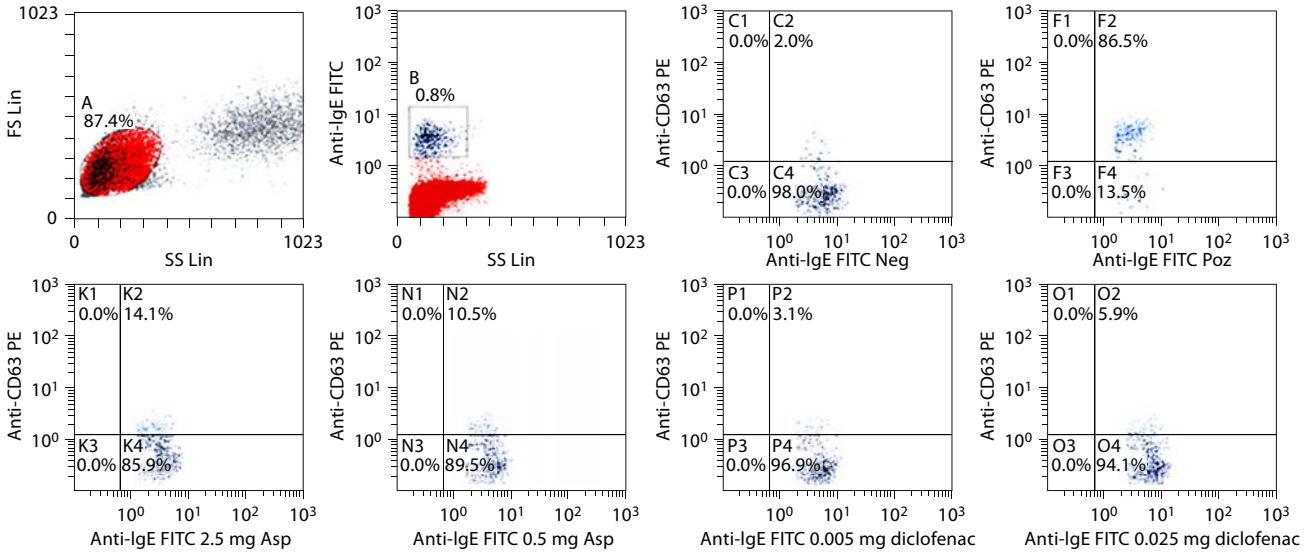

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Fig. 2. Expression of CD63 on basophils in a patient with ASA intolerance. Basophils were stimulated with stimulation buffer containing IL-3 as negative control, with monoclonal anti-IgE receptor antibody as positive control, and lysine-ASA at final concentrations of 2.5 and 0.5 mg/ml, and diclofenac at final concentrations of 0.025 and 0.005 mg/ml as allergens stimulation. FS Lin = Forward scatter (linear); SS Lin = side scatter (linear); Neg = negative control; Poz = positive control; Asp = aspirin.

centage of CD63 and CD203c expression to stimulation with 2 concentrations of ASA and diclofenac (data not shown).

According to the positivity criteria defined, the results of CD63 and CD203c measurements were compared between ASA-sensitive and ASA-tolerant individuals plus healthy controls, and the sensitivity and specificity of these assays were measured against the results of the oral ASA provocation testing. The diagnostic values of CD63 and CD203c measurements for A and B concentrations of ASA and diclofenac are detailed in table 2. In general, although a lower concentration of ASA yields equal sensitivity but higher specificity for CD63 expression than a higher concentration of ASA, the lower concentration of diclofenac did not detect any patient with ASA sensitivity. In terms of the CD203c molecule, 2 concentrations of ASA yield almost the same sensitivity and specificity, but the sensitivity is lower with a lower concentration of diclofenac, without showing any significant changes in specificity.

Neither the addition of CD203c to CD63, nor the addition of diclofenac improves the overall sensitivity and specificity of CD63 to ASA (data not shown). The positive likelihood ratio was calculated to be below 2 for all sensitivity and specificity values for both of the drugs and molecules, and thus the data is not shown.

Relationship between Oral ASA Provocation Test and Flow Cytometry Results

The distribution of CD63 and CD203c expressions according to the type of response to oral ASA provocation are summarized in table 3. Patients with either a systemic or classic response to ASA provocation had a higher number of activated basophils than patients with a nasal response to ASA provocation. However, we did not evaluate the correlation between the severity of the reaction and the basophil activation since the number of patients in the different groups of reaction to ASA provocation was quite small (fig. 3, 4).

Discussion

In this study, we established the sensitivity of CD63 measurement for ASA to be 33.3%, the specificity to be 75–79%, the positive predictive value to be 14.1–16.5%

Table 2. Diagnostic value of CD63, CD203c expression and both according to the positivity criteria (expression of related molecules >5%; stimulation index >2)

	CD63 (Flow-CAST)	CD203c (allergenicity)	CD63 and CD203c (Flow-CAST and allergenicity)
ASA (2.5 mg/ml)			
Sensitivity	33.3	16.7	33.3
Specificity	75	100	75
PPV	14.1	100	14.1
NPV	90.1	90.7	90.1
ASA (0.5 mg/ml)			
Sensitivity	33.3	11.1	33.3
Specificity	79.2	100	79.2
PPV	16.5	100	16.5
NPV	90.6	90.1	90.6
Diclofenac (0.025 mg/ml)			
Sensitivity	16.7	22.2	22.2
Specificity	83.3	100	83.3
PPV	11.0	100	14.1
NPV	89.0	91.2	89.7
Diclofenac (0.005 mg/ml)			
Sensitivity	0	11.1	11.1
Specificity	91.7	100	91.7
PPV	0	100	14.1
NPV	88.1	90.1	89.3

PPV = Positive predictive value; NPV = negative predictive value.

Table 3. CD63 and CD203c expressions in patients with a positive response to oral ASA provocation

Oral ASA provocation	CD63 %	CD203c %
In vitro lys-ASA stimulation (2.5 mg)		
Systemic reaction (n = 3)	3.6–22.4	3.4–20.3
Nasal response (n = 1)	2.8	2.5
Classic response (n = 12)	1.0–24.1	1.2–23.2
In vitro diclofenac stimulation (0.025 mg)		
Systemic reaction (n = 3)	2.4–7.5	2.3–15.6
Nasal response (n = 1)	1.7	1.9
Classic response (n = 12)	1.1–6.6	1.1–15.6

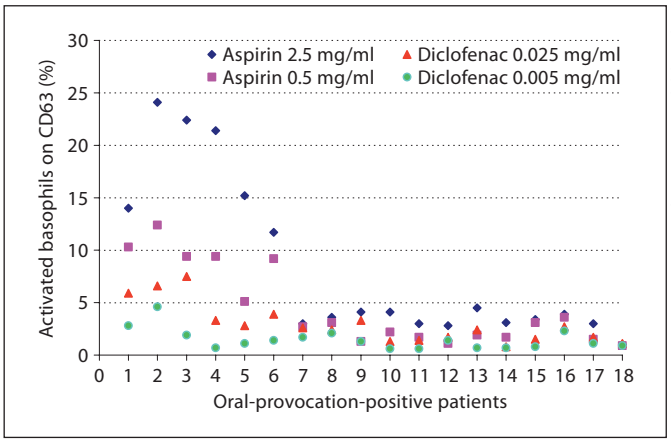

Fig. 3. Individual basophil activation test results for CD63 in a patient with a positive oral ASA provocation test. Patients 1, 3 and 8 had a systemic response; patient 12 had a nasal response; the others had a classic response.

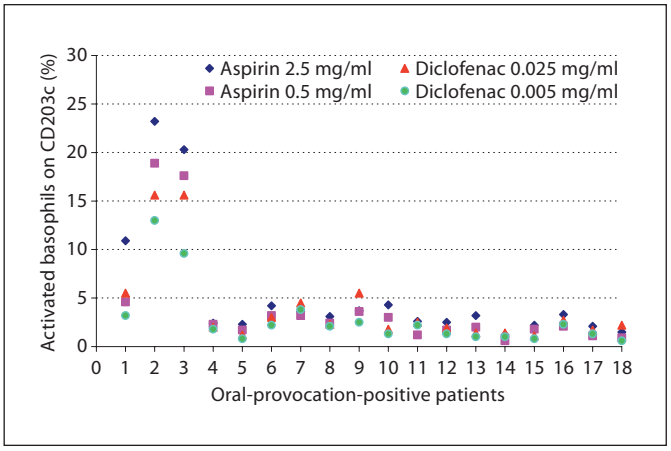

Fig. 4. Individual basophil activation test results for CD203c in a patient with positive oral ASA provocation test. Patients 1, 3 and 8 had a systemic response; patient 12 had a nasal response; the others had a classic response.

and negative predictive value to be 90.1–90.6%. The sensitivity of CD203c measurement for ASA was 11–16.7%, the specificity was 100%, the positive predictive value was 100% and the negative predictive value was 90.1–90.7%. Neither the measurement of CD203c as a second basophil activation marker, nor the use of diclofenac as a second NSAID to stimulate basophils, improved the overall sensitivity or specificity of CD63 measurement for ASA. We can therefore suggest that the use of flow cytometric analysis of in vitro basophil activation with CD63 or CD203c either alone or in combination does not seem to be useful in the diagnosis of ASA sensitivity. However, despite the low number of patients on which we based our research limiting the strength of our statistical analysis,

in this subject of significant controversy, we do believe that our study provides additional data specifically because almost all the patients had ASA provocation.

The usefulness of flow cytometry is mostly dependent on the allergen type. A number of studies have already demonstrated that flow cytometric basophil activation via CD63 expression is a highly reliable procedure for the diagnosis of IgE-mediated allergic reactions to inhalant or food allergens, latex and venom, with a high sensitivity and specificity [8, 10, 11, 19–21]. However, the clinical reliability of flow cytometry for the diagnosis of drug allergy remains to be established [8, 11, 19, 22]. Trials with neuromuscular blocking agents [12, 23] and β-lactams [13, 24] indicated that the measurement of CD63 is promising and complementary to the measurement of specific IgE in patients with IgE-mediated reactions to these drugs.

Studies concerning the validity of flow cytometry in the diagnosis of ASA sensitivity are even scarcer [25, 26]. Following the preliminary reports, the first comprehensive study appeared with reference to a group of exclusively metamizole-allergic patients with positive skin tests to metamizole [27]. The diagnostic reliability of CD63 expression has been compared with the measurement of leukotriene release and specific IgE to metamizole, and the sensitivity and the specificity of the technique have been reported to be 42.3 and 100%, respectively. Two studies from the same group have assessed the usefulness of the CD63 molecule in the in vitro diagnosis of ASA/NSAID sensitivity [16, 28]. In these studies, 60 ASA/NSAID-sensitive patients, 20 of whom had asthma, were considered and the diagnosis of ASA sensitivity was based on the history of these patients. The CD63 measurement demonstrated a sensitivity of 43.3%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 99.4% for ASA. The global sensitivity increased to 66.7%, while specificity remained at 93.3% upon consideration of the 4 NSAID, including paracetamol, metamizole and diclofenac collectively. More recently, a large multicenter study conducted in 8 centers reported that the sensitivity of CD63 expression varies between 43 and 90%, and the specificity between 42 and 92%. The study group concluded that both precise dose-response curves and a standardized technique for leukocyte isolation are required to improve the diagnostic reliability of the assay [29]. In our trial, the measurement of CD63 in challenge-proved ASA-sensitive patients with asthma/nasal polyp resulted in the lowest sensitivity reported so far, but a comparable specificity with that of the multicenter study.

In recent years, CD203c, being a novel basophil activation marker, has received increasing attention and has been evaluated for in vitro allergy diagnosis. In vitro activation of basophils assessed by CD203c has some advantages compared with CD63 assessment. It should be noted, however, that the presence of cells other than basophils expressing CD63 may interfere with the test results. Nevertheless, the expression of CD203c is restricted to basophils, and thus flow cytometric analysis of CD203c does not require the use of anti-IgE to identify basophils [8, 11, 19, 21].

Until now, only a few studies have compared the diagnostic usefulness of allergen-induced CD63 and CD203c molecules [18, 29, 30]. A significant correlation between recombinant pollen-allergen-induced CD63 and CD203c upregulation in patients with a pollen allergy has been reported [31]. This data was further supported by the demonstration of a marked improvement in the basophil activation test through the use of CD203c instead of CD63 in patients with IgE-mediated reactions to latex [32]. This conclusion has, however, been criticized by some groups [19, 33].

To the best of our knowledge, there has been no prior study evaluating CD203c as a basophil activation marker in the diagnosis of drug intolerance encompassing ASA. In our study, the measurement of CD203c yielded less sensitivity but perfect specificity for both ASA and diclofenac compared to the CD63 measurement. We applied the same concentrations of ASA and diclofenac used in the CD63 measurement for quantification of CD203c, which was the major limitation of our study. These doses may not be appropriate for CD203c upregulation, and optimal allergen concentration may vary from one protocol to another [8]. A dose-dependent increase in the expression of CD203c has been demonstrated in pollen and venom sensitivity [31]. Considering the perfect specificity obtained from CD203c measurement for ASA and diclofenac, further dose-ranging studies should be conducted to see whether a higher drug concentration would yield better sensitivity.

Recently, the ability of diclofenac to induce CD63 expression was tested by flow cytometry and basophil degranulation using light microscopy in diclofenac-sensitive patients [34]. Diclofenac activated basophils in a way that did not induce CD63 expression, but rather promoted basophil degranulation, as concluded by the disappearance of metachromatic-stained cytoplasmic granules. This observation supports previous data concerning 2 different degranulation mechanisms for human basophils [35, 36]. The first is IgE-mediated anaphylactic de-

granulation, and the second is piecemeal degranulation which defines the release of granule contents in the absence of any CD63 expression. Considering the diagnostic superiority of flow cytometry in IgE-mediated reactions in comparison to non-IgE-mediated reactions, as in the case of ASA sensitivity, it may be speculated that ASA or other NSAID may activate basophils in a way that is different from IgE-mediated events. Further studies using flow cytometric and the microscopic evaluation of basophils after in vitro stimulation with these drugs are needed to clarify this issue.

In conclusion, in our view, flow cytometric analysis of basophil activation, be it detected by CD63 or CD203c expression alone or collectively, constitutes an insufficient diagnostic test for the diagnosis of ASA hypersensitivity.

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