

Upregulation of CD11b on Eosinophils in Aspirin Induced Asthma

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ABSTRACT

Background: Although a challenge test using non-steroidal anti-inflammatory drugs (NSAIDs) is crucial for diagnosis of aspirin-induced asthma (AIA), it also has drawbacks in terms of possible side effects. Therefore, alternative *in-vitro* diagnostic methods for AIA are awaited.

Methods: Nineteen stable non-AIA patients (9 males and 10 females; mean age, 49.4 ± 4.8 years), and 20 AIA patients (9 males and 11 females; mean age, 51.1 ± 4.8 years) were enrolled in this study. CD11b and CD16 expressions on the peripheral-blood granulocytes after administration of aspirin and different concentrations of PGE₂ *in vitro* were examined using flowcytometry.

Results: Aspirin induced a significant increase in CD11b expression on eosinophils (CD16 negative granulocytes) in 19 AIA patients and one non-AIA patient. Increase in CD11b expression on eosinophils by aspirin administration was suppressed by PGE₂ in a dose-dependent manner.

Conclusions: The measurement of CD11b expression on peripheral-blood eosinophils showed very high sensitivity and specificity of (-95%) in diagnosing AIA. Although this method requires laboratory facilities for flowcytometry, it may be very useful in diagnosis of AIA without side effects. In addition, PGE₂ may be involved in regulation of CD11b expression on eosinophils by aspirin administration.

KEY WORDS

adhesion molecule, aspirin, asthma, CD11b, eosinophils

INTRODUCTION

In patients with AIA, asthma attacks are induced by almost all NSAIDs having aspirin-like effects. Cyclooxygenase (COX) inhibition, which is a pharmacological action common to these NSAIDs, is considered to trigger this hypersensitivity reaction to aspirin. In other words, AIA is thought to accompany some abnormalities in the biological reaction associated with the pathway of arachidonic acid metabolism or arachidonic acid metabolite, and such abnormalities become obvious after administration of NSAIDs and manifest as hypersensitivity reactions.

Although a challenge test using NSAIDs is essential for the definitive diagnosis of AIA, it has problems both in terms of the diagnostic sensitivity and specificity and is associated with risks. Therefore, methods for *in vitro* diagnosis of AIA have been sought.

Several attempts have been made to develop *in vitro* diagnosis methods for AIA, and approaches based on peptide leukotriene (pLT) production, 15-hydroxy-eicosatetraenoic (HETE) production,^{1,2} and lipoxin production³ in peripheral blood polymorphonuclear leukocytes (PMNs) as indicators have been examined. However, adequate follow-up and verification have not been conducted, and their usefulness remains questionable.

Eosinophils are inflammatory cells that play central roles in the onset and pathology of AIA and are known to be activated by the administration of aspirin in AIA patients.⁴ If a reaction specific to AIA could be detected at the level of the peripheral blood eosinophils, it may be useful for the diagnosis and clinical research of AIA. Therefore, the potential usefulness of a method using the adhesion molecule CD11b expressed on the cell surface of eosinophils as a marker

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for the *in vitro* diagnosis of AIA was examined in this study.

METHODS

SUBJECTS

For the basic examination, 8 non-AIA patients (3 males and 5 females; mean age, 41.3 ± 16.0 years) and 8 AIA patients (4 males and 4 females; mean age, 42.9 ± 10.1 years) who were being treated at the outpatient unit of our hospital and whose symptoms were stable (mild or moderate by GINA [Global Initiative for Asthma] classification) at the time of enrollment in the study were selected as the subjects. The characteristics and background of AIA and non-AIA patients who participated in this study were shown in Table 1. All patients in this study were diagnosed as asthma because of common asthmatic symptoms and pulmonary function indicating the reversibility of airflow limitation, i.e.; 1) Post-bronchodilator improvement of $\geq 12\%$ and ≥ 200 ml in forced expiratory volume in 1 second (FEV_{1.0}) from the baseline, or 2) $>20\%$ increase in FEV_{1.0} following 2 weeks of treatment with inhaled or systemic corticosteroids. For the diagnosis of AIA, we used inhalation provocation test. Increasing doses of tolmetin and sulpyrine in geometric progression (tolmetin; 0.05, 0.5, 5.0, 50 mg/ml and sulpyrine; 1.0, 10, 100 mg/ml) were administered patients by inhalation from a dosimeter-controlled jet nebulizer. The provocation test was considered positive and stopped when FEV_{1.0} had fallen $>20\%$ from the post-saline baseline value. For examination of the *in vitro* diagnosis method, 19 non-AIA patients (9 males and 10 females; mean age, 49.4 ± 4.8 years) and 20 AIA patients (9 males and 11 females; mean age, 51.1 ± 4.8 years) who were being treated at the outpatient unit of our hospital and whose symptoms were stable at the time of enrollment in this study were selected as the subjects.

The significance of this study was explained to all the subjects, and informed consent was obtained from each of them. Then, basic pulmonary function tests and general hematology tests were conducted, and peripheral venous blood samples were collected. All the subjects were subjected to tolmetin and sulpyrine inhalation challenge tests for diagnosis of hypersensitivity to NSAIDs. Patients who were assessed as showing positive results in these inhalation challenge tests and who also had a history of NSAID-induced asthma attacks were regarded as having AIA. Patients who showed negative on all these tests were regarded as non-AIA.

Isolation of Peripheral Blood Eosinophils

Approximately 20 ml of blood was collected from a peripheral vein of the target patients, and PMNs were isolated from the blood by the specific gravity centrifuge method using mono-poly resolving medium

Table 1 Patient characteristics of the patients in this study

	AIA	non-AIA	
<i>n</i>	20	19	
Sex (M/F)	9/11	9/10	n.s.
Age (mean [SD])	51.1 (4.8)	49.4 (4.8)	n.s.
Atopic (YES/NO)	7/13	8/11	n.s.
WBC (/mm ³ ; mean [SD])	6400 (1300)	6800 (1900)	n.s.
Eosinophil (%; mean [SD])	8.3 (5.2)	6.7 (5.0)	n.s.
Severity (mild/moderate)	7/13	8/11	n.s.
Therapy			
ICS (μg; mean [SD])	350 (171)	310 (112)	n.s.
LABA (<i>n</i>)	6	5	n.s.
Theophylline (<i>n</i>)	7	9	n.s.
LTRA (<i>n</i>)	11	7	n.s.

n.s., not significant; ICS, inhaled corticosteroid; LABA, long acting beta agonist; LTRA, leukotriene receptor antagonist.

(Dainippon Pharmaceutical, Osaka, Japan). The cells were washed with phosphate-buffered saline (PBS), and the cell count was adjusted to 2×10^6 /mL with RPMI1640 culture solution containing 10% fetal bovine serum (FBS) (Sigma, St Louis, MO, USA) (Fig. 1).

Measurement of CD11b Expression

The suspension of isolated PMNs was stained for 45 minutes with fluorescein isothiocyanate (FITC)-labeled CD11b monoclonal antibody and phycoerythrin (PE)-labeled CD16 monoclonal antibody while being kept cool with ice. Then, the sample was immobilized with 1% paraformaldehyde. Thereafter, the eosinophil fraction (CD16-negative) and neutrophil fraction (CD16-positive) were separated using flowcytometer (FACScan, Becton Dickinson, NJ, USA) based on the presence/absence of CD16 expression, and the CD11b expression level was determined by mean fluorescence intensity (MFI). To investigate the effect of aspirin, aspirin was added to a suspension of the isolated PMNs at a concentration of 10^{-4} M- 10^{-7} M, followed by double stain for CD11b and CD16. Furthermore, the effect of addition of prostaglandin E₂ (PGE₂) (10^{-7} M- 10^{-5} M) on the aspirin-induced change of CD11b expression level was also examined. A sample of the non-stimulated cell suspension to which the vehicle alone was added was used as the control.

Ethical Considerations

As described above, the subjects were enrolled after the objectives, method, and significance of the study had been clearly explained and informed consent had been obtained and this study was approved by the ethics committee of Fujita Health University. The sulpyrine and tolmetin inhalation challenge tests were conducted to diagnose hypersensitivity to

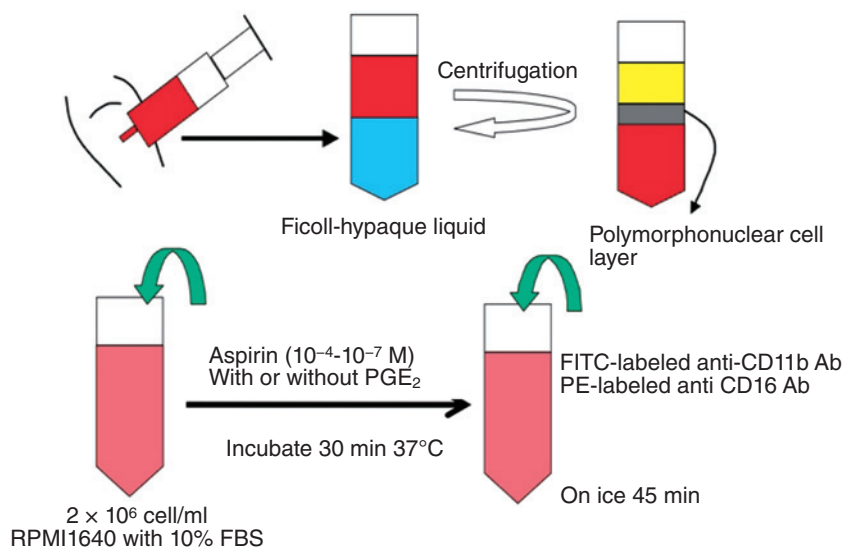


Fig. 1 A scheme of experimental procedures. Approximately 20 ml of peripheral blood was collected from each patient, and polymorphonuclear leukocytes (PMNs) were isolated by the specific gravity centrifuge method. Aspirin with or without PGE₂ was added to a suspension of the isolated PMNs. After centrifugation followed by re-suspension with PBS, the sample was stained with FITC-labeled anti-CD11b monoclonal antibody and PE-labeled anti-CD16 monoclonal antibody.

NSAIDs. These tests are routinely conducted in patients with bronchial asthma at our department, and therefore, were considered as being within the scope of routine clinical practice.

Statistics

All but a few figures were expressed as mean $\pm$ S.E. Concerning the results of measurements by flowcytometry, the value in the control was regarded as 100%, and the change rates were calculated according to the equation; change rate = (target MFI - control MFI)/control MFI. Mann-Whitney's *U* test was used for the statistical processing, and the level of significance was set at $p < 0.05$.

RESULTS

COMPARISON OF THE BASIC EXPRESSION LEVELS OF EOSINOPHIL CD11b IN THE NON-STIMULATED STATE

Flowcytometric analysis of PMNs with CD16 antibody clearly demonstrated two distinct cell populations (neutrophils as CD16-positive and eosinophils as CD16-negative) (Fig. 2a). Thus, we could identify the eosinophils from PMNs by gating for CD16 negative cell population (Fig. 2b) and used them in the following experiments. In this study, we analyzed the surface expression of CD11b quantitatively by fluorescence intensity. MFI representing CD11b expression in the non-stimulated state was 147.3 ± 12.1 in the non-AIA group and 130.4 ± 4.7 in the AIA group; the difference between the two groups was not sig-

nificant (Fig. 3).

EFFECTS OF ASPIRIN ADDITION ON THE CD11b EXPRESSION LEVEL

We investigated the effect of aspirin addition on the CD11b surface expression on eosinophils evaluated quantitatively by fluorescence intensity. The change of CD11b expression level from the baseline was $-5.7 \pm 3.2\%$ in the non-AIA group and $6.1 \pm 3.1\%$ in the AIA group after addition of 10^{-7} M aspirin, $-3.5 \pm 2.2\%$ in the non-AIA group and $9.9 \pm 3.1\%$ in the AIA group after addition of 10^{-6} M aspirin, $-1.2 \pm 2.2\%$ in the non-AIA group and $7.3 \pm 3.1\%$ in the AIA group after addition of 10^{-5} M aspirin, and $-8.0 \pm 2.6\%$ in the non-AIA group and $7.7 \pm 2.8\%$ in the AIA group after addition of 10^{-4} M aspirin (Fig. 4). These results demonstrated that CD11b expression on eosinophils from AIA patients was significantly increased from the baseline by aspirin stimulation ($p < 0.05$). However, intriguingly, aspirin stimulation significantly decreased the CD11b on eosinophils from non-AIA patients. A representative flowcytometric data of aspirin-induced enhancement of CD11b on eosinophils from an AIA patient was shown in Figure 2c, d.

EFFECTS OF PGE₂ ADDITION ON THE ASPIRIN-INDUCED INCREASE OF CD11b EXPRESSION

The increase of CD11b expression on the eosinophils induced by 10^{-4} M aspirin in the AIA group tended to be inhibited by the addition of PGE₂, in a concentration-dependent manner ($0.2 \pm 2.9\%$ at 10^{-7}

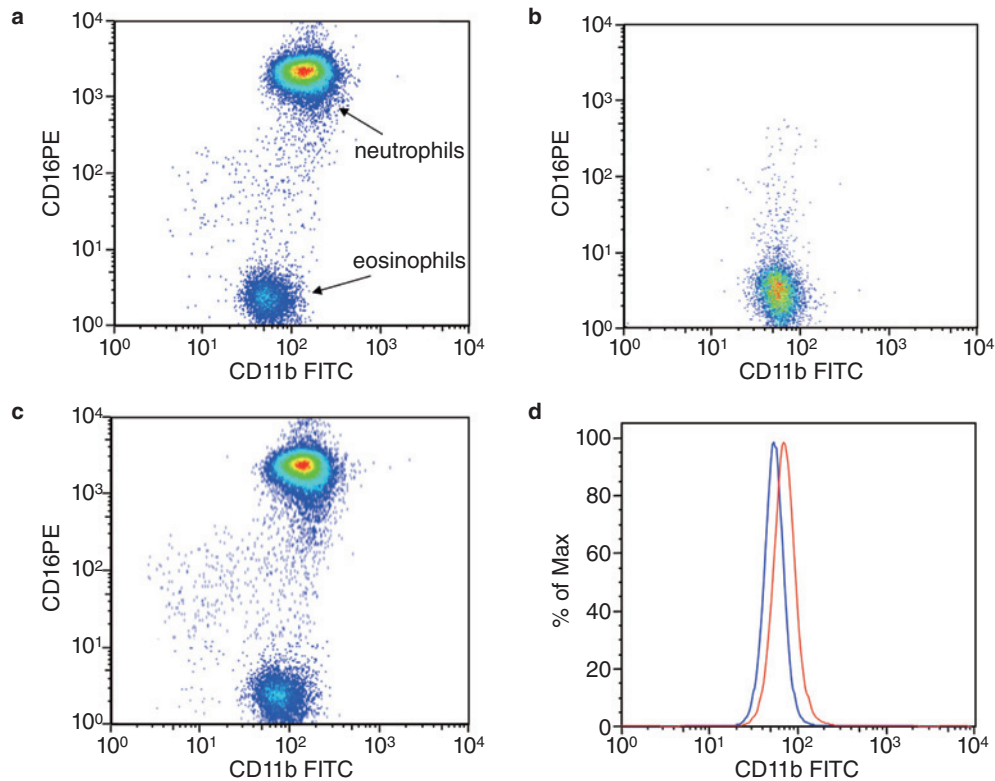


Fig. 2 Flowcytometric analysis of CD11b expression on eosinophils. PMNs from a non-AIA patient were double stained with anti-CD16 and anti-CD11b (a). The eosinophil fraction (CD16-negative) was separated from neutrophil fraction (CD16-positive) using flow cytometry (b). PMNs from an AIA patient under the aspirin stimulation were stained with anti-CD16 and anti-CD11b (c). The eosinophil fraction from an AIA patient increased CD11b expression by the stimulation of aspirin (10^{-4} M) (d). Data are representative of three independent experiments.

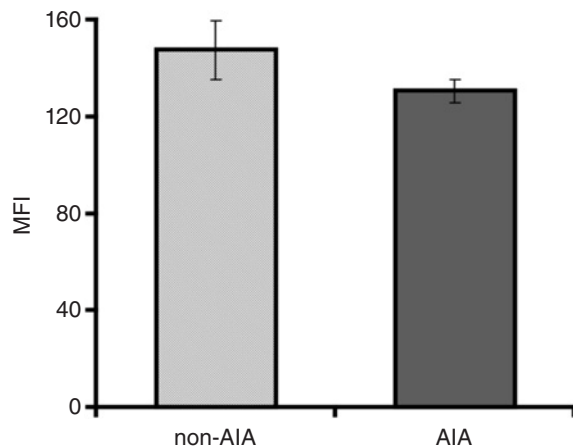


Fig. 3 Comparison of the CD11b expression on eosinophils in the aspirin non-stimulated condition (control) measured by mean fluorescence intensity (MFI) when stained with FITC-anti CD11b antibody. CD11b expression in the non-stimulated state was 147.3 ± 12.1 in the non-AIA group and 130.4 ± 4.7 in the AIA group; the difference between the 2 groups was not significant. Columns and vertical bars represent the mean $\pm$ SE.

M, $-0.6 \pm 2.9\%$ at 10^{-6} M, and $-3.5 \pm 4.0\%$ at 10^{-5} of PGE₂; a tendency toward a significant difference ($p = 0.07$) was seen after the addition of 10^{-5} M as compared with the value seen in the absence of addition of PGE₂ [$5.2 \pm 2.4\%$]. No obvious differences were observed in the non-AIA group (Fig. 5).

POSSIBILITY OF *in vitro* DIAGNOSIS OF AIA USING ASPIRIN-INDUCED CD11b EXPRESSION LEVEL ON THE PERIPHERAL BLOOD EOSINOPHILS AS AN INDICATOR

When cases showing increase of CD11b expression on the peripheral blood eosinophils after the addition of 10^{-4} M aspirin were regarded as positive, 19 out of 20 patients were assessed as positive in the AIA group (sensitivity 95.0%) and only 1 out of 19 patients was assessed as positive in the non-AIA group (specificity 94.7%) (Fig. 6).

DISCUSSION

AIA is an important condition that should always be borne in mind when treating patients with bronchial asthma. In regard to the mechanism of its onset, inhi-

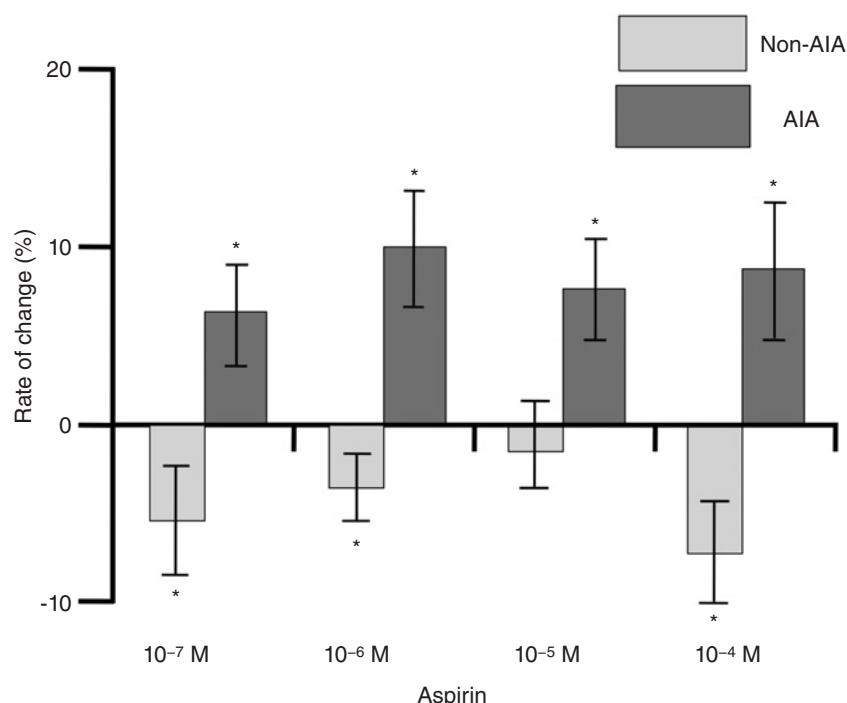


Fig. 4 Effects of aspirin (10^{-7} M to 10^{-4} M) on the CD11b expression of eosinophils. Eosinophils from AIA patients or non-AIA patients were stimulated with aspirin at various concentrations (10^{-4} M- 10^{-7} M), followed by assessment of CD11b expression by flowcytometer. With aspirin stimulation, CD11b expression level on eosinophils from AIA patients significantly increased from the baseline (non-stimulated condition), while it significantly decreased on the eosinophils from non-AIA patients. Columns and vertical bars represent the mean $\pm$ SE. * $p < 0.05$ compared to the non-stimulated baseline.

bition of COX in the pathway of arachidonic acid metabolism by NSAIDs is believed to play a major role. With regard to the arachidonic acid metabolites most closely involved in the pathology of AIA, there have been reports of elevated leukotriene E₄ (LTE₄) levels in the urine^{5,6} and elevated topical PGE₂ levels in the airways^{5,7} in AIA patients in a stable clinical state as compared with those in non-AIA patients. Also, following exposure to NSAIDs, the LTE₄ levels in the urine⁸⁻¹⁰ and topical pLT levels in the airways^{9,10} reportedly increase and the topical PGE₂ levels in the airways decrease significantly.⁹

The NSAID challenge tests for the diagnosis of AIA include systemic challenge tests and local challenge tests. The systemic challenge tests can be conducted by oral or intravenous administration. Being a systemic test, it is more reliable and symptoms outside of the airways can also be identified; however, the induced symptoms may become severe, increasing the risk levels of the tests.

The intravenous test can no longer be performed in Japan, because marketing of the reagent for the test, lysine-aspirin, has been discontinued here. Bronchial inhalation is used for local challenge tests; however,

this approach is not necessarily ideal because only local symptoms can be identified, and often, pseudo-negative results are obtained. The standard approach used at present is the oral aspirin challenge test.

However, challenge tests are associated with the risk of developing severe symptoms, and an *in vitro* method of diagnosis has been awaited. Several attempts have been made to develop methods for *in vitro* diagnosis of AIA, and approaches based on pLT production, 15-HETE production¹¹ and lipoxin production¹² in the peripheral blood PMNs as indicators have been examined. However, adequate follow-up and verification have not been conducted, and their usefulness remains questionable. Therefore, the potential usefulness of a method using the adhesion molecule CD11b expressed on the cell surface of eosinophils as a marker for the *in vitro* diagnosis of AIA was examined in this study.

CD11b is an adhesion molecule associated with local accumulation of eosinophils in the airways and is regarded as an indicator of activation of granulocytes. The expression level of this molecule increases following stimulation of the cells by various factors such as interleukin (IL)-4 and IL-5 and platelet activating

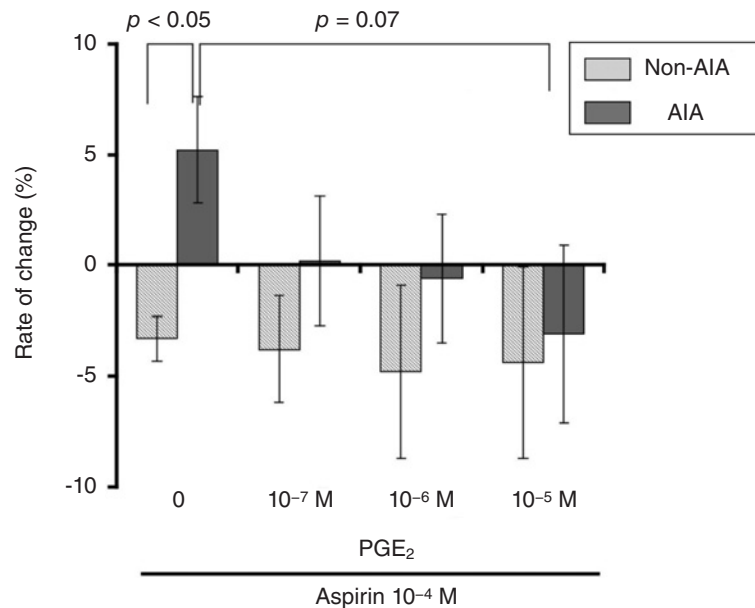


Fig. 5 Effects of PGE₂ on the CD11b expression on eosinophils induced by aspirin. The increase of CD11b expression induced by 10⁻⁴ M aspirin on the eosinophils from AIA patients was inhibited by the addition of PGE₂, in a concentration-dependent manner. No obvious differences were observed in the non-AIA group. Columns and vertical bars represent the mean $\pm$ SE.

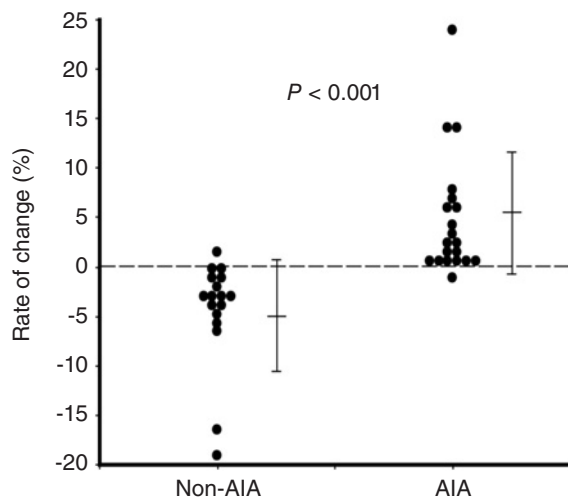


Fig. 6 Rate of changes in the CD11b expression induced by aspirin (10⁻⁴ M) on eosinophils may be useful for *in-vitro* diagnosis of AIA. When cases showing increase of CD11b expression of eosinophils by 10⁻⁴ M aspirin were regarded as positive, 19 out of 20 patients were assessed as positive in the AIA group (sensitivity 95.0%) and only 1 out of 19 patients was assessed as positive in the non-AIA group (specificity 94.7%). Bars indicate the mean $\pm$ SD.

factor (PAF).¹³⁻¹⁵ There have been no reports on the relationship between NSAID stimulation and CD11b expression on the eosinophils in AIA. Although stud-

ies on the gastrointestinal system have shown that aspirin and indomethacin up-regulated CD11b expression and/or increased its adhesion to the endothelial cells,^{16,17} the cells evaluated were neutrophils, and no examination of eosinophils has been conducted.

Measurement of CD11b expression by flowcytometry in this study enabled differentiation between an AIA group and non-AIA group. In other words, the CD11b expression level increased on the eosinophils of AIA patients after the addition of aspirin but decreased on the cells of the non-AIA patients, with a significant difference observed between the 2 groups. Moreover, the aspirin-induced increase of CD11b expression on the peripheral blood eosinophils of patients with AIA was no longer seen after the addition of PGE₂. These findings suggest that decrease of PGE₂ production may be involved in the aspirin-induced increase of CD11b expression on eosinophils.

When cases showing increased CD11b following exposure to NSAIDs were regarded as positive, 19 out of the 20 patients with AIA were assessed as positive (sensitivity 95.0%), whereas only 1 out of the 19 patients in the non-AIA group were assessed as positive (specificity 94.7%). For the diagnosis of AIA, it is inevitable at present to depend on the history and *in vivo* NSAIDs challenge tests. Although our method requires flowcytometry, it could become applicable as a method for *in vitro* diagnosis of AIA in the clinical setting if the procedure can be made simpler.

In conclusion, increase of CD11b expression on the surface of peripheral blood eosinophils of AIA patients was observed following the addition of aspirin. This may become applicable as a simple test in the clinical setting for *in vitro* diagnosis of AIA. Suppression of PGE₂ in AIA patients might be involved in regulation of CD11b expression on eosinophils by aspirin administration.

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