

Differential mediator release from basophils of allergic and non-allergic asthmatic patients after stimulation with anti-IgE and C5a

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Summary

Background The differentiation between allergic and non-allergic asthma is a common and important challenge for the clinician. Until now, no *in vitro* diagnostic characteristics have been described to distinguish between these types.

Objective To examine the diagnostic value of a basophil stimulation test, we compared anti-IgE- and C5a-induced mediator release from peripheral blood leucocytes in different types of bronchial asthma.

Methods Peripheral blood leucocytes (PBL) from 10 aspirin-sensitive asthmatics (ASA), 12 non-allergic asthmatics without aspirin intolerance (NAA), seven allergic asthmatics (AA), and nine healthy controls were prepared by dextran sedimentation. After priming with interleukin-3 (IL-3) PBL were stimulated with anti-IgE and C5a, and the release of histamine (HR) and sulfidoleukotrienes (LTR) in the supernatant was compared. Additionally, purified leucocyte fractions were studied to determine the cellular source of mediator release.

Results Upon stimulation with anti-IgE LTR was slightly, but not significantly, lower in ASA and NAA compared to AA and controls. In contrast, C5a-triggered LTR was significantly higher in ASA (14.4 ± 12.88 pg/ 10^5 cells) and NAA (22.9 ± 22.61 pg/ 10^5 cells) than in AA (9.6 ± 3.29 pg/ 10^5 cells) and controls (7.5 ± 7.19 pg/ 10^5 cells) ($P < 0.05$). This difference between ASA and NAA vs. AA and controls was even more pronounced when determining the quotient C5a-/anti-IgE-induced LTR ($P < 0.001$). At an optimal cut-off point of 1.0, calculated by relative operating characteristics (ROC) analysis, the positive predictive value for a donor to belong to ASA or NAA was 0.94. No significant differences could be found in HR between the asthmatic patient groups and healthy controls in either condition. As cellular source of LTR and HR the basophil could be determined.

Conclusion Determination of anti-IgE- and C5a-induced LTR from basophils allows us to discriminate between allergic and non-allergic asthmatic patients. For diagnostic purposes the quotient C5a-/anti-IgE-induced LTR is more significant than considering a single parameter. ASA cannot be distinguished from NAA.

Keywords: allergic asthma, non-allergic asthma, aspirin-sensitive asthma, basophil releasability, histamine, leukotrienes, anti-IgE, C5a, CAST-ELISA

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Introduction

Clinical experience with bronchial asthma advocates a division into an allergic (extrinsic) and a non-allergic (intrinsic) type of asthma. The differentiation is based on anamnestic characteristics and evidence of allergic sensitization being responsible for bronchospastic attacks [1,2]. Even though investigations point out immunological differences between allergic and non-allergic asthma [3], a clear diagnostic marker for differentiation does not exist.

Aspirin-sensitive asthma is a special type of bronchial asthma characterized by bronchospasm developing after ingestion of aspirin or other non-steroidal anti-inflammatory drugs. In aspirin-intolerant patients cutaneous or serological features indicating an allergic sensitization or specific immunoreaction towards aspirin could not be found [4,5]. Also other signs of an atopic diathesis are usually missing. Instead, aspirin-sensitive asthmatics, in addition to the intolerance reaction, often suffer from nasal polyps or chronic sinusitis (so-called aspirin-triad) and other symptoms placing them clinically among non-allergic asthma [4,5]. Numerous attempts to describe *in vitro* diagnostic characteristics of aspirin-sensitivity have not been successful [6].

The clinical picture of bronchial asthma is characterized by chronic airway inflammation and coherent bronchial hyperreactivity. Various triggers are able to induce the release of cellular mediators, such as histamine and *de novo* synthesized sulfidoleukotrienes, in the asthmatic airway resulting in acute attacks of bronchoconstriction and further inflammation [3]. The sulfidoleukotrienes are known to have an about 1000 times higher bronchoconstrictive potency than histamine [7]; they have been shown to play a crucial role in several types of bronchial asthma [8,9].

The cellular source of the released mediators in bronchial asthma is not completely characterized, though basophils and mast cells are major candidates [10–12]. Other cell-types like eosinophils pass for alternative candidates [10,11]. Basophils are found to accumulate in asthmatic human airways during episodes of bronchospasm induced by different stimuli [13]. Apart from the spasmogenic mediators such as histamine and sulfidoleukotrienes, basophils have been shown to produce cytokines and chemokines, which may contribute to influence the local inflammatory reaction [14–16].

In allergic asthma the cross-linking of membrane-bound IgE on basophils and mast cells by allergens triggers the release of bronchoconstrictive mediators. Accordingly, allergen-induced basophil mediator release allows us to identify relevant allergic sensitization *in vitro*, and it corresponds well to skin prick test and specific serum IgE-levels [17,18]. In non-allergic asthma mechanisms initiating the bronchospastic reaction are not fully understood. Bronchopulmonary infections, small chemicals, drugs like aspirin

and other triggers induce bronchoconstriction in this type of asthma [1], not knowing what kind of stimulating agent is finally responsible for cell activation and mediator release inside the asthmatic airway.

In vitro stimulation of basophils with anti-IgE antibodies leads to an allergen-like cross-linking of membrane-bound IgE and release of both histamine and sulfidoleukotrienes [19]. Similarly, anaphylatoxin C5a, a split-product deriving from complement activation, is known to be a strong *in vitro* stimulus for basophil mediator release via binding to the C5a-receptor on the cell surface [20]. C5a has also been shown to induce severe airway inflammation and hyper-reactivity [21]. However, the final role of complement activation and of C5a as a mediator-releasing stimulus in bronchial asthma is still not clear [22,23].

In vitro basophil mediator releasability in response to various stimuli has been shown to be altered in atopic and asthmatic diseases without considering asthmatic subtypes [24–26]. Recently, C5a-dependent mediator release from peripheral blood leucocytes was suspected to be elevated in aspirin-intolerant individuals compared to controls [27]. Following these observations and with regard to the supposed key-role of basophils for both initiating bronchoconstriction and supporting chronic airway inflammation in bronchial asthma, we hypothesized that basophil mediator release in response to anti-IgE and C5a could differ between allergic and non-allergic asthmatics. This would be of particular interest for both understanding the mechanisms especially of non-allergic asthma and improvement of diagnostic methods for identifying asthmatic subtypes.

Subjects and methods

Twenty-nine adult patients visiting the Medical Clinic of Borstel and diagnosed as having bronchial asthma according to the American Thoracic Society [28] were divided into the following groups (Table 1): aspirin-sensitive asthmatics (ASA, $n = 10$); non-allergic asthmatics without aspirin intolerance (NAA, $n = 12$), allergic asthmatics

Table 1 Age and sex distribution of studied individuals

	Age (year) Mean $\pm$ SD	Range	Sex M/F	<i>n</i>
ASA	44.5 $\pm$ 5.15	38–53	4/6	10
NAA	51.0 $\pm$ 7.20	41–64	2/10	12
AA	29.0 $\pm$ 12.95	18–52	3/4	7
Controls	40.3 $\pm$ 12.06	28–58	3/6	9

ASA, aspirin-sensitive asthmatics; NAA, non-allergic asthmatics; AA, allergic asthmatics; controls, healthy controls.

(AA, $n = 7$). They were compared with healthy controls ($n = 9$).

In ASA the history of bronchospasm induced by acetylsalicylic acid was confirmed by oral or bronchial provocation test with (lysine-)acetylsalicylic acid (Aspisol[®], Bayer AG, Leverkusen, Germany) carried out according to described proceedings [29]. AA were defined by a positive skin prick test (> 3 mm weal) and increased specific serum IgE-levels in radioallergosorbent test (RAST) to one or more common environmental allergens corresponding to anamnesticly named episodes of bronchoconstriction. In case of doubtful clinical relevance of sensitization, allergen-specific bronchial provocation was performed in addition. If a relevant allergic sensitization could be excluded and endogenous triggers were likely to initiate asthmatic exacerbation, patients were defined as non-allergic (NAA). Both NAA and AA showed negative results after provocation with acetylsalicylic acid. Patients' clinical characteristics are summarized in Table 2. In controls, anamnestic indications for an allergic sensitization or a chest disease were missing, total serum IgE showed normal levels and oral provocation test with acetylsalicylic acid was negative. Subjects were excluded from the study if there was any history of regular smoking (> 5 cigarettes per day), bronchial infection in the past 3 weeks, further severe diseases or any chest disease other than asthma. No systemic glucocorticoid or antihistaminic

medication was allowed for at least 2 weeks before bleeding.

Subjects were recruited on a voluntary basis and gave informed written consent to take part in the study, which was approved by the local ethical committee.

Materials

The following materials were used: EDTA (Fluca Biochemica AG, Buchs, Switzerland); Hanks' balanced salt solution (HBSS) without Ca^{++} , Mg^{++} and phenol red, adjusted to pH 7.35; HBSS(10x); RPMI with glutamine medium; stimulation buffer (SB) = tyrode's salt solution with Ca^{++} and Mg^{++} , adjusted to pH 7.35 (Gibco BRL, Life Technologies, Eggenstein, Germany); Hepes 25 mM (SERVA Feinbiochemica GmbH & CoKG, Heidelberg, Germany) was added to SB; Ficoll (Seromed, Biochrom KG, Berlin, Germany); Percoll (Pharmacia Biotech AB, Uppsala, Sweden); single Percoll density solutions were prepared according to the instructions from the manufacturer; bovine serum albumine (BSA); human recombinant C5a (Sigma-Chemie, Deisenhofen/Munich, Germany); dextran; interleukin-3 (IL-3); anti-IgE = mAb Le27 (contained in CAST-ELISA, Bühlmann Laboratories AG, Allschwil, Switzerland); MiniMACS separation columns and MACS CD16 microbeads (Miltenyi Biotech GmbH, Bergisch-Gladbach, Germany).

Table 2 Clinical characteristics of studied patients

	ASA	NAA	AA
<i>Age at onset of disease (yr)</i>			
Mean $\pm$ SD	40.0 $\pm$ 7.33	46.7 $\pm$ 7.22	21.1 $\pm$ 11.20
<i>Pulmonary function test¹</i>			
FEV ₁ (%pred) Mean $\pm$ SD	78.3 $\pm$ 14.81	72.2 $\pm$ 21.36	83.1 $\pm$ 18.01
R _{AW} (kPa/l/sec) Mean $\pm$ SD	0.34 $\pm$ 0.135	0.49 $\pm$ 0.309	0.30 $\pm$ 0.078
<i>Atopic features</i>			
Positive family history (%)	30.0	16.7	100.0
Allergic rhinitis (%)	10.0	0	100.0
Total IgE > 100 U/mL (%)	10.0	16.7	85.7
> 3 allergic sensitizations in skin prick test (%)	10.0	0	85.7
<i>Other symptoms:</i>			
Nasal polyps (%)	60.0	33.3	14.3
Chronic sinusitis (%)	40.0	25.0	14.3
Vasomotor rhinitis (%)	30.0	25.0	0
Alcohol intolerance (%)	60.0	41.7	0
History of migraine (%)	40.0	8.3	14.3

ASA, aspirin-sensitive asthmatics ($n = 10$); NAA, non-allergic asthmatics ($n = 12$); AA, allergic asthmatics ($n = 7$); ¹ carried out just before diagnostic provocation with aspirin; FEV₁, forced expiratory volume in 1st second; R_{AW}, specific airway resistance; %pred, per cent of predicted; %, per cent of total number of group patients.

Preparation of peripheral blood leucocytes

From each subject 9 mL peripheral venous blood was drawn into a syringe containing 1 mL 0.1 M EDTA 1 to 7 days before diagnostic provocation with aspirin. The EDTA blood was mixed with 0.25 volume of 6% dextran in saline. Erythrocytes were allowed to sediment at room temperature (RT) for 90 min, after which the leucocyte-rich plasma was removed. Leucocytes were pelleted by centrifugation at $130 \times g$ for 15 min at 4 °C, washed twice in HBSS and resuspended in SB.

After cell preparation total leucocyte counts and viability were determined by trypan blue exclusion using a Neubauer chamber. Basophil content was determined on May-Grünwald-stained cytocentrifuge preparations counting at least 400 cells per slide. Prior to stimulation, the cell number was adjusted to 1×10^7 cells/mL. The results for viability counting were $> 99\%$, the average over-all basophil purity was $0.9 \pm 0.54\%$ (mean $\pm$ SD) without significant differences between the subject groups.

Preparation of purified leucocyte subpopulations

To determine the cellular source of the released mediators 120 mL EDTA blood was obtained from randomly selected subjects of each group and purified leucocyte subpopulations were prepared simultaneously as follows:

Purified basophils were prepared in a three-step procedure [30]. After a Ficoll-Hypaque density centrifugation at $400 \times g$ and 4 °C for 30 min, cells from the resulting interphase were washed twice in HBSS and resuspended in elutriation buffer (HBSS + 0.25% BSA, adjusted to pH 7.35). During the following counter current elutriation at $1220 \times g$ and 4 °C (centrifuge type J2-21, rotor type JE-6B and standard elutriation chamber, Beckmann Instruments Inc., Palo Alto, CA, USA) buffer flow was gradually raised from 28 mL/min to 41 mL/min, and separate elutriation fractions were obtained [30]. Basophil-rich elutriation fractions were collected at a flow of 35–41 mL/min, washed in HBSS and carefully layered on a discontinuous percoll density gradient with densities of 1.070 and 1.077 g/mL. After centrifugation at $400 \times g$ and 20 °C for 30 min the resulting interphase contained basophils at purities between 81.3 and 93.6%.

Lymphocytes were prepared in a two-step procedure including Ficoll-Hypaque density centrifugation and subsequent counter current elutriation as described above. The elutriation fractions collected at a flow of 33–34 mL/min contained between 74.0 and 97.9% lymphocytes. For monocyte preparation, which also began in the same way, the last fraction of counter current elutriation obtained after stopping the centrifuge (so-called stop fraction) was washed in HBSS and carefully layered on a percoll solution

with a density of 1.065 g/mL. This time, gradient centrifugation took place at $400 \times g$ and 20 °C for 30 min and the resulting interphase contained between 75.6 and 91.8% monocytes.

Neutrophils were prepared by collecting the top-layer of cellular sediment resulting from initial Ficoll-Hypaque density centrifugation. This granulocyte-rich pellet was washed twice in HBSS, resuspended in RPMI and mixed with 0.25 volume of 6% dextran in saline. After sedimentation at RT for 90 min the upper phase contained between 90.1 and 93.7% neutrophils. To prepare eosinophils again the top-layer of the cellular sediment after Ficoll-Hypaque density centrifugation was collected and washed in HBSS. To lyse contaminating erythrocytes the pellet was resuspended in nine parts distilled water and waved for 30 s. Lysis reaction was stopped by adding one part HBSS(10x) immediately followed by another washing step. Erythrocyte lysis was repeated twice and afterwards cells were layered on a percoll gradient with a density of 1.092 g/mL. After gradient centrifugation at $400 \times g$ and RT for 30 min the cellular sediment containing the eosinophils was washed twice and resuspended in MACS buffer (HBSS + 0.5% BSA, adjusted to pH 7.35) for final magnetic cell separation (MACS). Contaminating neutrophils were marked with MACS CD16 microbeads and the MACS procedure was repeated once as previously described [31]. The resulting effluent contained between 92.3 and 99.4% eosinophils.

Total and differential leucocyte counts were determined as described above. For the stimulation procedure cells were transferred to SB and adjusted to 1×10^6 cells/mL.

Basophil remixing experiments

For remixing experiments of purified basophils the basophil-free peripheral blood mononuclear cell (PBMC) fractions obtained after counter current elutriation were pooled again. To obtain a series of cell preparations with decreasing basophil purity and a constant cell concentration the highly purified basophils (1×10^6 cells/mL) were serially (1 : 2) remixed with the pooled cells, which had also a concentration of 1×10^6 cells/mL. The last tube contained only the pooled cells without added basophils. Then the cells were stimulated as follows.

Cell stimulation procedure

Cell stimulation was performed at 37 °C in a shaking water bath. After a warming-up period of 5 min, interleukin-3 was added at a concentration of 10 ng/mL for cell priming [20]; 15 min later stimulating agents were added. Optimal conditions of stimulation time and concentration were 15 min and 1×10^{-7} M for C5a (data from our own

investigations) and 30 min and 1×10^{-6} M for anti-IgE [19,20]. Spontaneous mediator release was determined by adding an equal volume of SB to the cells after the priming with IL-3. For determination of total histamine content cells were alternately frozen and heated to 70 °C and this procedure was repeated five times. Stimulation was stopped by centrifugation at $1000 \times g$ and 4 °C for 5 min, and mediator release was determined in the supernatant. All stimulation was carried out in duplicates.

Leukotrienes and histamine assays

Sulfidoleukotrienes were determined by an ELISA technique (CAST-ELISA, Bühlmann Laboratories AG, Allschwil, Switzerland) according to the manufacturers' protocol. Histamine release was measured by radioimmunoassay (Methyl-Histamine RIA, Pharmacia Diagnostics AB, Uppsala, Sweden) according to the manufacturers' instructions for determination of non-methylated histamine.

Statistical analysis

Results are presented graphically as the group mean (+ standard error of the mean) if not indicated otherwise. Mean values of groups were analysed for significant differences using the Mann–Whitney *U*-test. For the calculated quotient C5a-/anti-IgE-induced leukotriene production a relative operating characteristics (ROC) curve

analysis has been performed for all patients to select an optimal cut-off point of discrimination [32]. For the purpose of calculation of ROC curve, patients with characteristics of both non-allergic and allergic asthma had to be excluded, as the test is designed only for bivariate outcomes. The ROC plot is demonstrated as a graph of sensitivity vs. $1 - \text{specificity}$ for diagnosing ASA or NAA at each possible cut-off point. We selected an optimal cut-off point on the ROC curve that corresponds to the best sensitivity and specificity (maximum sum of sensitivity and specificity). Additionally, the positive predictive value of a positive result and negative predictive value of a negative result for diagnosis of non-allergic asthma were determined at the optimal cut-off point of the test. Correlation analysis between mediator release and basophil purity was performed by calculating the correlation coefficient and the regression line from scatter diagram using sigma-plot software (version 5.0). *P*-values of less than 0.05 were considered statistically significant.

Results

Stimulation with C5a and anti-IgE

The release of sulfidoleukotrienes and histamine from peripheral blood leucocytes (PBL) after stimulation with C5a and anti-IgE is shown in Fig. 1. Spontaneous sulfidoleukotriene production was significantly increased

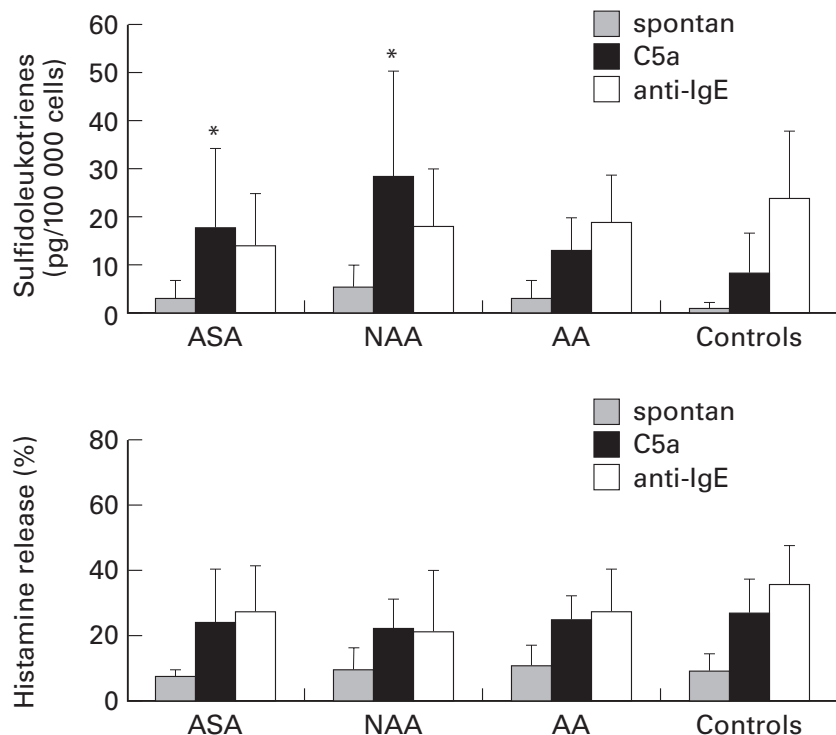


Fig. 1. Sulfidoleukotriene production (above) and % histamine release (below) from peripheral blood leucocytes after preincubation with IL-3 and stimulation with C5a or anti-IgE in the studied groups. ASA, aspirin-sensitive asthmatics ($n = 10$); NAA, non-allergic asthmatics ($n = 12$); AA, allergic asthmatics ($n = 7$); controls, healthy controls ($n = 9$); spontan, spontaneous mediator release, which is incubation with IL-3 alone. Results are shown as mean + SD, they are not corrected for spontaneous mediator release. Statistical comparison was made using the Mann–Whitney *U*-test. * $P < 0.05$ compared with AA and controls.

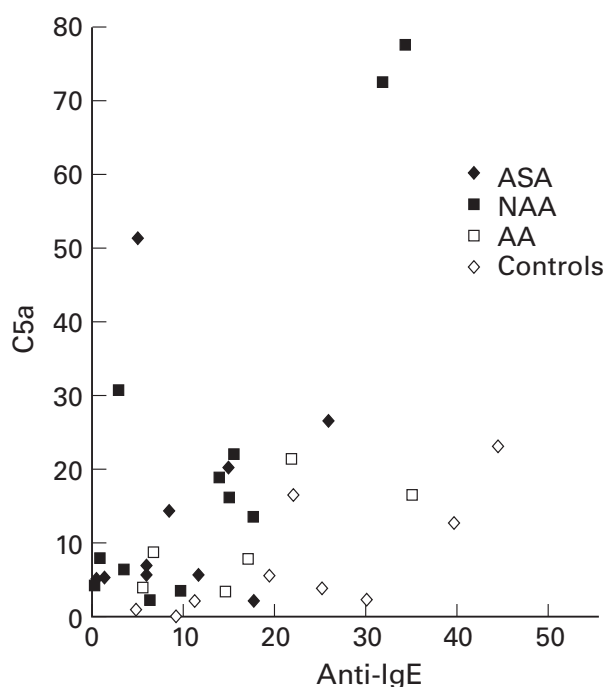


Fig. 2. Individual relation between C5a- and anti-IgE-induced sulfidoleukotriene production from peripheral blood leucocytes in respect to subjects' groups (ASA, aspirin-sensitive asthmatics; NAA, non-allergic asthmatics; AA, allergic asthmatics; controls, healthy controls). Values are corrected for spontaneous release, they are expressed in pg/100 000 cells.

($P < 0.05$) in all asthmatics compared to healthy controls. Stimulation of the cells with anti-IgE or C5a produced a marked enhancement of sulfidoleukotriene production over spontaneous levels in nearly all subjects. Sulfidoleukotriene production after stimulation with anti-IgE tended to be lower in ASA and NAA than in AA and healthy controls, but no significance could be observed. Sulfidoleukotriene production after stimulation with C5a was significantly higher in ASA and NAA as compared to AA and controls

($P < 0.05$). The individual relation between anti-IgE- and C5a-induced mediator release in each investigated subject is shown in Fig. 2, where ASA resp. NAA and AA resp. controls cluster separately. Whereas in AA and controls anti-IgE-induced sulfidoleukotriene production exceeded C5a-induced values in 6/7 and 9/9 individuals, respectively, the reverse was seen in ASA and NAA. Here, C5a-induced sulfidoleukotriene production exceeded anti-IgE-induced values in 7/10 and 9/12 individuals, respectively.

Calculating the quotient C5a/anti-IgE-induced sulfidoleukotriene production demonstrated highly significant differences between ASA and NAA vs. AA and controls ($P < 0.001$, Table 3). The optimal cut-off point of the quotient to discriminate between the groups, calculated from ROC curve analysis as shown in Fig. 3, is 1.0, with the interval of 0.7–1.3 being nearly as good as the optimal point. Taking the cut-off point of 1.0 the quotient C5a/anti-IgE-induced sulfidoleukotriene production allows a diagnostic differentiation of aspirin-sensitive and non-allergic asthmatics from allergic asthmatics and healthy controls with a sensitivity of 0.73 and a specificity of 0.94. The predictive value of a positive result for an enhanced C5a/anti-IgE quotient to belong to ASA or NAA was 0.94, the predictive value of a negative result was 0.71.

With regard to histamine release, no differences were found in spontaneous mediator release between asthmatics and healthy controls. Mean total histamine content in the basophils was 116.4 ± 63.68 ng/10⁵ basophils without noticeable differences between the groups. Again, stimulation of the cells with anti-IgE or C5a produced a marked enhancement of histamine release over spontaneous levels in all subjects. However, neither anti-IgE-induced nor C5a-induced histamine release differed significantly between the investigated groups (Fig. 1). Even calculating the quotient C5a/anti-IgE-induced histamine release did not allow significant differentiation of aspirin-sensitive and non-allergic asthmatics from allergic asthmatics and healthy controls.

Table 3 Quotient C5a-/anti-IgE-induced mediator release from human PBL

	ASA	NAA	AA	Controls
Sulfidoleukotrienes (Mean $\pm$ SD)	$3.1 \pm 3.83^*$	$3.3 \pm 3.45^*$	0.7 ± 0.36	0.3 ± 0.23
Histamine (Mean $\pm$ SD)	1.1 ± 0.80	2.9 ± 3.56	1.0 ± 0.49	0.7 ± 0.24

All values are corrected for spontaneous release before dividing C5a-dependent mediator release by anti-IgE-dependent mediator release. PBL, peripheral blood leucocytes; ASA, aspirin-sensitive asthmatics ($n = 10$); NAA, non-allergic asthmatics ($n = 12$); AA, allergic asthmatics ($n = 7$); controls, healthy controls ($n = 9$); *, $P < 0.001$ compared to AA and controls.

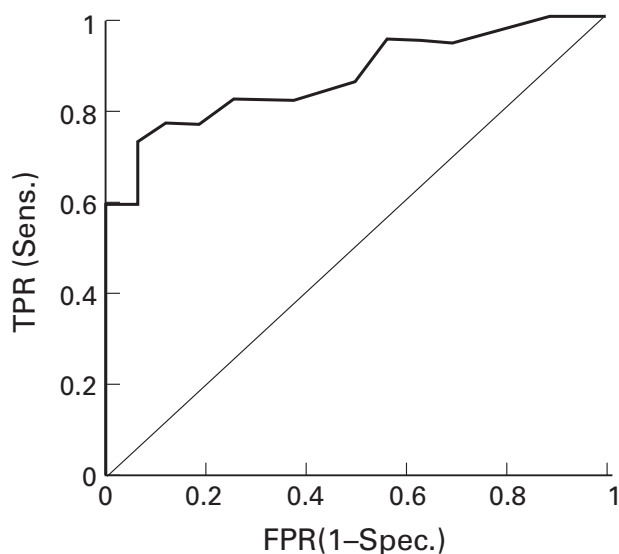


Fig. 3. ROC plot of C5a/anti-IgE-induced sulfidoleukotriene release from the studied patients to determine the optimal cut-off point for differentiation between ASA resp. NAA and AA resp. controls according to Beck *et al.* [32]. FPR, false-positive ratio; sens., sensitivity; spec., specificity; TPR, true-positive ratio.

Cellular source of C5a-induced mediator release

Whereas the cellular IgE-receptor ($Fc_\epsilon RI$) is expressed mainly on basophils, the cellular receptor for C5a (CD88) is known to be expressed on monocytes, basophils, neutrophils, and eosinophils. Since all of these cells are capable of producing sulfidoleukotrienes too, several cell types might be involved in the C5a-induced leukotriene production in dextran sedimented cell suspensions. To address this point, purified leucocyte subpopulations were prepared from the peripheral blood of single subjects from all investigated groups. As shown in Fig. 4 basophils were the main source of C5a-induced sulfidoleukotriene production, whereas eosinophils released only small sulfidoleukotriene amounts in response to C5a. Monocytes, lymphocytes and neutrophils showed no C5a-dependent leukotriene production. When highly purified basophils were remixed with PBMC at various concentrations, C5a-dependent leukotriene production significantly correlated with the basophil purity ($r = 0.991$, $P < 0.001$; Fig. 4) additionally confirming the basophils as cellular source of released sulfidoleukotrienes.

Analogous results were obtained concerning C5a-dependent histamine release. Only basophils released histamine upon stimulation with C5a (377.7 ± 196.58 ng/ 10^6 cells, corrected for spontaneous histamine release), all the other purified cell fractions remained unaffected after incubation with C5a. Again, a significant correlation was observed between C5a-induced histamine release and basophil

purity in remixing experiments with PBMC ($r = 0.996$, $P < 0.001$; data not shown). These results indicate that the cellular source of C5a-induced mediator release from dextran sedimented PBL is the basophil.

Discussion

In this study, we demonstrate that non-allergic asthmatics may be distinguished from allergic asthmatics by calculating the quotient C5a/anti-IgE-induced sulfidoleukotriene production from peripheral blood leucocytes. The concept and study of basophil releasability was originally proposed by L.M. Lichtenstein and his colleagues in the 1970s. It says that, apart from surface receptor density and extracellular influencing factors, intracellular factors deriving from genetic and pathogenic conditions are responsible for an individually characteristic amount of stimulus-induced basophil mediator release [24,26,33]. Previous studies have shown that basophil mediator release in response to various stimuli changes under circumstances of disease, especially in atopic and asthmatic subjects [24,25]. However, until now, no suitable marker of diagnostic relevance could be established from that observation [19,26].

Anti-IgE-induced basophil mediator release via cross-linking of cell-bound IgE and $Fc_\epsilon RI$ activation does not correlate with the amount of surface IgE molecules or serum IgE levels [19,34]. It is increased by, but does not necessarily depend on, pre-incubation with the maturing and priming cytokine IL-3 [20]. In contrast, basophil stimulation with C5a via C5a-receptor activation leads to a concentration-dependent mediator release, which necessarily requires initial cell priming with IL-3 [20]. As it is obvious that basophil stimulation via C5a-receptor and $Fc_\epsilon RI$ activation follow different signalling pathways [20,25], the extent of mediators released by these stimuli can be regulated either in parallel or differently [26]. Our data show that an increased C5a-induced mediator release in non-allergic asthmatics is accompanied by a mild decrease in mediator release after stimulation with anti-IgE, suggesting an inverse regulation of these two types of cell activation. Plotting C5a-induced vs. anti-IgE-induced leukotriene release (Fig. 2) disclosed two different patient populations either emphasizing C5a-induced or anti-IgE-induced mediator release. For this reason, the quotient C5a/anti-IgE-induced mediator release more precisely describes the differential basophil releasability in the asthmatic patient groups than the single parameters. ASA and NAA show high values of the quotient, while AA and healthy controls demonstrated low levels with a calculated optimal cut-off point at 1.0. As seen in Fig. 2 an overlapping area could be found between the patient populations around the cut-off line, which parallels the observation of a fluent transit between allergic and non-allergic asthma

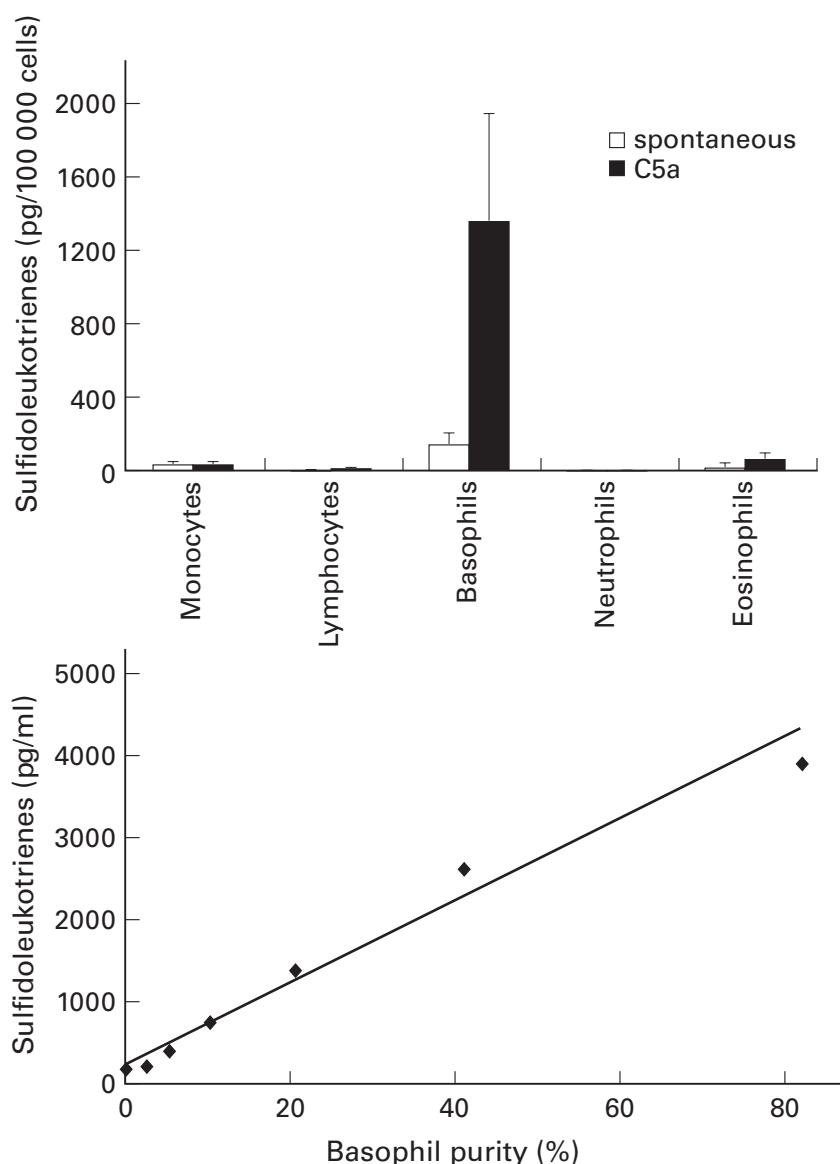


Fig. 4. Above: Sulfidoleukotriene production from highly purified leucocyte subpopulations after preincubation with IL-3 and stimulation with C5a (10^{-7} M) in randomly selected subjects from the investigated groups ($n = 4$). Results are shown as mean + SD, they are not corrected for spontaneous leukotriene release. Below: Correlation between C5a-induced sulfidoleukotriene production and basophil purity in mixed peripheral blood mononuclear cell (PBMC) suspensions with a constant total cell number. Values are corrected for spontaneous leukotriene production. One representative out of three experiments is shown ($r = 0.991$, $P < 0.001$). No significant difference was found between the investigated subjects. Analogous results were obtained for histamine release (data not shown).

found in a proportion of patients. Therefore, diagnostic decisions should carefully be made from quotient values localized in this overlapping area between 0.7 and 1.3 of the quotient value.

A large number of factors are described as influencing basophil releasability *in vivo* and *in vitro*, such as patients' age, bacterial and viral infections and individual medication [25,26]. The number of these factors was considerably reduced by the chosen inclusion criteria for patients in this study. Also, inter-individual variability in patients' releasability studies is known to be high [19,24]. Calculating the quotient C5a-/anti-IgE-induced mediator release has the advantage of obtaining a parameter of differentiation that is statistically less affected by inter-individual variability and other non-systematically influencing factors.

Thus, the calculated quotient should be favoured for diagnostic decisions.

Immunological events of allergic and non-allergic asthma are known to be different. In allergic asthma a predominant TH2-like pattern of lymphocyte activation and cytokine production could be demonstrated [3,35], whereas in non-allergic asthma these qualities are missing [36]. The cytokine spectrum found in the intrinsic type of bronchial asthma is incompatible with a clear-cut TH1- or TH2-like pattern. In our study, this difference is reflected by the predominant basophil mediator release in response to anti-IgE in allergic asthmatics and a more pronounced releasability in response to C5a in non-allergic asthmatics. Although in non-allergic asthma stimulating factors for basophil and mast cell activation initiating acute bronchoconstriction are unknown,

the anaphylatoxin deriving from complement activation is said to be a good candidate for being involved [23]. An altered basophil releasability in response to C5a underlines this hypothesis and offers further possibilities for investigation.

Diagnosis of aspirin-sensitive asthma as a subtype of non-allergic asthma is very important because of possibly life-threatening asthmatic attacks induced by analgesics. Until now, only risky provocation procedures allowed the confirmation of aspirin intolerance [4]. Stimulation of basophils with acetylsalicylic acid induced no reliable mediator release [5,37], and even other *in vitro* diagnostic proposals were unsuccessful [6,37]. In the present study, basophil mediator releasing characteristics of aspirin-sensitive asthmatics do not differ from those of non-allergic asthmatics without aspirin intolerance. Thus, basophil stimulation with C5a and anti-IgE is not suitable for diagnosing aspirin intolerance, as was proposed in a previous study [27]. On the other hand, it is the first time that data from studying basophil releasability *in vitro* can confirm the clinical experience of aspirin-sensitive asthmatics belonging mainly to the intrinsic type of asthma.

The cellular antigen stimulation test (CAST) is a new and promising diagnostic tool in allergic diseases and asthma [18]. Apart from histamine release, determination of stimulus-induced sulfidoleukotriene production gains additional importance for interpreting clinical relevance of suspected antigens in allergic and non-allergic diseases [18,33]. The present description of a differential C5a-/anti-IgE-dependent mediator release from basophils in non-allergic and allergic asthmatics can only be demonstrated in the case of sulfidoleukotriene production and not in the case of histamine release, similar to previous observations [27]. A different intracellular regulation of sulfidoleukotriene production and histamine release may explain this discrepancy [7,19,20]. The results correspond with the known predominant role of sulfidoleukotrienes as bronchoconstrictive mediators in asthma compared to histamine [7,8], and they underline the importance of eicosanoid mediators even for *in vitro* investigations of mechanisms of bronchial asthma.

While the cellular source of histamine release in PBL is well known to be the basophil, the origin of sulfidoleukotriene production especially in response to C5a-receptor activation could be manifold [7,20]. Other authors have already proposed the basophil as the cellular source of C5a-induced leukotriene production from PBL [20], although definite proof was missing. By stimulating highly purified leucocyte subpopulations we also found basophils to be the major source of leukotriene release, an observation which was further supported by additional remixing experiments with PBMC. When considering the other leucocyte populations only eosinophils produce an about 50 times smaller

amount of leukotrienes, suggesting a very high eosinophil count in PBL is required for an equivalent leukotriene production after dextran sedimentation. Although peripheral blood eosinophilia is a typical feature of bronchial asthma [1,38], such dimensions are rarely achieved.

Another variable of the dextran sedimented stimulation system that should be mentioned is the total number of C5a-receptor positive cells, which can compete with the basophils for C5a and diminish the mediator release by binding the anaphylatoxin to their high-affinity C5a-receptors [39]. However, considering the enormous values of stimulus-induced mediator release in the purified basophil fraction compared to the other leucocyte subpopulations the influence seems to be trivial. Especially, a parameter of a somewhat coarse valuation like the C5a-/anti-IgE-quotient is unlikely to be falsified for diagnostic differentiation between non-allergic and allergic asthma. Nevertheless, confirmation of the data presented here in basophil-rich cell fractions and in a larger number of patients is necessary.

The present study emphasizes the role of basophils for the understanding of bronchial asthma. It is generally accepted that basophil and mast cell derived mediators play a major role for initiating acute bronchoconstriction [10–12]. Here, we show the basophil being the only quantitative source of mediator release from peripheral blood leucocytes after C5a-dependent stimulation. It is not known if this also applies to the *in vivo* situation, where other C5a-receptor positive cell types such as mast cells and epithelial cells [40] may be involved in the bronchoconstrictive reaction of the lung. However, the particular importance of basophils for bronchial asthma is stressed not only by the high mediator releasing capacity of these cells even after stimulation with C5a, but also by the fact that basophils accumulate in human airways during airway narrowing and that their number in bronchial tissue correlates with bronchial responsiveness in asthmatics [12,13,41]. As mentioned before, an altered basophil releasability is suspected to play a crucial role in asthmatic and allergic disease, though it is not clear what causes the altered intracellular response after cell stimulation. The involvement of cytokines, chemokines or specific auto-antibodies as additional factors in the pathophysiology of asthma have already been proposed elsewhere [42–44]. Thus, basophils, which can be easily prepared, provide an attractive target cell for future investigations concerning basic mechanisms in bronchial asthma.

In conclusion, this study suggests that an *in vitro* differentiation between non-allergic and allergic asthma is feasible by comparing basophil mediator release following stimulation with anti-IgE and C5a. The special diagnostic value of the quotient C5a-/anti-IgE-induced sulfidoleukotriene production is described. However, to evaluate its

general applicability, this diagnostic approach has to be examined in a larger number of patients. New aspects for exploring the trigger mechanisms initiating acute bronchospasm and supporting chronic airway inflammation especially in non-allergic asthma result from the presented observations and basophils provide an attractive target cell for future investigations in this field.

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