

# Means of increasing sensitivity of an *in vitro* diagnostic test for aspirin intolerance

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## Summary

**Background** Pseudo-allergic reactions caused by aspirin (acetyl salicylic acid; ASA) often resemble immediate-type hypersensitivity reactions consisting of urticaria and angioedema or rhinoconjunctivitis, asthma and nasal polyps. In the last few years, a new *in vitro* assay based on determination of sulfidoleucotrienes from isolated leucocytes (cellular allergen stimulation test – CAST) has been introduced for type I allergies and pseudoallergic reactions. In ASA intolerance, there is only limited experience using this assay with – in some studies – only moderate sensitivity. Furthermore, the necessity to use freshly isolated leucocytes from untreated patients is inconvenient for routine settings.

**Objective** The purpose of our study was to search for possibilities of increasing the sensitivity of the test and to use stored blood samples which would permit shipping, two requirements for the clinical suitability of this test.

**Patients and methods** Leucotriene release in response to ASA and other non-steroidal anti-inflammatory drugs (NSAIDs) was analysed in 38 ASA-intolerant patients (predominantly airway-related symptoms  $n = 22$ ; predominantly cutaneous symptoms  $n = 16$ ) and 50 controls. The diagnosis of ASA intolerance was established by history and placebo-controlled oral challenge tests.

**Results** Using 24 h-stored leucocytes obtained from 10 ASA-intolerant patients and 10 healthy controls there were no significant differences of leucotriene release by resting, ionomycin-, and anti Fc $\epsilon$ RI $\alpha$ -stimulated leucocytes compared with freshly isolated leucocytes. Analysis of ASA + C5a-mediated leucotriene release by stored blood samples in combination with indomethacin- and diclofenac-mediated leucotriene release in ASA-intolerant patients ( $n = 38$ ) resulted in an increased sensitivity (from 50 to 72.7% in ASA-intolerant patients with predominantly airway-related symptoms and from 81 to 100% in ASA-intolerant patients with predominantly skin symptoms) compared with assays in which only ASA + C5a-mediated leucotriene release has been determined. Moreover, the specificity of the assay remained high (96.7% when analysing different NSAIDs compared with >99% when analysing only ASA + C5a-mediated leucotriene release).

**Conclusion** *In vitro* stimulation with ASA + C5a leucocyte stimulation with other NSAIDs should be performed to achieve a higher sensitivity. This finding can be explained by the clinical observation of a high ratio of cross-reactivities between the mentioned NSAIDs.

**Keywords:** aspirin, asthma, diclofenac, indomethacin, intolerance, leucotriene, nasal polyps, non-steroidal anti-inflammatory drugs, urticaria

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## Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin (acetyl salicylic acid; ASA) belong to one of the most frequently used groups of pharmacologically active compounds. Although only a small percentage of individuals suffer from pseudoallergic reactions such as urticaria, angioedema, nasal polyps, asthma, or anaphylactic shock after the intake of these compounds, these side-effects are of particular importance to allergists due to the frequency of use [1–4]. In recent years, evidence has accumulated that changes in the arachidonic acid metabolism are of pathogenic importance because substances known to be responsible for intolerance reactions such as ASA, indomethacin, diclofenac, and ibuprofen all inhibit the cyclooxygenase pathway. Consequently, concentrations of anti-inflammatory compounds such as prostaglandin (PG) $E_2$  decrease whereas sulfidoleucotriene (sLT) concentrations rise [5–8]. This has been shown *in vivo* in animals [5] and humans [6–10]. Furthermore, not only different leucocyte subsets [11–14] but also platelets [15,16] have been claimed to be responsible for LT production in ASA-intolerant patients in response to ASA *in vitro*. *In vivo*, LTC $_4$  synthase in the bronchial mucosa from ASA-intolerant asthma patients was over-represented compared with ASA-tolerant patients [17]. Expression of LTC $_4$  synthase forming the precursors of the receptor-active LTD $_4$  and LTE $_4$  correlated with bronchial responsiveness to inhaled ASA [17]. These data provide the basis that determination of LTs should be of benefit in the *in vitro* diagnosis of ASA intolerance.

Indeed, the rise of LTE $_4$  in urine after natural or experimental exposure to ASA has been shown [9]. However, for technical reasons it is not routinely used in allergic or intolerance reactions. In the last few years, a new, fast and simple ELISA-based assay (CAST-ELISA, DPC Biemann, Bad Nauheim, Germany) which allows the simultaneous measurement of leucotrienes C $_4$ , D $_4$  and E $_4$  in the supernatants of isolated leucocytes has been established [11–14]. This test has been performed in 63 patients with ASA intolerance and the results have been reviewed recently [18]. Using freshly isolated leucocytes obtained from untreated, symptom-free, ASA-intolerant patients the sensitivity was reportedly >66% with high specificity.

The purpose of this study was to analyse the suitability of this assay for clinical use. Therefore, based on the observation of high cross-intolerance among different NSAIDs LT release by leucocytes obtained from ASA-intolerant and -tolerant individuals was analysed in response to different NSAIDs aiming at an increased sensitivity of the assay. We report here that a large proportion of ASA-intolerant patients do not tolerate other NSAIDs *in vitro* which resulted in a marked increase of the sensitivity of this test.

## Materials and methods

### Patients and control subjects

The study included four groups with a total number of 88 individuals. Since it is known that ASA-intolerant patients only rarely show respiratory and cutaneous symptoms [1,19], patients with pseudoallergy to ASA were divided into two groups: group 1 (ASA-RS) with predominantly nasal and/or asthmatic symptoms ( $n = 22$ , 13 female; mean age  $44.0 \pm 12.9$  years, range 19–67 years; time since first occurrence of nasal polyps or asthma until testing:  $16.2 \pm 8.9$  years). 'ASA triad' (defined by ASA-induced asthma, nasal polyps and intolerance reaction to ASA) occurred in 18/22 patients, the remaining 4/22 patients were suffering only from rhinosinusitis and ASA intolerance. Eleven of the 22 patients were on local glucocorticosteroid therapy [20,21] (intranasal application of budesonide 200  $\mu$ g/day) which was interrupted 24 h before venipuncture. One of 11 suffering from ASA triad was additionally receiving 4 mg per day methylprednisolone. Atopy was present in 8/22 patients (8/8 allergic rhinitis with elevated total IgE and specific IgE; no atopic dermatitis; total IgE  $200.9 \pm 338.1$  kU/L, eosinophil count  $4.8 \pm 3.1\%$  and  $340 \pm 150$  eosinophils/nL). One of 22 patients was allergic to amoxicillin. Group 2 (ASA-URT;  $n = 16$ , eight female; mean age  $34.5 \pm 10.4$  years, range 22–59 years; time since first episode of urticaria until testing:  $17.9 \pm 11.9$  months) consisted of patients exclusively suffering from skin symptoms such as urticaria and/or Quincke oedema without asthma or polyps. Six of 16 patients were atopic (2/6 with history but not presence of atopic dermatitis, 5/6 with allergic rhinitis; total IgE  $133 \pm 128$  kU/L, eosinophil count  $4.8 \pm 2.3\%$  and  $332 \pm 180$  eosinophils/nL). In ASA-URT patients, the diagnosis of pseudoallergy to ASA was confirmed by history and placebo-controlled oral challenge tests with ASA using a cumulative amount of ASA up to 1900 mg (50, 100, 250, 500, 1000 mg). Oral challenge tests were defined as positive if the patient showed objective clinical symptoms after challenge, i.e. urticaria, angioedema, rhinitis, conjunctivitis or hypotension. The amount administered was increased every 2 h if the patient did not show any objective symptoms. Oral challenge tests were performed in the ASA-URT patients after a symptom-free interval of at least 4 weeks. In ASA-RS patients, bronchial challenge tests with inhaled lysine-ASA (5, 7.5, 10, 12.5, 15, 20, 25 mg; Bayer Vital, Leverkusen, Germany) was performed. FEV $_1$  was measured spirometrically using Vitalograph 2170 (Vitalograph GmbH, Hamburg, Germany) every 30 min. Additional measurements were performed at the time the patients recorded symptoms. The challenge test was defined as positive if the fall in FEV $_1$  was >25% below the baseline associated with

subjective symptoms such as cough, dyspnea, rhinitis or conjunctivitis. In ASA-RS patients, challenge tests were performed after a period of at least 3 months without an asthma attack. However, most ASA-RS patients were suffering from polyposis nasi at the time of the challenge test. None of the patients included in this study showed symptoms after placebo 'challenge'. Group 3 (NC) consisted of 30 nonatopic healthy volunteers (15 female; mean age  $32 \pm 11.3$  years) with no previous histories of drug allergy or intolerance. Atopy was excluded by history, determination of total IgE ( $< 140$  kU/L; CAP IgE FEIA, Pharmacia-Upjohn, Freiburg, Germany) and specific IgE (birch, mugwort, timothy grass, *Dermatophagoides pteronyssinus* and *D. farinae*; CAP-RAST, Pharmacia-Upjohn). Group 4 (AC) consisted of 20 patients suffering from atopic rhinitis (12 female; mean age  $37 \pm 12.2$  years; all patients had a positive history of atopy; specific sensitization was proven by skin and specific IgE tests; IgE  $> 140$  kU/L, mean IgE  $347.5 \pm 154.5$  kU/L).

#### *Collection of blood samples, isolation of leucocytes, and analysis of sulfidoleucotriene release in vitro*

Leucotriene measurements were performed up to 6 months after oral challenge. Two weeks prior to venipuncture, ASA-URT patients, AC, and NC individuals received no glucocorticosteroids, antihistaminic compounds, or NSAIDs. In contrast, 11/22 ASA-RS patients were on local or systemic ( $n = 1$ ) glucocorticosteroids which was interrupted only 24 h prior to venipuncture. Interruption of anti-inflammatory compounds for a longer time period was not possible due to the severity of the patients' symptoms.

Additionally, measurement of leucotriene release for routine purposes most often requires shipping of blood samples. Therefore, after collection of blood specimens into EDTA-containing CAST venipuncture tubes (DPC Biermann), the tubes were stored for 24 h at room temperature before leucocytes were isolated by dextran sedimentation of whole blood for 90 min as previously described [11]. Subsequently, the cellular allergen stimulation test was performed as described according to the manufacturer's instructions [11]. Leucocytes were harvested, resuspended in 4 mL of an IL-3-containing stimulation buffer (IL-3: 20 ng/mL; DPC Biermann) and the leucocytes (200  $\mu$ L/test) were stimulated in duplicates for 40 min at 37 °C with NSAIDs (Lys-ASA [20  $\mu$ g/mL = 111  $\mu$ mol/L and 200  $\mu$ g/mL = 1.1 mmol/L], diclofenac [20  $\mu$ g/mL = 62.9  $\mu$ mol/L], ibuprofen [20  $\mu$ g/mL = 97.1  $\mu$ mol/L], and indomethacin [20  $\mu$ g/mL = 55.9  $\mu$ mol/L] DPC Biermann) or stimulation buffer as control of spontaneous leucotriene release. In the case of ASA, tests were performed with or without C5a ( $10^{-7}$  mol/L; Sigma, München, Germany) because C5a induction of leucotriene release by leucocytes of ASA-intolerant patients has already been published [12].

Leucotriene release induced by ionomycin (0.2  $\mu$ mol/L; DPC Biermann) and by a mAb directed against the high affinity IgE receptor Fc $\epsilon$ RI $\alpha$  (2.75  $\mu$ g/mL, DPC Biermann) served as positive controls. Finally, sLT concentration in the supernatants was detected by ELISA (CAST-ELISA, DPC Biermann) according to the manufacturer's instruction.

#### *Definition of normal sLT release and statistical analysis*

The means of sLT release in NSAID-stimulated leucocytes from NC individuals ( $n = 30$ ) and AC individuals ( $n = 20$ ) were calculated for each compound. There were no significant differences in sLT concentrations from unstimulated or NSAID-stimulated samples obtained from NC compared with AC individuals. In general, sLT concentrations in AC slightly exceeded sLT concentrations from NC individuals. To obtain highest specificity, sLT release obtained from AC and not from NC was chosen. Finally, sLT concentrations exceeding the mean + 3SD (cutoff) of AC were defined as abnormal, because mean + 2SD resulted in a marked loss of specificity.

Differences between stimulated and unstimulated release of sLT in one individual were calculated using the paired Student's *t*-test after analysis of Gaussian distribution with bias 4.0 computer program (H. Ackermann, Frankfurt, Germany). Differences between patients and controls were evaluated using Welch's *t*-test.

## **Results**

#### *Leucotriene release by leucocytes remains unchanged during a 1 day storing period*

Since determination of sLT release by leucocytes for routine diagnostic purposes most often requires shipping of blood samples, we analysed unstimulated, ionomycin- and anti-Fc $\epsilon$ RI $\alpha$ -induced sLT release by leucocytes of 10 NC individuals and 10 ASA-RS patients immediately after and 24 h after venipuncture. In both groups, there were no significant differences between sLT concentrations from freshly isolated leucocytes compared with those stored for 24 h (see Fig. 1). Therefore, leucocyte isolation 24 h after venipuncture was chosen for the study.

#### *Effect of ASA on sLT release in ASA-intolerant and healthy individuals*

sLT release by the leucocytes of 88 individuals was investigated using different ASA concentrations in the presence and absence of C5a to find optimal stimulation procedures for diagnostic purposes. There were no significant differences between sLT releases of unstimulated leucocytes in

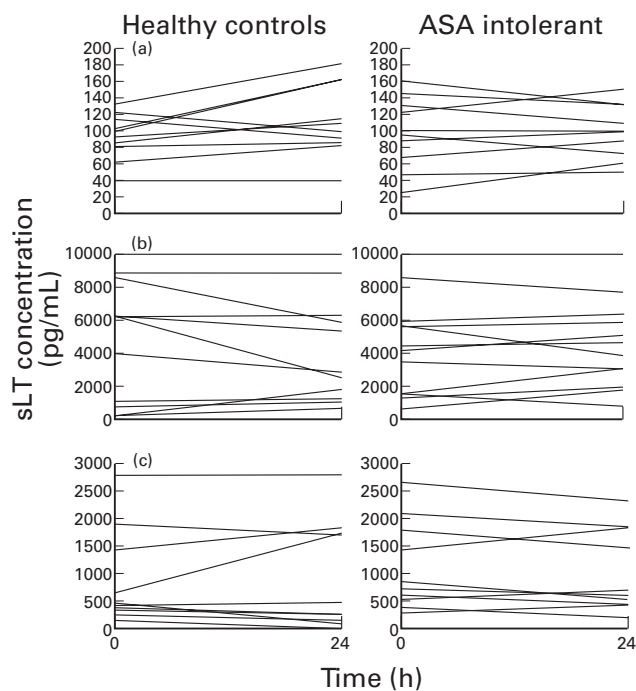


**Fig. 1.** sLT release by freshly isolated and 24 h stored leucocytes *in vitro*. Leucocytes from 10 NC and 10 ASA-RS patients were isolated either immediately or 24 h after venipuncture. Thereafter, sLT release was analysed in resting (A) and ionomycin- (B) or FcεRIα- (C) stimulated cells. The results obtained from a storage period of 0 h and 24 h are presented as paired data points to show how sLT synthesis changed in individual patients during storage.

the four groups. In nonatopic and atopic controls ASA induced a slight but significant increase in sLT concentration both at 200 and 20 µg/mL ( $P < 0.05$ ). C5a did not further enhance sLT in ASA-tolerant controls (NC and AC). In both groups of ASA-intolerant patients (ASA-RS and

ASA-URT), ASA at both concentrations induced a more prominent increase in sLT concentration compared with both control groups. ASA-induced sLT levels were higher in both groups of ASA-intolerant patients compared with NC and AC individuals. Furthermore, in both groups of ASA-intolerant patients C5a induced a significant increase in sLT concentrations which was not observable in ASA-tolerant controls and which was most prominent at a concentration of ASA 200 µg/mL ( $P = 0.01$  in ASA-RS and  $P < 0.05$  in ASA-URT compared with AC). sLT concentrations in ASA (200 µg/mL) + C5a were significantly higher in both groups of ASA-intolerant patients compared with AC individuals (AC:  $152.6 \pm 46.1$ ; ASA-RS:  $309.3 \pm 198.3$ ; ASA-URT:  $440.4 \pm 202.4$ ; ASA-RS vs AC:  $P < 0.001$ ; ASA-URT vs AC:  $P < 0.0001$ ; Table 1).

#### Sensitivity and specificity of CAST-ELISA in ASA intolerance

The gold standard in the diagnosis of ASA intolerance is to present the combination of a positive history and a positive placebo-controlled challenge test. In both control groups, the mean sLT concentration + 3SD in response to NSAIDs was calculated (e.g. mean + 3SD in ASA 200 µg/mL + C5a: 260.3 pg/mL for AC). None of the 50 controls (NC and AC) exceeded these values as shown in Fig. 2 (specificity > 99%). However, in ASA-intolerant patients a high proportion of individuals was above this concentration. The proportion depended predominantly on the stimulation procedure and reached the highest percentage in ASA (200 µg/mL) + C5a stimulated leucocytes. In patients with skin symptoms after ASA-ingestion (ASA-URT), up to 13/16 patients were positive in the CAST-ELISA (sensitivity 81.3%; Fig. 2). The sensitivity was (in decreasing order) ASA 200 µg/mL + C5a (13/16) < ASA 200 µg/mL (10/16) <

**Table 1.** Release of sLT in ASA-intolerant patients and controls *in vitro*<sup>a</sup>

|                        | sLT concentration (pg/mL) <sup>1</sup> |                 |              |              |
|------------------------|----------------------------------------|-----------------|--------------|--------------|
|                        | ASA-RS                                 | ASA-URT         | NC           | AC           |
| Unstimulated           | 105.3 ± 75.3                           | 111.1 ± 148.9   | 99.3 ± 44.5  | 104.3 ± 42.7 |
| ASA <sup>2</sup>       | 221.8 ± 167.7                          | 308.7 ± 155.1   | 132.5 ± 39.3 | 141.8 ± 51.3 |
| ASA + C5a <sup>3</sup> | 309.3 ± 198.3*                         | 440.4 ± 202.4** | 133.9 ± 35.7 | 152.6 ± 46.1 |

<sup>a</sup>Leucocytes were obtained from nonatopic controls (NC,  $n = 30$ ), atopic controls (AC,  $n = 20$ ), ASA-intolerant patients with predominantly airway-related symptoms (ASA-RS,  $n = 22$ ), and ASA-intolerant patients with predominantly skin symptoms (ASA-URT,  $n = 16$ ). sLT release by leucocytes was analysed in response to the mentioned stimuli. (Data for stimulations with ASA 20 µg/mL and ASA 20 µg/mL + C5a: not shown.)

<sup>1</sup>sLT: mean sulfidoleucotriene concentration ± SD. <sup>2</sup>ASA: acetyl salicylic acid (200 µg/mL).

<sup>3</sup>ASA + C5a: ASA (200 µg/mL) + C5a ( $10^{-7}$  mol/L). \*ASA-RS vs AC:  $P < 0.001$ . \*\*ASA-URT vs AC:  $P < 0.0001$ .

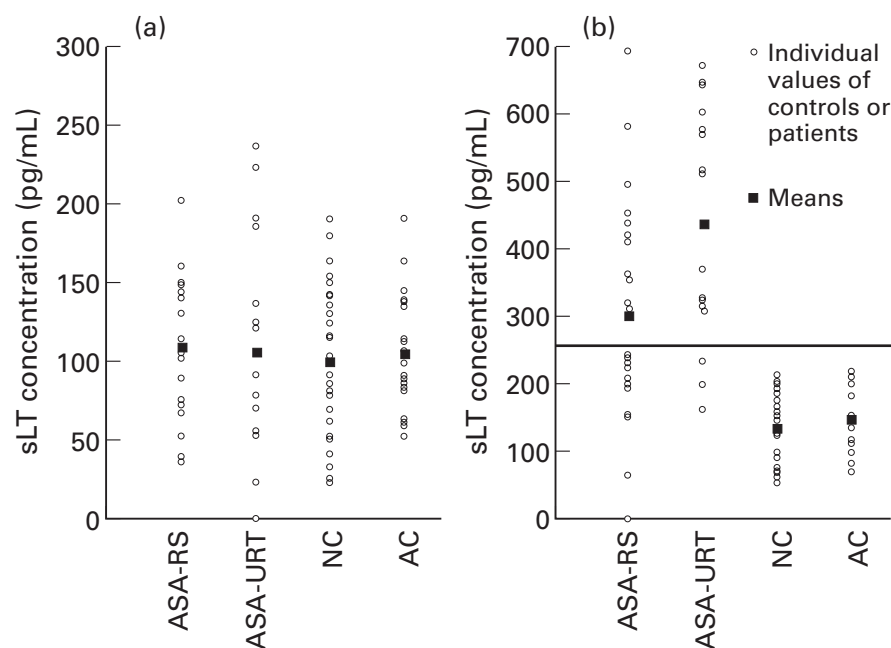


**Fig. 2.** sLT release in resting leucocytes (a) and in response to ASA (200 µg/mL) + C5a (b) in ASA-intolerant patients (ASA-RS and ASA-URT) and in controls (NC and AC). Note that none of AC or NC individuals exceeds the mean + 3SD of AC or NC group. The horizontal line in Fig. 1(b) represents the mean + 3SD of the control group (cutoff). Since in general mean + 3SD in AC individuals slightly exceeded the one in NC individuals, the AC group was used for the calculation of the cutoff.

ASA 20 µg/mL + C5a (8/16) < ASA 20 µg/mL (6/16). In patients with predominantly airway-related symptoms (ASA-RS), however, only up to 11/22 had sLT concentrations above the mean + 3SD. The sensitivities in decreasing order: ASA 200 µg/mL + C5a (11/22) < ASA 20 µg/mL (6/22) < ASA 200 µg/mL (4/22) < ASA 20 µg/mL + C5a (3/22). If all ASA-stimulation procedures are considered simultaneously, in ASA-URT patients 15/16 patients had a positive CAST-ELISA (two more positive CAST in ASA 200 µg/mL), whereas in ASA-RS patients only one more patient have a positive CAST-ELISA in the other stimulation procedures

(ASA 20 µg/mL + C5a). None of the NC or AC individuals had a positive CAST in any of the four tested ASA conditions.

*Increase in sensitivity can be achieved by simultaneous measurements of sLT release induced by different NSAIDs in vitro*

Although there is a general consensus that a positive CAST correlates well with oral challenge tests using ASA, the sensitivity of the CAST-ELISA in the diagnosis of NSAID intolerance is limited which is demonstrated by our data: 11/

|                            | ASA-RS (total)     |                    | ASA-RS (budesonide therapy) |       | ASA-URT |       |
|----------------------------|--------------------|--------------------|-----------------------------|-------|---------|-------|
|                            | Sens. <sup>1</sup> | Spec. <sup>1</sup> | Sens.                       | Spec. | Sens.   | Spec. |
| ASA 200 <sup>2</sup>       | 18.2               | >99.0              | 9.1                         | >99.0 | 62.5    | >99.0 |
| ASA 200 + C5a <sup>3</sup> | 50.0               | >99.0              | 45.5                        | >99.0 | 81.3    | >99.0 |
| ASA 20 <sup>2</sup>        | 27.3               | >99.0              | 9.1                         | >99.0 | 37.5    | >99.0 |
| ASA 20 + C5a <sup>3</sup>  | 13.6               | >99.0              | 9.1                         | >99.0 | 50.0    | >99.0 |
| Diclofenac <sup>4</sup>    | 31.8               | >99.0              | 27.3                        | >99.0 | 25.0    | >99.0 |
| Indomethacin <sup>4</sup>  | 40.9               | 96.7               | 27.3                        | 96.7  | 31.3    | 96.7  |
| Ibuprofen <sup>4</sup>     | 0.0                | >99.0              | 0.0                         | >99.0 | 0.0     | >99.0 |
| All tests                  | 72.7               | 96.7               | 72.7                        | 96.7  | 100.0   | 96.7  |

<sup>1</sup>Sens. = sensitivity (%), Spec. = specificity (%). <sup>2</sup>ASA was used in concentrations of 200 µg/mL and 20 µg/mL, respectively. <sup>3</sup>C5a was used in a concentration of 10<sup>-7</sup> mol/L. <sup>4</sup>Diclofenac, indomethacin and ibuprofen were used in concentrations of 20 µg/mL.

**Table 2.** Sensitivity and specificity of *in vitro* sLT release in response to different NSAIDs

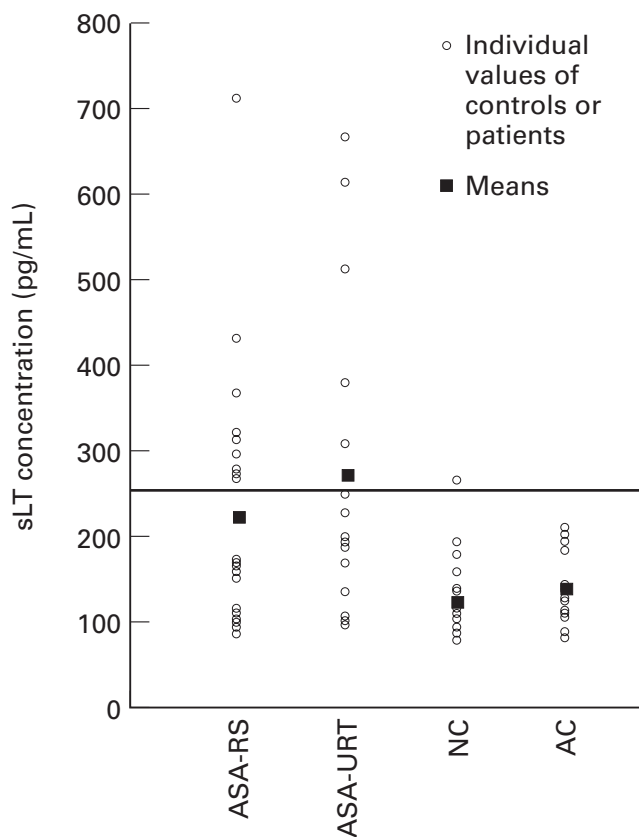


**Fig. 3.** sLT release in response to indomethacin (20  $\mu$ g/mL) in ASA-intolerant patients (ASA-RS and ASA-URT) and in controls (NC and AC). Note that one control from NC individuals exceeds the mean + 3SD of the NC and AC group. The horizontal line represents the mean + 3SD of the AC group (cutoff). Since in general mean + 3SD in the AC group slightly exceeded the one in the NC group, the AC group was used for the calculation of the cutoff.

22 ASA-RS patients and 3/16 ASA-URT patients are below the mean + 3SD (cutoff) of AC individuals when stimulated with ASA + C5a (Fig. 2). Since various drugs, most of which are prostaglandin inhibitors, 'cross-react' with ASA in intolerant patients [2,4], and 'cross-reactivity' with indomethacin up to 100% has been reported [2], we wondered whether *in vitro* sLT release in response to NSAIDs other than ASA could increase the sensitivity of the CAST-ELISA. Therefore, we tested *in vitro* sLT release by leucocytes obtained from ASA-tolerant individuals (NC and AC), and from ASA-intolerant patients (ASA-RS and ASA-URT) in response to indomethacin, diclofenac, and ibuprofen. Means + 3SD (cutoff) were calculated for each NSAID. One out of 50 controls (one NC individual) exceeded this sLT level in response to indomethacin, whereas 50/50 controls (NC and AC) were below this limit in response to diclofenac and ibuprofen. Table 2

shows the sensitivity and specificity of all stimulation procedures in the different patient groups for the tested NSAIDs. The most frequent *in vitro* cross-reacting compound was indomethacin followed by diclofenac. In ASA-intolerant patients, 40.9% (9/22) of ASA-RS patients and 31.3% (5/16) of ASA-URT patients had a positive response to indomethacin (Table 2 and Fig. 3). Interestingly, none of the ASA-intolerant patients had a positive CAST in response to ibuprofen. Due to the measurement of sLT release in response to indomethacin and diclofenac, 5/22 additional ASA-RS patients resulting in a total number of 16/22 had a positive CAST (i.e. sLT release  $\geq$  mean + 3SD). In ASA-URT patients, 3/16 additional patients were positive resulting in 16/16 patients with positive CAST results (Fig. 3, Table 2). This indicates that analysis of sLT release in response to indomethacin and diclofenac markedly increases the sensitivity in the diagnosis of ASA intolerance (from 11/22, 50% to 16/22, 72.7% in ASA-RS patients and from 13/16, 81% to 16/16, 100% in ASA-URT patients). The decrease in specificity, however, was very limited. Table 3 shows the results for 14 ASA-intolerant patients from Fig. 2 who were negative in the CAST in response to ASA (200  $\mu$ g/mL) + C5a. In this subgroup again, sLT release in response to indomethacin caused the most pronounced increase in the sensitivity of the CAST compared with stimulation with diclofenac, ibuprofen or stimulation with the lower ASA concentration.

The degree of ASA sensitivity as judged by *in vivo* ASA challenge was compared with the result of *in vitro* sLT release in response to ASA. ASA-URT patients showed objective symptoms after a cumulative dose 1.087–512 mg ASA (median: 900 mg). In ASA-URT patients, the cumulative oral dose in patients with a positive CAST-ELISA in response to ASA (13/16) tended to be lower ( $977 \pm 449$ , median: 900 mg) compared with CAST-ELISA negative patients (3/16)  $1566 \pm 577$  (median: 1900 mg). ASA-RS patients showed objective symptoms in the bronchial challenge tests after  $8.9 \pm 2.7$  mg ASA. In ASA-RS patients with a positive CAST-ELISA in response to ASA (11/22) the cumulative dose which was necessary to induce objective symptoms was not significantly lower compared with CAST-ELISA negative ASA-RS patients ( $7.9 \pm 2.2$  mg, median: 7.5 mg vs  $10.5 \pm 2.7$  mg, median: 10 mg).

Absolute peripheral eosinophil and basophil number was measured and correlated with the result of the CAST-ELISA. There was no significant difference in the number of eosinophils or basophils in CAST-ELISA positive patients (e.g. in response to ASA 200  $\mu$ g/mL + C5a) compared with negative patients (data not shown). However, there was a trend of higher eosinophil numbers in CAST-ELISA positive patients (ASA 200  $\mu$ g/mL + C5a) compared with CAST-ELISA negative patients in the ASA-RS subgroup.

**Table 3.** CAST results in response to different NSAIDs<sup>a</sup>

| Stimulation group<br>Pat. # | ASA200 + C5a <sup>1</sup> | ASA20 + C5a <sup>1</sup> | Indo <sup>2</sup> | Diclo <sup>2</sup> | Therapy <sup>3</sup> |
|-----------------------------|---------------------------|--------------------------|-------------------|--------------------|----------------------|
| <i>ASA-RS</i> <sup>4</sup>  |                           |                          |                   |                    |                      |
| 1                           | Neg. <sup>5</sup>         | Neg.                     | Pos. <sup>5</sup> | Neg.               | No                   |
| 2                           | Neg.                      | Neg.                     | Neg.              | Neg.               | Yes                  |
| 3                           | Neg.                      | Pos.                     | Pos.              | Neg.               | No                   |
| 4                           | Neg.                      | Neg.                     | Neg.              | Pos.               | Yes                  |
| 5                           | Neg.                      | Neg.                     | Pos.              | Neg.               | Yes                  |
| 6                           | Neg.                      | Neg.                     | Pos.              | Neg.               | Yes                  |
| 7                           | Neg.                      | Neg.                     | Neg.              | Neg.               | No                   |
| 8                           | Neg.                      | Neg.                     | Neg.              | Neg.               | Yes                  |
| 9                           | Neg.                      | Neg.                     | Neg.              | Neg.               | No                   |
| 10                          | Neg.                      | Neg.                     | Neg.              | Neg.               | No                   |
| 11                          | Neg.                      | Neg.                     | Neg.              | Neg.               | Yes                  |
| <i>ASA-URT</i> <sup>4</sup> |                           |                          |                   |                    |                      |
| 1                           | Neg.                      | Neg.                     | Pos.              | Neg.               | No                   |
| 2                           | Neg.                      | Neg.                     | Neg.              | Pos.               | No                   |
| 3                           | Neg.                      | Pos.                     | Pos.              | Neg.               | No                   |

<sup>a</sup>All patients and controls were stimulated with NSAIDs as mentioned in the Materials and methods section. The results of 14 ASA-intolerant patients with a negative test result (i.e. below the mean + 3sd) in response to ASA (200 µg/mL) + C5a (10<sup>-7</sup> mol/L) as shown in Fig. 2 are displayed here. The *in vitro* cross-intolerance increases the sensitivity of the CAST-ELISA for the establishment of the diagnosis.

<sup>1</sup>ASA200 + C5a/ASA20 + C5a: acetyl salicylic acid 200 and 20 µg/mL, respectively. C5a 10<sup>-7</sup> mol/L.

<sup>2</sup>Diclo/indo: diclofenac or indomethacin, 20 µg/mL.

<sup>3</sup>Therapy: yes = local budesonide 24 h before venipuncture; no = no therapy.

<sup>4</sup>ASA-RS/ASA-URT: ASA-intolerant patients with predominantly airway-related symptoms (ASA-RS) or with predominantly skin symptoms (ASA-URT).

<sup>5</sup>Neg./pos.: sLT release by leucocytes in response to NSAIDs: < mean + 3sd of atopic controls—negative, ≥ mean + 3sd of atopic control—positive.

## Discussion

The diagnosis of ASA intolerance is based mainly on history, clinical manifestation and verification by potentially harmful challenge tests. Since immune mechanisms are not involved in intolerance reactions, skin tests and determination of IgE antibodies are not suitable diagnostic procedures. Other tests, such as basophil histamine release by platelet-activating factor [22], determination of platelet hydrogen peroxide metabolism [23] and urine LTE<sub>4</sub> determination [8,9] have not found a place in routine diagnostic procedures. In contrast, there is growing, but still limited experience in determination of sLT release in the supernatants of resting and NSAID-challenged leucocytes in intolerance reactions which has been reviewed recently [18]. In six studies, a total of 63 patients with intolerance to ASA were examined. In these studies, a sensitivity of >66% with a high specificity was observed. The largest study consisted of 15 patients and 10 controls [24]. However, the necessity to use freshly isolated

leucocytes from untreated patients as well as the limited sensitivity of the test in ASA intolerance are still major disadvantages that reduce its diagnostic use for routine purposes.

In the present study we analysed a larger group of ASA-intolerant patients (*n* = 38) and nonatopic and atopic controls (*n* = 30 and *n* = 20, respectively). We showed that storing blood samples for 24 h at room temperature has no influence on baseline and ionomycin- and FcεRIα-stimulated sLT release. This indicates that the cells remain viable and retain an active metabolism. We therefore conclude that the shipping of blood samples to specialized laboratories does not interfere with the test and increases the suitability of the test for routine purposes.

ASA itself significantly increased sLT release by leucocytes from controls and ASA-intolerant patients compared with unstimulated cells. This has not been shown previously which is probably due to the larger number of individuals analysed in this study compared with others [12]. In the

presence of ASA and C5a a more pronounced and highly significant increase of sLT release was observed in both groups of pseudoallergic patients compared with both control groups. The sensitivity and specificity of the test was in the range published by others when an ASA concentration of 200 µg/mL was chosen (sensitivity up to 81.3%, specificity >99%). In one subgroup (ASA-RS), however, the sensitivity of the assay was below the published data [18] which may be due to differences in the severity of the disease in the two ASA-intolerant groups.

It has been shown that different, chemically unrelated NSAIDs such as ASA, indomethacin and diclofenac have the inhibition of cyclooxygenase in common [6–10]. Furthermore, a high degree of 'cross-reactivity' among several NSAIDs has been reported which may be a consequence of the shared inhibition of the cyclooxygenase pathway [1,2,4]. Even ASA-pseudoallergic patients who have not taken indomethacin before developed intolerance symptoms after indomethacin provocation [1]. Finally, after tolerance induction by ASA in patients with intolerance to ASA, indomethacin and diclofenac, a state of tolerance to ASA and to the other NSAIDs was achieved [25]. Therefore, we decided to perform *in vitro* tests with NSAIDs other than ASA in ASA-intolerant patients. Using this approach the sensitivity of the test was highly increased (from 50 to 72.7% in ASA-RS patients and from 81.3 to 100% in ASA-URT patients). Most importantly, the specificity of the test was only slightly reduced (from >99 to 96.7% based on the comparison with nonatopic controls; based on atopic controls it remained unaffected, i.e. >99%). We therefore believe that the analysis of sLT release in response to diclofenac and indomethacin will be of benefit in establishing the diagnosis of ASA intolerance. This is emphasized by the problem that the reproducibility of oral challenge tests as so-called gold standard in ASA intolerance is limited [26], indicating the necessity of additional test systems.

Interestingly, ibuprofen failed to induce sLT release by peripheral blood leucocytes in ASA-intolerant patients. The reason for this remains elusive. IC<sub>50</sub> values for COX-1 are 3.2 µmol/L for ASA, 0.037 µmol/L for diclofenac, 0.013 µmol/L for indomethacin, and 3.0 µmol/L for ibuprofen [27]. The NSAID concentrations used in this study exceed these by far (ASA concentration 111 µmol/L and 1.1 mmol/L, diclofenac 62.9 µmol/L, indomethacin 55.9 µmol/L, and ibuprofen 97.1 µmol/L), and therefore should be sufficient to inhibit COX-1. However, the ratio 'NSAID concentration divided by IC<sub>50</sub> concentration' for ibuprofen is 10–100 times lower compared with the other NSAIDs which were capable of sLT release *in vitro*. This may indicate that ibuprofen in this setting may have a lower COX-1-inhibiting potential compared with the other NSAIDs. Furthermore, ASA and indomethacin are time-dependent inhibitors of COX-1 whereas ibuprofen is a

time-independent inhibitor of COX-1 [28]. Prolonged incubations (e.g. 40 min as in this assay) of the enzyme with its inhibitor will increase the inhibitory effect of a time-dependent inhibitor, whereas the effect of a time-independent inhibitor will remain almost unaffected [28]. This may additionally account for a lower COX-1 inhibiting potential of ibuprofen compared with the other NSAIDs. Finally, a predominant COX-2 selectivity as the cause for the failure of ibuprofen to release sLT *in vitro* by leucocytes is not probable since ASA, indomethacin, and ibuprofen are all more potent inhibitors of COX-1 than COX-2 whereas diclofenac which was able to induce sLT release is an approximately equipotent inhibitor of COX-1 and COX-2 [29].

Several lines of evidence support the central role of leucotriene release in ASA intolerance, although their cellular source is unknown. The two most likely cells are the mast cell and the eosinophil [30–33]. In ASA-intolerant patients, bronchial, nasal, or oral ASA challenge induced mast cell infiltration, increased mast cell tryptase concentration in bronchoalveolar lavage, nasal fluid, and/or serum [30,32,33]. Furthermore, prevention of ASA-induced bronchoconstriction by pretreatment with cromolyn sodium or nedocromil sodium is compatible with an important role of mast cells in ASA intolerance [33]. However, increased numbers of eosinophils in the tissue after local ASA challenge, increased local or serum concentration of ECP demonstrate that eosinophils are also of major importance in ASA intolerance [30,31,34]. Since we found an increased sLT release by peripheral blood leucocytes in response to ASA in the majority of ASA-intolerant patients, mast cells can be excluded as the major sLT source in our experimental setting. Therefore, eosinophils or potentially basophils must be the predominant source of sLT. In our study, we have no statistically significant evidence that the number of peripheral blood eosinophils or basophils correlates with sLT release in response to NSAIDs. Depletion experiments may help to further characterize the leucocyte subset(s) responsible for ASA-induced sLT release *in vitro*.

It has been reported that most of the ASA-intolerant patients suffer either from asthma and/or rhinitis and/or nasal polyps or from urticaria and/or angioedema [1,19,35]. This was also the case with the majority of our patients which allowed us to divide them into two groups with airway-related or cutaneous symptoms. Interestingly, the sensitivity in patients suffering from cutaneous symptoms (ASA-URT) was much higher (up to 100%) compared with patients with airway symptoms (ASA-RS; up to 72.7%). The reason for this difference remains unclear but may indicate that the CAST-ELISA is superior in ASA intolerance associated with cutaneous to airway-related symptoms. Another possibility is a different distribution of disease severity in the two groups. Fifteen out of 16 ASA-URT patients were suffering from urticaria and angioedema.

Shock fragments (oedema of the tongue and shock index > 1.5) were additionally observed in 5/16 patients whereas in 1/22 ASA-RS patients from group 1 shock fragments have been documented. Glucocorticosteroid therapy was not responsible for the reduced sensitivity in ASA-RS patients because the sensitivity in patients with and without therapy was identical (Table 2). The identical sensitivity of the test in these two subgroups was unexpected even though the patients were on local glucocorticosteroids. Local glucocorticosteroid therapy is known to be associated with no or limited systemic effects depending on the substance and concentration used [36]. However, despite local application glucocorticosteroids are rapidly found in the systemic circulation even after nasal application [37]. This may be responsible for reductions in plasma and urinary cortisol concentrations that have been demonstrated in patients on intranasal glucocorticoid therapy [20,21].

We conclude that analysis of sLT release by isolated leucocytes is a highly sensitive and specific assay for the diagnosis of ASA intolerance even when stored blood samples are used. Simultaneous stimulations with NSAIDs other than ASA have to be performed to achieve the highest sensitivity of this assay. Since the results of this study—performed with the largest published, but still limited number of ASA-intolerant patients—are very promising, more ASA-intolerant patients in a multicentre study should be analysed to investigate whether this assay can substitute for the sometimes risky oral challenge tests.

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### References

- Samter M, Beers RF. Intolerance to aspirin. Clinical studies and consideration of its pathogenesis. *Ann Int Med* 1968; 68:975–83.
- Settipane GA. Aspirin and allergic diseases: a review. *Am J Med* 1983; 74:102–9.
- Szczeklik A. Mechanism of aspirin-induced asthma. *Allergy* 1997; 52:613–9.
- Gall H, Sterry W. Gibt es eine Analgetika-Intoleranz? *Dtsch Med Wschr* 1994; 119:480–1.
- Robinson DR, Skoskiewicz M, Bloch KJ et al. Cyclooxygenase blockade elevates leukotriene E4 production during acute anaphylaxis in sheep. *J Exp Med* 1986; 163:1509–17.
- Szczeklik A, Gryglewski RJ, Czerniawska-Mysik G. Relationship of inhibition of prostaglandin biosynthesis by analgesics to asthma attacks in aspirin-sensitive patients. *Br Med J* 1975; 1:67–9.
- Pinto S, Gallo O, Polli G et al. Cyclooxygenase and lipoxygenase metabolite generation in nasal polyps. *Prostaglandins Leukot Essent Fatty Acids* 1997; 57:533–7.
- Oosaki R, Mizushima Y, Mita H, Shida T, Akiyama K, Kobayashi M. Urinary leukotriene E4 and 11-dehydrothromboxane B2 in patients with aspirin-sensitive asthma. *Allergy* 1997; 52:470–3.
- Kumlin M, Stensvad F, Larsson L, Dahlen B, Dahlen S-E. Validation and application of a new simple strategy for measurements of urinary leukotriene E4 in humans. *Clin Exp Allergy* 1995; 25:467–79.
- Mewes T, Riechelmann H, Klimek L. Increased in vitro cysteinyl leukotriene release from blood leukocytes in patients with asthma, nasal polyps, and aspirin intolerance. *Allergy* 1996; 51:506–10.
- De Weck AL, Stadler BM, Urwyler A, Wehner HU, Bühlmann RP. Cellular allergen stimulation test (CAST): a new dimension in allergy diagnostics. *Allergy Clin Immunol News* 1993; 5:9–14.
- Czech W, Schöpf E, Kapp A. Release of sulfidoleukotrienes in vitro: Its relevance in the diagnosis of pseudoallergy to acetylsalicylic acid. *Inflamm Res* 1995; 44:291–5.
- Juergens UR, Christiansen SC, Stevenson DD, Zuraw BL. Inhibition of monocyte leukotriene B4 production after aspirin desensitization. *J Allergy Clin Immunol* 1995; 96:148–56.
- Wedi B, Elsner J, Kapp A. In vitro diagnostic of pseudo-allergic reactions – new aspects. *Allergy Clin Immunol Int* 1996; 8:113–5.
- Ameisen JC, Capron A, Joseph M et al. Aspirin-sensitive asthma: abnormal platelet response to drugs inducing asthma attacks. Diagnostic and physiopathological implications. *Int Arch Allergy Appl Immunol* 1985; 78:438–48.
- Wüthrich B. Acetylsalicylic acid pseudoallergy: an anomaly of thrombocyte function? *Hautarzt* 1988; 39:631–4.
- Cowburn AS, Sladek K, Soja J et al. Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998; 101:834–46.
- De Weck AL. Cellular allergen stimulation test (CAST) – an overview and critical evaluation of clinical application in allergy diagnosis. *Allergologie* 1997; 20:487–502.
- Szczeklik A, Gryglewski RJ, Czerniawska-Mysik G. Clinical patterns of hypersensitivity to non-steroidal and anti-inflammatory drugs and their pathogenesis. *J Allergy Clin Immunol* 1977; 60:276–84.
- Scadding GK, Lund VJ, Jaques LA. A placebo-controlled study of fluticasone propionate aqueous nasal spray and beclomethasone dipropionate in perennial rhinitis: efficacy in allergic and non-allergic perennial rhinitis. *Clin Exp Allergy* 1995; 25:737–43.
- Möller C, Mygind N. Nasal blockage in children with non-infectious rhinitis: consequences and treatment. *Allergy* 1997; 52 (Suppl. 40):45–51.
- Okuda Y, Hattori H, Takashima T et al. Basophil histamine release by platelet-activating factor in aspirin-sensitive subjects with asthma. *J Allergy Clin Immunol* 1990; 86:548–53.
- Pearson DJ, Suarez-Mendez VJ. Abnormal platelet hydrogen

- peroxide metabolism in aspirin hypersensitivity. *Clin Exp Allergy* 1990; 20:157–63.
- 24 Wolanczyk-Medrała A, Medrała W, Mtolepsy J, Liebhart J, Pietrzak E. CAST-ELISA test in diagnosis of aspirin-induced asthma. *J Invest Allergy Clin Immunol* 1997; 7:13–7.
- 25 Szmidt M, Grzelewska-Rzymowska I, Rznieski J. Tolerance to aspirin in aspirin-sensitive asthmatics. Methods of inducing the tolerance state and its influence on the course of asthma and rhinosinusitis. *J Invest Allergy Clin Immunol* 1993; 3:156–9.
- 26 Pleskow W, Stevenson DD, Mathison DA, Simon RA, Schatz M, Zeiger RS. Aspirin-sensitive rhinosinusitis/asthma: spectrum of adverse reactions to aspirin. *J Allergy Clin Immunol* 1983; 71:574–9.
- 27 Kawai S. Cyclooxygenase selectivity and the risk of gastrointestinal complications of various non-steroidal anti-inflammatory drugs: a clinical consideration. *Inflamm Res* 1998; 47:S102–S106.
- 28 Noreen Y, Ringbom T, Perera P, Danielson H, Bohlin L. Development of a radiochemical cyclooxygenase-1 and -2 in vitro assay for identification of natural products as inhibitors of prostaglandin biosynthesis. *J Nat Prod* 1998; 61:2–7.
- 29 Mitchell JA, Akaraseenont P, Thiernemann C, Flower RJ, Vane JR. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci USA* 1993; 90:11693–7.
- 30 Sladek K, Szczeklik A. Cysteinyl leukotrienes overproduction and mast cell activation in aspirin-provoked bronchospasm in asthma. *Eur Respir J* 1993; 6:391–9.
- 31 Sladek K, Dworski R, Soja J et al. Eicosanoids in bronchoalveolar lavage fluid of aspirin-intolerant patients with asthma after aspirin challenge. *Am J Respir Crit Care Med* 1994; 149:940–6.
- 32 Fischer AR, Rosenberg MA, Lilly CM et al. Direct evidence for a role of the mast cell in the nasal response to aspirin in aspirin-sensitive asthma. *J Allergy Clin Immunol* 1994; 94:1046–56.
- 33 Lane SJ, Lee TH. Mast cell effector mechanisms. *J Allergy Clin Immunol* 1996; 98:S67–S71.
- 34 Nasser S, Christie PE, Pfister R et al. Effect of endobronchial aspirin challenge on inflammatory cells in bronchial biopsy samples from aspirin-sensitive asthmatic subjects. *Thorax* 1996; 51:64–70.
- 35 Lumry WR, Curd JG, Zeiger RS, Pleskow WW, Stevenson DD. Aspirin-sensitive rhinosinusitis: the clinical syndrome and effects of aspirin administration. *J Allergy Clin Immunol* 1983; 71:580–7.
- 36 Barnes PJ. Inhaled glucocorticosteroids for asthma. *N Engl J Med* 1995; 332:868–75.
- 37 Akerlund A, Bende M, Intaglietta M, Arfors KE. The effect of oxymetazoline on nasal and mucosal blood flow in the rabbit as measured with laser-Doppler flowmeter. *Ann Otol Rhinol Laryngol* 1993; 102:123–6.