

Basophil Responsiveness and Clinical Picture of Acetylsalicylic Acid Intolerance

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Key Words

Acetylsalicylic acid hypersensitivity · Basophil activation test

Abstract

Background: Exposure to acetylsalicylic acid (ASA) may exacerbate respiratory or skin diseases or induce anaphylactoid reactions in apparently healthy individuals. We wanted to evaluate specific responsiveness of basophils to ASA in correlation with the clinical picture. **Methods:** We performed a prospective single-blind study of 59 subjects involved in clinical evaluation and/or ASA provocation testing. Whole blood basophils were stained with anti-CD63/CD123/HLA-DR mAbs after stimulation with 0.25 or 1 mg/ml ASA. **Results:** We found that 40 subjects were ASA tolerant and 19 were ASA intolerant. Both groups had comparable manifestations of asthma and/or rhinitis (13 in the tolerant and 9 in the intolerant group). Intolerant subjects showed significantly higher basophil responsiveness to ASA in comparison to tolerant subjects, which was concentration-dependent in both groups. The ratio between responses at 1 mg/ml of ASA and at baseline (activation index) was analyzed according to the clinical picture. We demonstrate that the activation index was higher only in the intolerant subjects with anaphylactoid reactions, but not in a subgroup of subjects with asthma/rhinitis. The ROC calculations show that the optimal threshold activation index was more than 2.18. The sensitiv-

ity was 80% and the specificity was 83% in the subgroup with anaphylactoid reactions. In the asthma/rhinitis subgroup, the sensitivity was 78% and the specificity was 50%. **Conclusions:** Our study demonstrates that there is a significantly higher in vitro basophil response to ASA in intolerant as compared to tolerant subjects. ROC analyses suggest that this measurement might only have a diagnostic value in subjects without asthma and/or rhinitis.

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Introduction

Exposure to acetylsalicylic acid (ASA) may exacerbate nasal and/or respiratory symptoms in sensitive individuals with asthma or cutaneous symptoms in patients with chronic urticaria [1]. Urticaria and angioedema may also occur in apparently healthy individuals [2]. The intolerance may manifest itself in a series of anaphylactoid reactions that can be life-threatening [3, 4].

At present, the mechanism of intolerance is not fully understood. ASA intolerance is believed to be due to its pharmacological effects and is not thought to be an immunological phenomenon [2, 5]. ASA inhibits the cyclooxygenase enzyme, which catalyzes the transformation of arachidonic acid to prostaglandins. Following the reduction cyclooxygenase activity after ASA exposure, ara-

chidonic acid is primarily metabolized to form leukotrienes, which are stronger inflammatory mediators and bronchoconstrictors than prostaglandins [5–7]. It has been shown that patients with ASA intolerance synthesize more leukotrienes and are more susceptible to the effects of leukotrienes as compared to individuals who tolerate ASA [5, 6, 8]. Clinical studies have shown that mast cell and basophil-derived mediators are also increased during ASA-induced reactions [9]. Other cells, such as monocytes, could also be involved in these reactions [10].

The diagnosis of ASA intolerance can be confirmed by patient history or with a provocation test, which can be oral, nasal or bronchial. The test usually takes 2 days and has some important limitations. First, previous studies have reported rather low reproducibility for positive provocation tests in patients with a positive history [11, 12]. Second, in vivo testing might be dangerous and uncomfortable for some patients [13]; therefore, in vitro methods are being developed.

One such method is the basophil CD63 activation test. This test is based on ASA-induced activation of basophils, followed by the measurement of the abundance of the CD63 surface marker. The results of previous publications conflict with those of some authors showing that ASA basophil testing is useful [14–17] and others showing that it is not [18, 19]. Because of these conflicting results, we conducted a prospective single-blind study to evaluate the specificity of basophil responsiveness to ASA, as well as to diagnostically compare these results between clinically different patients and matched tolerant subjects.

Materials and Methods

Study Subjects

We performed a prospective single-blind study in 59 subjects (mean age 46.8 years, 24 women, 35 men) recruited in the last 2 years. The majority of subjects were recruited for the assessment of hypersensitivity to nonsteroidal anti-inflammatory drugs (NSAIDs), and subjects with chronic urticaria were excluded from recruitment. All participants were subjected to the detailed anamnestic examination and the following tests: a standard skin prick test, optional functional pulmonary and methacholine testing and ASA basophil activation assay testing. In 80% of subjects we performed an oral provocation test with different NSAIDs, and in 71% of subjects we performed an oral provocation test with up to 933 mg of ASA. All subjects gave their written consent, and the study was approved by the National Medical Ethics Committee.

Basophil Activation Assay

A basophil activation test was performed using a BD FastImmune kit (BD Biosciences, San Jose, Calif., USA). Briefly, whole blood (with heparin anticoagulant) was preincubated with basophil stimulation buffer (Bühlmann Laboratories, Basel, Switzerland) containing different concentrations of ASA or with the positive controls, anti-FcεRI mAb (0.55 µg/ml; Bühlmann Laboratories) and fMLP (2 µM Sigma, St. Louis, Mo., USA) at 37°C in a water bath. For testing optimization, we first performed preliminary dose-response experiments with 6 different final concentrations of ASA: 10, 5, 2, 1, 0.5 and 0.25 mg/ml. We selected 2 optimal final concentrations of 1 and 0.25 mg/ml. These 2 concentrations demonstrated dose-response activation and no unspecific stimulation, which was observed for higher concentrations. We also preliminarily tested 3 different incubation times: 15, 30 and 60 min. Because there were no differences in specific activations after longer incubations, we selected an incubation time of 15 min, followed by chilling in a 4°C water bath to stop the degranulation. Thereafter, FITC anti-CD63 mAb, PE anti-CD123 mAb and PerCP-conjugated anti-HLA-DR mAb (BD Biosciences) were added and incubated with the sample for 20 min. Finally, the whole blood samples were lysed, washed, fixed and analyzed. At least 500 CD123-positive cells were acquired. Results are given as the percentage of basophils expressing CD63 [20, 21].

Statistical Analysis

The distribution of the data was analyzed by a Shapiro-Wilk test. Because the majority of the data showed a non-Gaussian distribution, we used nonparametric tests, such as the Mann-Whitney or Wilcoxon tests. To evaluate the possible diagnostic importance of basophil testing, we also performed ROC curve analysis. Probability values of $p < 0.05$ were accepted as significant. The results are presented as the median and interquartile range. Analyses were performed with GraphPad Prism 5.

Results

We found that 40 subjects were ASA tolerant and 19 subjects were ASA intolerant. In the majority of subjects, tolerance was confirmed by a negative provocation test (fig. 1a). In more than one third of the intolerant subjects, the diagnosis was confirmed with a positive provocation test, while in others, the diagnosis was based on a strong positive history of ASA intolerance, such as a clinical reaction clearly documented by a physician or recording of more than one event (fig. 1b). In the intolerant group, 13 patients also had a history of intolerance to other NSAIDs, whereas in the ASA-tolerant group, only 2 subjects demonstrated NSAID intolerance (fig. 1c). Both groups had comparable manifestations of asthma and/or rhinitis (13 in the tolerant group and 9 in the intolerant group; fig. 1d).

The detailed data on the CD63 activation test are presented in figure 2. There were no differences in baseline basophil CD63 responses or in the responses after fMLP

Fig. 1. Diagnostic evaluation of ASA intolerance (**a, b**), other NSAID intolerances (**c**) and chronic conditions (**d**) in all study subjects (n = 59).

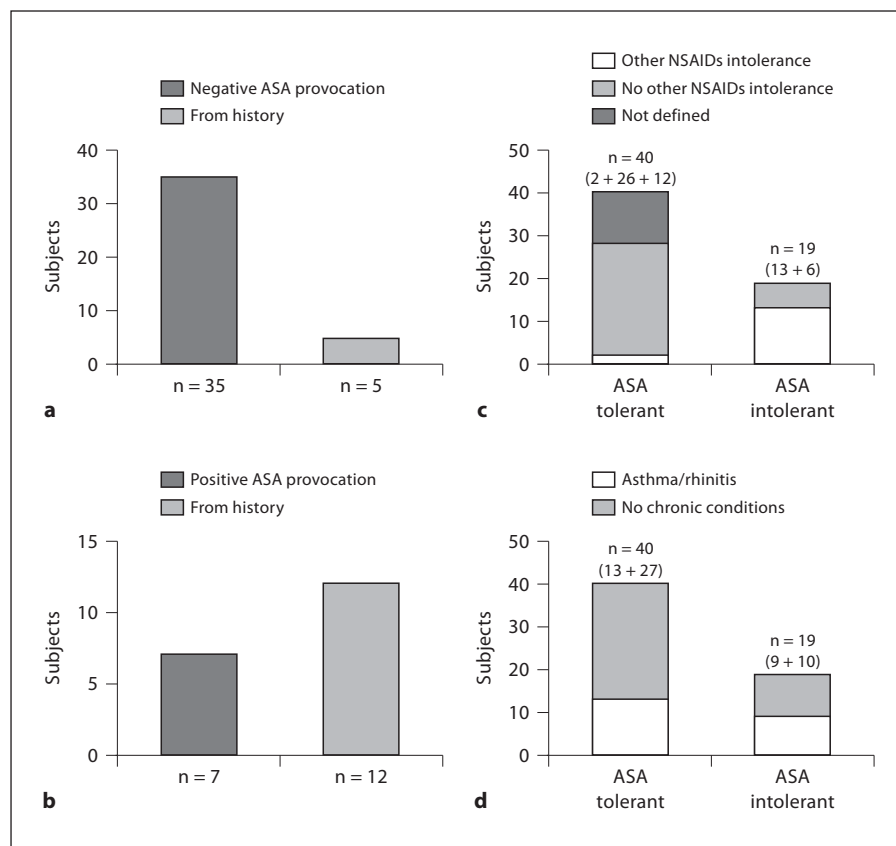


Fig. 2. In vitro basophil response determined by the percentage of surface CD63-expressing CD123+HLA-DR⁺ cells in all study subjects (n = 59). We found significantly higher responses to ASA in intolerant subjects (n = 19) than in tolerant (n = 40) subjects ($p \leq 0.01$; Mann-Whitney). This response was concentration dependent (0.25 vs. 1 mg/ml; $p \leq 0.001$; Wilcoxon test). Data are presented as the median and interquartile range. Baseline: stimulation with IL-3-containing buffer alone.

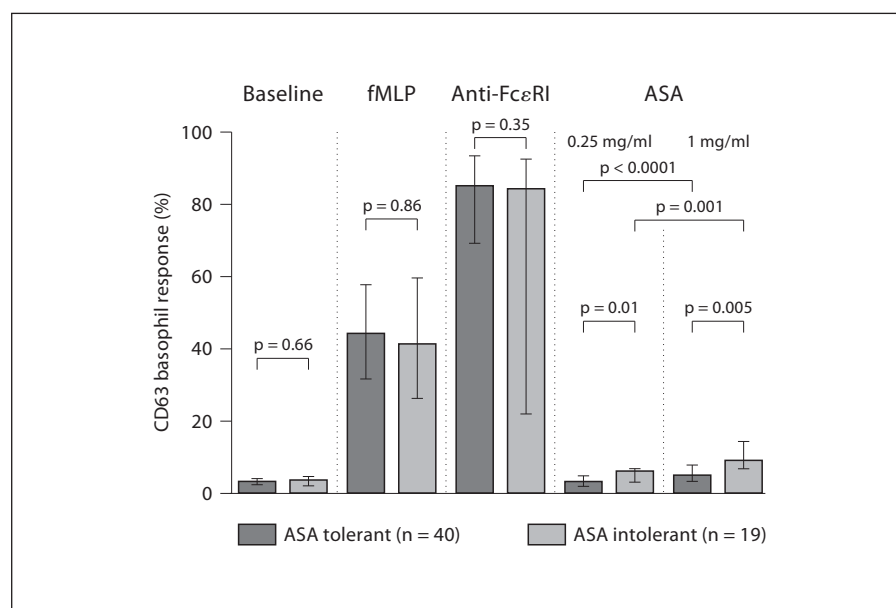
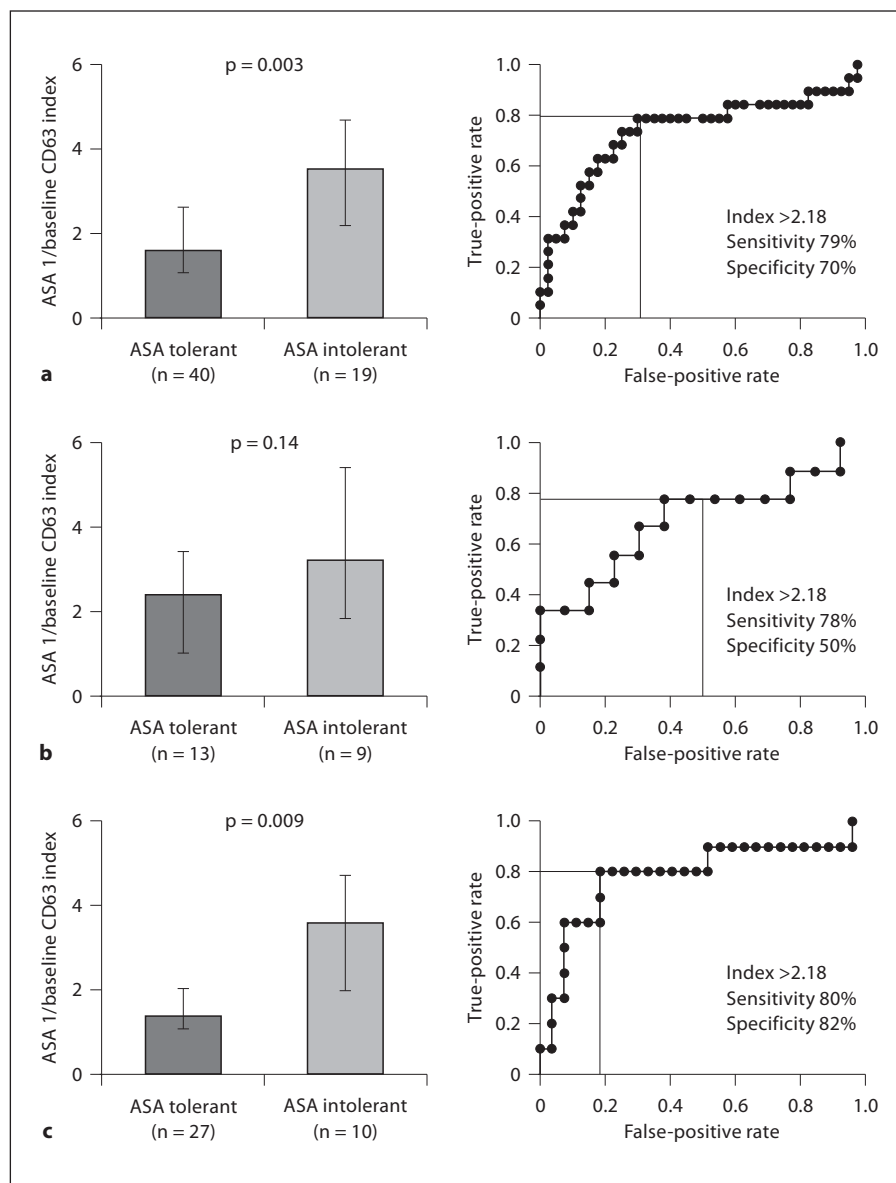


Fig. 3. In vitro basophil CD63 response determined as an index between the response to 1 mg/ml ASA and the baseline response and ROC curve analysis in all study subjects (**a**, $n = 59$), in the subgroup of asthma/rhinitis subjects (**b**, $n = 21$) and in apparently healthy subjects without chronic conditions (**c**, $n = 37$). The index was significantly higher in all intolerant subjects (**a**, $p = 0.003$; Mann-Whitney) and in intolerant subjects with anaphylactoid reactions (**c**, $p = 0.008$; Mann-Whitney). These differences were not significant in intolerant subjects with asthma/rhinitis (**b**). The areas under the ROC curves were 0.73 (**a**), 0.69 (**b**) and 0.79 (**c**), and the p values were 0.003, 0.13 and 0.008, respectively.



or anti-Fc ϵ RI mAb stimulation between the ASA-tolerant and ASA-intolerant groups. On the other hand, we found a significantly higher response to ASA stimulation in the intolerant group at both 0.25 and 1 mg/ml ASA concentrations in comparison to the tolerant group ($p \leq 0.01$). Moreover, these CD63 responses were concentration dependent and, therefore, cell specific, as we demonstrated higher responses at 1 than at 0.25 mg/ml of ASA ($p \leq 0.001$).

In the next step, we recalculated the index between the response to 1 mg/ml ASA and the baseline basophil response (activation index) according to previously pub-

lished reports [14–19]. We performed ROC curve analyses for this recalculation (fig. 3a–c). To evaluate possible diagnostic usefulness in different clinical presentations, we further divided the intolerant and tolerant groups in asthma/rhinitis and no chronic condition subgroups. We found higher activation indices in the intolerant subjects as a whole group ($p = 0.003$; fig. 3a); these differences were not due to intolerant asthma/rhinitis subjects ($p = 0.14$; fig. 3b) but only to intolerant subjects with anaphylactoid reactions ($p = 0.009$; fig. 3c). Moreover, ROC curve analyses confirmed this discrepancy, as the area under the ROC curve was 0.79 ($p = 0.008$) for the sub-

group without asthma/rhinitis (fig. 3c); the area under the ROC curve was 0.69 ($p = 0.13$) for the asthma/rhinitis subgroup (fig. 3b). The overall ROC area was 0.73 ($p = 0.003$; fig. 3a). The optimal recalculated activation threshold index was >2.18 , with a true-positive rate of 79% (sensitivity) and a false-positive rate of 30% (specificity of 70%) in all study subjects (fig. 3a). At this threshold (>2.18), the asthma/rhinitis subgroup had a true-positive rate of 78%, with a very poor false-positive rate of 50% (specificity of 50%; fig. 3b). In contrast, in the subjects with anaphylactoid reactions, the true-positive rate was 80% and the false-positive rate was 18% (specificity of 82%; fig. 3c).

Discussion

In this study, we demonstrated significantly higher *in vitro* basophil responsiveness to ASA stimulation in ASA-intolerant subjects than in ASA-tolerant subjects. We further performed ROC curve analyses to evaluate the possible diagnostic usefulness of ASA basophil testing. The results show that this testing is diagnostically useful only in apparently healthy subjects without chronic conditions, who can experience an anaphylactoid reaction after exposure to ASA. However, this did not prove to be useful in asthma/rhinitis subjects who experience exacerbation of nasal and/or respiratory symptoms after exposure to ASA. The reason for this discrepancy was the poor diagnostic specificity for the intolerant asthma/rhinitis subgroup in comparison to tolerant asthma/rhinitis subjects. The main purpose of this study was to compare, prospectively and in a blind manner, the results of routine clinical evaluation of ASA hypersensitivity with ASA basophile testing. Hence, there was no retrospective recall of previously defined patients and no recall for additional testing.

Basophil responsiveness to ASA is a controversial issue [14–19]. The main reasons for this controversy are poor understanding of the mechanisms of ASA-mediated basophil activation and the fact that the level of activation is markedly lower than in IgE-mediated responses to allergens. In our report, we clearly show that basophil responses to ASA are dose dependent, which has also been suggested by recent observations of De Weck et al. [17]. If the response is dose dependent, then ASA or its intermediates are targeting basophils in a cell-specific way. Notably, we know that beyond IgE, cells also have other types of basophil receptors, such as G protein-coupled receptors for complement component C5a.

What is of particular importance in the basophil activation assay is the ASA concentration; higher concentrations produced nonspecific activation. We found that an optimal final ASA concentration was 1 mg/ml, as the higher concentrations induce nonspecific activation. This optimal final concentration (1 or 1.25 mg/ml) was also used in recent reports by Celik et al. [19] and Rodríguez-Trabado et al. [16] and in two initial reports by Gamboa et al. [14] and Sanz et al. [15]. Celik et al. [19] and Sanz et al. [15] also demonstrated that 5 mg/ml ASA was obviously too high a concentration to induce specific responses. Therefore, we might conclude that higher concentrations eliminate the usefulness of the basophil response as a diagnostic test for ASA intolerance. Nevertheless, the optimal ASA final concentrations used in two recent reports were higher, with 2 mg/ml used by De Weck et al. [17] and 2.5 mg/ml used by Bavbek et al. [18].

The major inconsistency concerning previous reports is the diagnostic usefulness of ASA basophil testing. We suggest that this inconsistency might be associated with the different clinical presentations of ASA intolerance. Previous reports mainly dealt with heterogeneous groups with different manifestations of ASA intolerance and preferentially focused on ASA-exacerbated respiratory disease [14–19]. Importantly, basophils from subjects with asthma showed a primed phenotype with a significantly higher response to a rise in intracellular free calcium (i.e., thapsigargin stimulation) in comparison to basophils from healthy subjects [22]. In other words, the basophil phenotype in asthma/rhinitis subjects is different than in subjects without chronic conditions; therefore, to diagnostically evaluate ASA basophil responsiveness in intolerant asthma/rhinitis subjects, one should compare these subjects only to matched tolerant subjects with asthma/rhinitis. We demonstrate that ASA basophil testing only has a satisfactory diagnostic capability (sensitivity and specificity was more than 80%) in subjects with anaphylactoid reactions. This diagnostic utility was superior to that demonstrated in previous reports, which demonstrated sensitivities from 33 up to 43% and specificities from 79 up to 100% [14–16, 18]. However, the authors of these previous reports analyzed and compared various groups of ASA- and NSAID-intolerant patients. On the other hand, we found that due to very poor specificity (50%), basophil testing is useless in the diagnosis of ASA intolerance in asthma/rhinitis subjects. Similarly poor specificity (40%) concerning ASA intolerance in subjects with asthma was observed by Celik et al. [19].

In conclusion, this study provides further support for the evaluation of in vitro basophil ASA response testing for diagnostic and research purposes. We confirmed that this responsiveness is cell specific and that it is significantly higher in intolerant subjects. However, we clear-

ly demonstrated that this responsiveness measurement might have reasonable diagnostic value only in the subgroup of apparently healthy subjects who experience anaphylactoid reactions after exposure to ASA.

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