

## In vitro Tests: Basophil Activation Tests

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### Abstract

In recent years, the quantification of basophil activation by flow cytometry (basophil activation test, BAT) has proven to be a useful tool for the assessment of immediate-type responses to allergens mediated by IgE or other mechanisms in drug-allergic patients. To date, most BAT studies reported in the literature have used CD69 or CD203c as markers to quantify basophil activation after antigen-specific stimulation. Technical variations, such as the use of whole blood or isolated leukocytes, the addition of IL-3, the conditions of storage of the blood sample, the time of incubation with allergens and their concentration, can affect the results of the BATs. The BAT is more sensitive and specific than other in vitro diagnostic techniques in drug allergy. In various studies, its sensitivity in allergy to muscle-relaxant drugs ranges between 36 and 97.7%, with a specificity of around 95%. For  $\beta$ -lactam antibiotics, BAT sensitivity is 50% and its specificity 90%. For NSAIDs, sensitivity varies between 66 and 75%; specificity is about 93%. BAT is also a useful technique in the diagnosis of isolated cases of hypersensitivity to various other drugs and substances used in some therapeutic and diagnostic procedures.

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other kinds of allergic or pseudo-allergic reactions in which other activation mechanisms such as complement activation, non-IgE-mediated stimulation or non-immunological mechanisms are implicated [1].

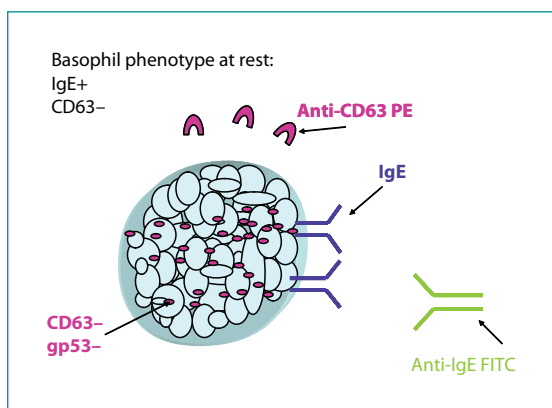
Basophils represent less than 0.5% of the total leukocytes in peripheral blood, which makes their purification difficult. Since these cells play an important role in immediate allergic reactions, some functional in vitro tests have been developed which detect their activation. One of the first was the histamine release test, a technique that did not find extensive clinical application due to its insufficient sensitivity and specificity [2]. This is why in the past few years several groups have taken advantage of flow cytometry and developed new tools to monitor basophil activation after antigen-specific stimulation using the expression of various membrane surface markers [3, 4].

### Introduction

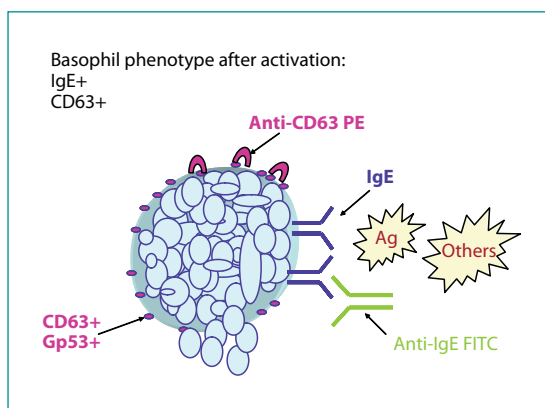
Peripheral blood basophils and tissue mast cells are primary effector cells in IgE-mediated immediate allergic reactions such as rhinitis, asthma and anaphylaxis. They may also be involved in

### Historical Background

The background of the flow cytometric basophil activation test (BAT) goes back to early studies performed by Nakagawa et al. [5], Knol et al. [6], Gane et al. [7], among others, and its application to allergy diagnosis by Sainte-Laudy et al. [3].



**Fig. 1.** Basophil phenotype at rest.



**Fig. 2.** Basophil phenotype after activation.

### Basis of the Basophil Activation Test

Flow cytometry is a useful tool for the analysis of different cellular types and can be used to identify specific cell populations, even those which are present in low amounts. It has proven to be useful in the study of allergen-induced activation [8]. In a first step the basis of these assays is the identification of basophils by specific fluorescent antibodies such as anti-IgE, anti-CD123 (IL-3 receptor) and anti-HLA-DR or anti-CCR3, and in a second step the demonstration of certain membrane phenotypes that appear after exposure to allergen. Upregulation has been described for expression of CD45 [7], CD11b and CD11c molecules, but downregulation in CD62L expression [3], as well as a decrease in the mean fluorescent intensity of IgE-carrying basophils after in vitro stimulus with the allergen [9].

However, most studies in the literature make reference to the expression of CD63 [10–12] or CD203c [13] on basophils after their in vitro activation. CD203c antibodies recognize a type-II transmembrane protein which is increased on the surface after activation.

Whether in the future the novel basophil identification antigen, CRTH2 (DP2) and the activation markers CD13, CD164 and CD107a, associ-

ated with degranulation, can be applied in flow-assisted allergy diagnosis and/or improve the technique remains to be established [8].

The basophils are able to release the content of their granules after an activation process dependent on the antigenic stimulus. Bridging of IgE receptors by the action of di- or plurivalent allergens provokes the intracytoplasmic fusion of the granules and the fusion of their membrane with the plasmatic cell membrane, followed by exocytosis of the granules (degranulation). Molecules present on the granule membrane, such as the CD63 molecule, are then expressed on the basophil membrane upon activation (fig. 1, 2).

CD63 is a tetraspan, 53-kDa granular protein that is expressed not only on basophil granules but also on monocytes, macrophages and platelets. The expression of this marker correlates with degranulation and histamine release, which makes it an ideal marker of basophil activation [14]. For in vitro stimulation with allergen, the peripheral blood cells are incubated with the suspected allergen for 15–40 min at 37°C. After stopping the reaction, the cells are labelled with anti-CD63-PE and anti-IgE-FITC monoclonal antibodies. Two controls are used: a negative control in which the cells are incubated with the stimulation buffer used in the assay and that often con-

tains IL-3 (negative control alias basal stimulation), and as a positive control, an anti-IgE or an anti-IgE receptor antibody can be used.

With the help of receiver-operated curves (ROC; optimal sensitivity versus specificity), the determination of a positivity cutoff must be made for each allergen in order to evaluate the results.

The BAT technique has generally been considered to be useful for in vitro allergologic diagnosis [8, 15, 16] and has been validated clinically for various allergens: such as inhalants [12, 17, 18], hymenoptera venoms [11, 19], latex [20–22], muscle relaxants [9, 23],  $\beta$ -lactam antibiotics [24–26], pyrazolones [27, 28], and NSAIDs [28, 29].

Recently, its diagnostic reliability has also been studied using recombinant allergens [30] in the monitoring of antigen-specific immunotherapy [31], in food allergy diagnosis [10, 32, 33] and in chronic urticaria [34, 35].

## Some Technical Aspects

### *Use of Whole Blood or Isolated Leukocytes*

The flow cytometry technique can be used with either whole blood or isolated leukocytes. Whole blood simplifies the manipulation (fewer centrifugation steps), but it has several disadvantages such as a decreased basophil recovery, possible interference with serum components (e.g. allergen-specific IgE and IgG antibodies) that can lead to decreased sensitivity, or serum components causing unspecific activation in controls [36], and interference by aggregated platelets also expressing CD63 markers, which can be responsible for inaccurate flow cytometric counting [3, 37, 38]. For protein allergens, these differences have not appeared to markedly affect the clinical diagnostic results. For drug allergy, on the other hand, several reports of results with a whole blood technique show a lower sensitivity than those obtained with isolated leukocytes (table 3).

### *Factors Affecting the Negative Control*

It is desirable to obtain an as low as possible negative control, particularly when investigating allergens causing a low specific stimulation, as is the case with drugs. In general, the negative control remains below 5% in 80% of the cases (of 504 cases: stimulation 0–5% in 79.9%; 5–10% in 13.6%; >10% in 6.5% of the cases).

Natural exposure in vivo to the allergen can cause high basal activation, for example in a pollen-allergic patient studied during the pollen season [39], although some authors disagree [18]. In the latter case, the use of whole blood with the corresponding increase in pollen-specific IgG during the season may have led to misinterpretation. High basal values have also been observed when a food-allergic patient has suffered a recent reaction or is presumably continuously exposed [10] and in patients with venom allergy undergoing immunotherapy [31].

There are also several causes likely to be responsible in vitro for a high basal value, particularly pyrogens and endotoxins that could contaminate the water used in the technique or other reactives such as heparin, preservatives or even some plastic tubes or microplates. It is therefore important to use ultrapure water and cell culture grade plastic material [40].

### *Factors Affecting the Positive Control*

Most studies use mono- or polyclonal anti-IgE in the assay, but it is known that a percentage of patients do not react to anti-IgE, either by histamine release [3] or sulfidoleukotriene production [41, 42]. Polyclonal anti-IgE is recommended because many monoclonal anti-IgE antibodies are poor activators. The percentage of nonreactors ranges between 15 and 25% depending on the authors [10, 43, 44], but this seems to apply essentially to histamine release. For BAT, the percentage of non-responders reported is usually lower, near or below 10% [8].

The sensitivity of the positive control can be improved using a monoclonal anti-IgE Fc recep-

tor (FcεR1) antibody instead of anti-IgE: this increases the activation percentage and the number of reactors [9]. In the largest series of patients investigated so far (n = 504) with the same reagents (FLOW CAST) within the frame of multicentric studies [45, 46], the percentage of true non-responders (negative for BAT and CAST) was 3.2% for CAST and 2.8% for BAT. An additional 10.5% were found to be negative for BAT but positive for CAST: they were then not true non-responders. It was later found that this dichotomy was due to dilution of the anti-IgE receptor antibody in a buffer not containing Ca<sup>+</sup> and Mg<sup>+</sup>. It was then confirmed that BAT is more sensitive than CAST to a low external Ca<sup>+</sup> concentration (results not shown).

If the positive control with anti-IgE is negative, a negative result with antigen cannot be interpreted properly. A negative control with anti-IgE seems to be more frequent in non-atopic patients [12].

However, there is no apparent correlation between the total IgE levels and the degree of basophil activation by anti-IgE (n = 104; r = 0.002; p = n.s., results not shown), which reflects that there is no relation between the basophil reactivity determined by BAT and the IgE level. This finding is in contradiction with what has been reported for histamine release and shows once more that both manifestations of basophil activation should not be entirely amalgamated.

#### *Effect of Blood Storage*

Blood sampling and storage for cellular tests, such as BAT, require some special conditions in order to obtain good cellular viability and functionality. The recovery of an acceptable number of reactive basophils depends on the medium, the storage time and the temperature of the blood sample. It has been shown that EDTA and ACD blood samples kept at 4°C maintain a suitable viability for at least 24 h, which is less the case for heparinized blood. In normal conditions at room

temperature, IgE-mediated reactions decrease markedly faster, probably due to the release of IgE suffered by the cells obtained ex vivo [10, 40]. At 48 h and 4°C, a sizeable response can still be observed, but for lower sensitivities, as it is the case with drugs, a greater number of false-negatives can be observed [10, 40].

#### *Effect of Time of Incubation with Allergen*

IgE-mediated activation is a relatively short process which reaches its peak within 10–15 min. Therefore, maximum activation of the basophils can be expected within this time range. However, if BAT is to be performed concomitantly with sulfidoleukotriene determination in the supernatant, a longer incubation time is recommended [47]. Some other manifestations of basophil activation, such as expression of CD203c [8] or activation by non-IgE mechanisms (e.g. C5a, fMLP) are faster and require only a few minutes to reach their peak.

#### *Effect of Allergen Concentration*

It is very important to determine the optimal concentrations that provoke the maximum cellular activation for each allergen by means of dose-response curves. If the concentration that provokes antigen-specific stimulation is narrow but shows great individual variations, it will be necessary to use several concentrations per allergen in diagnostic tests. If, on the contrary, the activation by allergen takes place over a broad concentration range, with few individual variations, it becomes possible to use a single protein allergen concentration. Here too, there seems to be some contrast between BAT and what has been recommended for histamine release tests. In contrast to histamine release, the dose-response curves for BAT appear to be sigmoid in most instances and not bell-shaped.

For small molecule-like drugs, the range of concentrations to which the cells respond is usually narrow; for example, 0.001–0.0001 mM for muscle relaxants [9]. In any case, for drugs, the

use of at least two consecutive concentrations around the optimal range is recommended.

It is essential to use allergens or drugs free of preservative agents, such as glycerol, pyrogens, and any substances that could provoke unspecific activation or, on the contrary, cytotoxicity. For each new drug to be tested, it is necessary to include appropriate healthy controls tolerating the drug. ROC must be established in order to determine the correct cutoff points.

#### *Preactivation with IL-3*

Pre- or simultaneous incubation with IL-3 has been found essential for optimal sulfidoleukotriene production (CAST) [47] and also increases histamine release [47], but there is no general consensus on its need for improving the sensitivity of BAT. Some authors have reported that it increases CD63 expression, increasing thereby the assay's sensitivity, which is relevant for allergens causing little specific stimulation, such as drugs [8].

#### *Reproducibility*

Intra-assay reproducibility assessed by duplicate determinations for different allergens is very high, which allows performance of a single test for each allergen concentration [48]. The reproducibility of BAT was found in drug allergy to be quite remarkable with an intra-assay variation coefficient of 0.89 ( $p < 0.0001$ ) for  $\beta$ -lactams, 0.99 ( $p < 0.0001$ ) for metamizol and 0.92 ( $p < 0.0001$ ) for NSAIDs.

#### *Evaluation of Results*

For appropriate evaluation of the results, two values should be taken into account: (1) the absolute number of basophils evaluated, which should be over 150, and (2) the percentage of activated basophils. In the negative (non-stimulated) control, the percentage of activated basophils is usually below 5%. The positive control after activation of the cells with anti-IgE anti-receptor has been discussed above.

Some authors also describe a decreased mean fluorescence emitted by the cells activated in vitro by allergen [9, 19, 49]; a phenomenon which does not seem to affect the percentage of basophils expressing CD63.

#### *BAT Positivity Criteria*

To establish cutoff points for each allergen, it is necessary to set up ROC. It is also important to take into account the following considerations: (1) the negative control can show variable values, although it is usually  $< 5\%$ , and (2) some allergens, particularly foods, can provoke unspecific stimulations.

Other allergens, such as drugs, usually cause lower responses than those obtained with protein allergens.

In our experience, the cutoff points offering the highest specificity and sensitivity values determined by ROC [25, 28] are the following: for inhalant allergens  $> 15\%$ ; food allergens  $> 15\%$ ; latex  $> 10\%$ , hymenoptera venoms  $> 10\%$ ;  $\beta$ -lactam antibiotics  $> 5\%$  and SI  $> 2$ ; metamizol  $> 5\%$  and SI  $> 5$ , and aspirin (ASA) and NSAIDs  $> 5\%$  and SI  $> 2$  (SI: stimulation index = stimulation by allergen/basal stimulation or negative control).

Some authors found a correlation between the degree of clinical sensitivity and the percentage of activated basophils [36], but other authors have not confirmed this observation [10]. For muscle relaxants, a correlation between the degree of skin sensitivity and CD63 expression has been reported [9].

#### *Comparison with Other in vitro Diagnostic Techniques*

Comparisons among various in vitro tests have been made for several allergens. The BAT usually shows a good correlation with histamine release and/or sulfidoleukotriene determinations (CAST) [10, 12, 25, 50], but BAT seems more sensitive and specific than the other tests [10, 51].

For diagnosis of immediate reactions to drugs in general [49] and for immediate reactions to  $\beta$ -

**Table 1.** BAT in allergy to muscle relaxants

Reference	Clinical manifestations	Number of patients	BAT sensitivity, %	BAT specificity, %
Abuaf et al. [9]	Anaphylaxis	41	64	93
Monneret et al. [53]	Anaphylaxis	39	54	100
Sudheer et al. [55]	Anaphylaxis	21	79	100
Kvedariene et al. [56]	Anaphylaxis	47	36	93
Ebo et al. [57]	Anaphylaxis	14	91.7	100

lactams in particular, the joint use of BAT and CAST offers better results and allows detection of up to 80% of the cases. The same holds true for pyrazolones [51].

### Clinical Applications of BAT to Diagnosis of Drug Allergy

#### *BAT in Diagnosis of Hypersensitivity Reactions to Neuromuscular-Blocking Agents*

Several reports have been published relating the usefulness of the BAT technique in the diagnosis of hypersensitivity reactions to muscle relaxants (table 1).

Abuaf et al. [9] studied basophil activation to muscle relaxants, such as suxamethonium, gallamine, vecuronium, and pancuronium. In a series of 41 patients, they observed a characteristic dose-response curve, but indicated that high drug concentrations can act as unspecific basophil activators. The sensitivity of the technique reported by them is 64% and the specificity 93%.

Monneret et al. [4] applied a three-colored flow cytometry assay (IgE, CD45, CD63) in 4 patients with muscle relaxant-induced anaphylaxis, observing that the results correlate with intradermal tests. In a subsequent study in 39 patients with reactions to muscle relaxants, the same authors found a sensitivity of BAT of 54% with a specificity of 100% [23, 53].

Aly Hassan et al. [54] published good results of the BAT technique in suxamethonium allergy. More recently, Sudheer et al. [55] compared the CD63 and CD203c expressions and measured histamine release in 21 patients who were referred with possible perioperative anaphylaxis. The sensitivity of CD63, CD203c, basophil histamine release and skin prick test for the muscle relaxants was found to be 79, 36, 36 and 64%, respectively.

In 47 patients Kvedariene et al. [56] observed a sensitivity of 36% and a specificity of 93% when performing the Basotest (CD63 in whole blood). On the other hand, Ebo et al. [57] reported the sensitivity of a similar BAT technique to be 91.7% and 100% specificity for rocuronium in a study including 14 patients with anaphylaxis and positive skin tests to rocuronium.

#### *BAT in $\beta$ -Lactam Allergy*

We studied the diagnostic reliability of the BAT and CAST techniques in a series of 81 patients with immediate allergy to  $\beta$ -lactams and in 30 healthy controls [25, 48]. BAT (FLOW-CAST) and sulfidoleukotriene determination by CAST ELISA (Bühlmann Laboratories, Allschwil, Switzerland), as well as specific IgE determination (CAP-FEIA, Phadia, Uppsala, Sweden) were performed. The results are discussed in detail below.

#### BAT in $\beta$ -Lactam-Allergic Patients with Positive Skin Tests

The patients included in this study presented with anaphylaxis or urticaria-angioedema with-