

Basophil Activation Test in Allergy: Time for an Update?

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Key Words

Allergen challenge • Allergy • Anti-IgE • Basophil activation test • CD203c • CD63 • Flow cytometry

Abstract

A wide range of reported evidence in the literature has shown that 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 allergic patients. The usefulness of BAT in anaphylactic adverse reactions, late-onset allergy and immunotherapy follow-up has also been demonstrated. To date, most BAT studies reported in the literature involved the capture of basophils only with a fluorochrome-labeled anti-IgE antibody and application of CD63 upregulation in order to evaluate the basophil response to allergens. Many issues need to be addressed, such as optimizing the analytical performance of the test, checking preanalytical conditions, the selection of the flow cytometry best gating protocol, the introduction of new algorithms and parameters, the search for new activation markers and the introduction of anti-IgE controls. BAT is certainly a useful technique, also for isolated cases of hypersensitivity to various other compounds and drugs, and an update of its application is certainly an interesting topic to expand the debate on allergy diagnosis.

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Introduction

Although more than 10 years have elapsed since the first basophil activation test (BAT) was successfully established, and although it is still the only routine cellular assay for allergy diagnosis, its use shows difficulty in having an appeal. In the diagnosis of allergy, skin testing and quantification of serum levels of allergen-specific IgE are considered the gold standards for routine laboratory investigations, even though variability in results and discrepancies between interpretation and clinical assessment have been clearly demonstrated. For example, levels of serum IgE (sIgE) tend to decrease over time, as has been shown with drug hypersensitivity, and this may affect the correlation with cellular tests, although a decrease in sensitivity has also been reported for BAT in certain circumstances [1]. These issues clearly illustrate the difficulty in defining a consensus in the investigation of allergy, which is largely explained by the multiple factors which have to be taken into account. This demonstrates why there is still a need for reliable in vitro methods to complement the usual investigations into allergy when they show discrepancies or are not feasible.

Functional in vitro tests are focused on basophils since, in contrast to mast cells, basophils are circulating cells and are easy to manipulate. Nevertheless, their very low number constitutes a true limitation to research.

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Concentration steps may be a good alternative, but they are time-consuming, expensive and may directly increase the variability of the results. A possible preliminary functional evaluation would be to measure mediator release, such as histamine or leukotrienes, following allergen-induced activation, but this approach involves a number of difficulties, mainly due to cell releasability, which depends on several experimental issues.

The attempt to standardize basophil activation elicited by allergens for pharmacological investigation [2] and the discovery of the first monoclonal antibodies able to target membrane molecules upregulated upon allergic activation [3] led some authors to use flow cytometry to investigate the basophil response to agents able to trigger an IgE-mediated response [4–6]. Different activation markers (CD63 or CD203c) with distinct modes of regulation have been used after stimulation with various allergens. Sensitized basophils express a very high amount of surface IgE immunoglobulins bound to FcεRI. When cells are triggered by different stimulants, for example allergens, they upregulate several molecules on the cell membrane, for example the tetraspanin CD63 or the ectoenzyme CD203c.

One of the first applications of BAT was in IgE-mediated food allergy diagnosis; in a work by Moneret-Vautrin et al. [7], only about 60% of basophils released LTC₄ and upregulated CD63, and probably these percentages would be lower if one excludes those subjects with negative sIgE replaced by passive sensitization. It was already clear by then that there was no correlation among skin tests, provocation tests, sIgE levels and BAT. Some attempt to address this issue was made by optimizing the BAT technical approach, for example by improving basophil capture in flow cytometry by adding a CD45 marker [8]. Although some improvements were made, several experiments showed that there was not always a linear relationship between allergen type and/or dose and basophil response measured as CD63 upregulation; furthermore, in vitro spontaneous priming of basophils by handling the cells can easily occur, and the time interval between venous blood withdrawal and sampling, the working temperature and buffer pH and ionic strength play a critical role in CD63 upregulation [9]. So, when functional assays are performed on basophils, no flow cytometry parameter currently used in BAT is able to fully highlight the activation stage and potential responsiveness of tested basophils. Interleukin-3 (IL-3) used in BAT might even worsen this scenario. Basophils are able to produce IL-3, which induces autocrine priming, an event that may represent a problem for the handling and interpretation of

BAT [10, 11]. Previous reports have shown that very low doses of IL-3 are able to induce basophil degranulation [12].

For BAT, the different experimental conditions have not yet been standardized, as they depend on different allergens [13]. A lot of factors contribute to differences in BAT predictivity and analytical performance. For example, during pollen season, basophils respond less to pollen allergens by upregulating CD63 in subjects monosensitized to grass pollen, thus dramatically modifying CD63-based BAT sensitivity [14]. The European Interest Group for the Evaluation of BAT in clinical routine is still focusing on harmonization of the existing different BAT protocols (even establishing a reference BAT protocol, if possible) to render results comparable across international laboratories. A possibility to optimize BAT would come from the introduction of new phenotyping methods to capture basophils in flow cytometry and new activation markers. Also, the application and performance of BAT should be reappraised. Most CD63-based BATs (more than 40%) are used to diagnose drug hypersensitivity [15]; their sensitivity could be around 60%, while specificity is very high (80–100%), a range clearly confirmed by other recent studies [16, 17]. Due to the potential hazards of drug allergies, an early and reliable diagnosis is crucial; this might explain why the BAT was immediately considered as a possible surrogate for drug hypersensitivity diagnosis, while replacing other routine assays [18]. A reliable clinical approach, as well as a detailed examination of the drug intake, remains obligatory to diagnose drug allergy and, hence, research to improve the existing tests and to develop new diagnostic tools is still of paramount importance. However, there is still a long path ahead.

The Use of Basophil Flow Cytometry in Allergy Diagnosis: State of the Art

Table 1 summarizes the main reports from the literature in which a BAT approach has been applied for allergy diagnosis. Most BATs use anti-IgE as the capturing marker in flow cytometry and CD63 to measure basophil function.

The first application of CD63-based BAT occurred in the 1990s; the percentage of CD63 upregulation in basophils triggered with an IgE-mediated stimulus was of the same order of magnitude as histamine release [19] and appeared to be suitable for diagnosing main allergy manifestations, even as preliminary attempts [20]. In these first attempts, correlation between expression of a mem-

brane molecule and a degranulation event was of the utmost interest. A very good correlation between the histamine release test (HRT) and CD63-positive basophils was observed in latex [21], hymenoptera venom [22, 23], grass pollen [6], food [7] and drug allergy [24], so that flow cytometry was proposed for routine diagnosis of allergic reactions [25, 26]. In any case, basophil flow cytometry also presented an opportunity to study basophilic cells in general and to aid in separating them from peripheral blood [27, 28]. Attempts to standardize in vitro flow cytometry of basophils to provide a reliable diagnostic tool have also been made [3]. Most of the so-called 'Basotests' or 'basophil activation tests' or flow-cytometric allergen stimulation tests, available also as affordable commercial kits, used CD63 activation markers, as the anti-ENPP3 (CD203c) antibody, 97A6, was only discovered a few years later [28]. Difficulty in finding and enrolling specific allergic subjects in BAT studies has always been a hindering issue, which may have prevented optimization of the analytical performance of the cellular test. Sanz et al. [29], in a study carried out on 58 subjects allergic to β -lactams, confirmed that with CD63-based BAT, the allergic patients showed a statistically higher number of activated basophils than controls but, although BAT showed higher sensitivity (50%) and specificity (93.3%) than specific sIgE (37.9 and 86.7%, respectively), the test sensitivity was quite a lot lower than expected and differences did not reach statistical significance. Moreover, BATs do not always show a higher sensitivity than serological tests. The use of BAT in diagnosing allergy to β -lactams has been reviewed recently [30]. In the intervening years, some authors attempted to introduce the newly discovered 97A6 antibody to highlight the basophil response in hymenoptera venom allergy by investigating CD203c upregulation; the test showed a very good correlation with HRT and CD63 upregulation and proved able to successfully diagnose 20 of 22 allergic patients (91%) [31]. This high sensitivity was also confirmed by other authors [32]. A good correlation with CD63 ($r = 0.76$) was also reported by other authors in birch and grass pollen allergy [33]. A comparison between CD63 and CD203c in basophil flow cytometry for the diagnosis of insect venom allergy has reported a higher sensitivity of CD203c (97%) compared to CD63 (89%) [34], while in cat allergy, other contributors have found BAT performance to be quite similar when using either CD63 or CD203c as activation markers, either with or without the introduction of IL-3 [35]. What is interesting is that in this report, the CD63 BAT included an IL-3-containing buffer, while the CD203c BAT did not. In an amoxicillin allergy model, Abuaf et

al. [36] showed that CD203c correlated better with the β -lactam anaphylactic group (60%) than did CD63 (20%), evidence that provoked several comments [37, 38], as the authors also used CD45, together with anti-IgE, to phenotype basophils in flow cytometry. Actually, the question whether CD203c and other gating strategies are truly useful in ameliorating the capture of basophils and their functional investigation in flow cytometry has become a fundamental issue. Marked improvements in CD203c BAT were obtained just by ameliorating basophil electronic separation, for example by simply adding CD45 [39], and even in this case many controversial comments arose [40, 41]. Probably, CD63 will have had more account due to its relationship with the degranulation event and because it is not a constitutive membrane marker. After all, not all the evidence suggesting CD203c as a marker to optimize BATs was unambiguous, as controversial results were reported [42]. Although in some tests the sensitivity of CD63-based BAT appeared rather low, other authors have described a detailed study on the analytical performance of this test, reaching very different results. Based on the receiver operating characteristics-generated cutoff value of 17% for CD63-positive basophils, as captured by the usual anti-IgE-FITC method, Ebo et al. [43] estimated a BAT sensitivity and specificity of 93.1 and 91.7%, respectively, as evaluated in a population of subjects allergic to latex, encouraging results that were then confirmed in subsequent reports by others [44]. Several reviews about this promising test began to appear, aimed at achieving possible acceptance while describing the state of the art of its application in allergy diagnosis [45–51], issues that are still being faced nowadays [52–74]. However, despite promising initial studies and some encouraging evidence, many authors agreed that CD63-based BAT remained disappointing in terms of sensitivity, while CD203c-based BAT showed a higher sensitivity (75 vs. 50%), though not as exciting as some reports suggested [39]. Where's the catch? The problem pertains mainly to the different molecular and cellular behaviors of the two activation markers when related to the basophil response to an allergen; this could be an arguable reason why this issue has never ended up with two separate challenging teams among researchers, but on the contrary has always involved the possibility of a constructive debate, although CD63-based BAT has long dominated the scene. The report by Boumiza et al. [39] on CD203c-based BAT in latex allergy, in which a lower sensitivity compared to other studies with CD63-based BAT was estimated, raised many comments [40, 41]. Both Ebo's and Boumiza's groups used heparinized blood for

their investigations and used samples 3–4 h following blood withdrawal. The paper by Ebo et al. [43] was well planned with regard to sample design and subject enrolment, but, unlike Boumiza et al. [39], the authors used IL-3 to prime basophils, which might explain the encouraging results reached for CD63 expression on basophils [43]; perhaps if they had used CD203c as the activation marker in that preactivating condition, there may not have been any significant increase in this marker, thus resulting in quite a low sensitivity of BAT. This group did not find any correlation between serum-specific IgE and BAT positivity. In the work by Boumiza et al. [39], the authors did not use IL-3, but the experimental conditions would have elicited an autocrine release of IL-3 [9, 10]. CD203c is sensitive to IL-3 priming, and a subsequent challenge with allergen can result in the absence of a response of this marker, when its membrane expression reaches a plateau, while CD63 is dragged out from vesicles by the contribution of allergen to the primed state. CD63 membrane overexpression due to an IL-3 priming mechanism is a mechanism distinct from histamine release; in these circumstances, HRT and CD63 upload to the basophil membrane may show low correlation [75]. Some years ago, a paper by Gamboa et al. [76] described the application of the flow-cytometric allergen stimulation test (a commercial CD63-based BAT) on basophils from individuals affected by allergy to pyrazolones; the reported sensitivity was particularly low (42.3%) compared to the specificity (100%), but the authors did not use IL-3. Moreover, they used acid citrate dextrose tubes for blood withdrawal and did not use the samples within a few hours of collection but rather stored them at +4°C for at least 24 h [76]. In the field of pollen-associated food allergy, Erdmann et al. [77] found high sensitivity values (80–85%) for BAT with very good agreement with sIgE and skin prick testing (SPT); the authors used fresh heparinized blood samples primed with 2 ng/ml IL-3. Questions about the use of IL-3 to enhance BAT performance have recently been raised [11], but maybe this is just one of the many problems in dealing with BAT. The goal of achieving optimal performance of BAT leads many researchers to attempt to optimize the test to the maximum possible values of sensitivity and specificity, although this target might be realistically unattainable in most circumstances [78]. In certain cases, the sensitivity values of BAT were very low, less than 50%, while a target of 60% could be reached by combined use of SPT, sIgE and BAT [79]. In a study on hymenoptera sting allergy, the concordance between BAT and SPT was 42.9%, while the concordance between BAT and sIgE was higher (57.1%), as well as the

concordance among all three diagnostic tests [80]. Relatively low sensitivity values for BAT were also observed for drug allergy (48.6% [52], 41.7% [81], 39.1% [82], 36.1% [83]). The overall sensitivity estimation of a routinely used CD63-based BAT should be around 60%, with a higher specificity of close to 90–100%, but sensitivity and also specificity vary widely depending on the different allergens considered [53, 79, 84] or simply the allergen dose [86] and are influenced by possible cross-reaction with different allergens, as in the case of hymenoptera venoms [86]. Furthermore, one report found a lower sensitivity for BAT (76%) compared to SPT (100%) and sIgE or RAST (92%) in wasp venom allergy [87], evidence not confirmed by others who found, on the contrary, comparable sensitivity between BAT (87.7%), SPT (93%) and specific sIgE (91.2%) [88]. Why these contradictory results when both studies were carried out in subjects with wasp sting allergy? It is very interesting, for example, to note that Sturm et al. [88] physically purified basophils in the Histopaque-derived mononuclear cell layer and phenotyped cells using an HLA-DR/CD123 protocol, rather than anti-IgE-FITC or anti-IgE-phycoerythrin, while using CD63 as in other routine BAT. Physical separation of basophils from other blood cells can increase the analytical parameters of BAT performance, but this approach is still too expensive and time-consuming for the routine diagnostic evaluation of allergy. In this regard, there was recent debate about the different phenotyping methods used in basophil flow cytometry aimed at ameliorating BAT technical performance [89]; although newly discovered markers have been introduced, most BATs are based on an IgE/CD63 method panel (table 1). Ebo et al. [90] applied an HLA-DR/CD123 gating protocol to validate flow-assisted diagnostic management of anaphylaxis caused by rocuronium compounds; the sensitivity of BAT was 91.7% and its specificity 100%. This good performance has not been confirmed by more recently reported contributions, in which the usual BAT gating protocol was applied on the same allergic panel [91]. Frezzolini et al. [92] used an HLA-DR/CD123 gating protocol to evaluate CD63 expression in the diagnosis of chronic urticaria; the sensitivity and specificity of the basophil activation assay in flow cytometry, performed on cells from atopic donors, were 95.5 and 90.5%, respectively. Thus, the application of a gating strategy other than anti-IgE seems to greatly improve BAT performance. Eberlein et al. [17] used a CCR3 marker instead of anti-IgE to gate basophils in the new BAT called FlowCAST-2, for the diagnosis of allergy to antibiotics, showing a higher sensitivity compared to the classical routine BAT. This marker

Table 1. Application of the BAT

Allergy	Phenotyping markers		Activation markers		Results and technical notes	Report
	anti-IgE	others	CD63	CD203c		
<i>Food allergy</i>						
Food allergens	■		■		BAT positive 18/31 (58%), LRT = 65%	Moneret-Vautrin et al. [7]
Pollen-derived food allergy	■		■		29 BAT: sensitivity and specificity >80–85%	Erdmann et al. [77]
Pollen-derived food allergy	■		■		BAT sensitivity and specificity 100%	Ebo et al. [78]
Pollen-derived food allergy	■		■		BAT sensitivity and specificity dependent on allergens	Erdmann et al. [87]
Peach allergy	■		■		BAT with peach and other food allergens	Gamboa et al. [132]
Cedar pollen	■			■	rush immunotherapy	Nagao et al. [133]
Wheat allergy	■			■	BAT sensitivity 85%, specificity 77%, CD203c	Tokuda et al. [134]
Pollen-derived food allergy	■		■		heat processing reduces allergenicity	Worm et al. [135]
Beef	■			■	BAT in beef meat anaphylaxis	Kim et al. [136]
<i>Drug hypersensitivity</i>						
Muscle relaxants	■	CD45	■		BAT: low sensitivity, good specificity	Abuaf et al. [137]
Antibiotics, m. rel.	■		■		BAT sensitivity (71%) = SPT	Monneret et al. [138]
β-Lactams	■		■		58 BAT: sensitivity = 50%, specificity = 93.3%	Sanz et al. [29]
Metamizole	■		■		26 BAT: allergic sensitivity 42.3%, specificity 100%	Gamboa et al. [76]
NSAIDs	■		■		different sensitivity/allergens; total = 63.3%	Gamboa et al. [82]
NMBA	■		■		BAT sensitivity low 39.1%, high after 3 years	Kvedariene et al. [83]
Rocuronium bromide		HLA-DR/ CD123	■		high sensitivity (91.7%) and specificity (100%)	Ebo et al. [90]
Amoxicillin	■	CD45	■		CD203c better than CD63	Abuaf et al. [36]
Clavulanic acid	■		■		usefulness of BAT with CAST	Longo et al. [139]
Rocuronium	■		■		BAT positive predictive value 98%, sIgE 83%	Leysen et al. [91]
<i>Hymenoptera venom and insect sting</i>						
Honey bee venom	■		■		high correlation of CD63+ with HRT, LRT	Sabbah et al. [22]
Mosquito allergy	■		■		BAT correlates with anaphylaxis	Hassoun et al. [140]
Hymenoptera venom	■		■		BAT sensitivity 88%, sIgE = 85%, HRT, LPT = 100%	Sainte-Laudy [141]
Hymenoptera venom	■				BAT 20/22 (91%) CD203c upregulated	Platz et al. [31]
Wasp and bee venom	■				BAT positive 35/39 allergic subjects (89.7%)	Binder et al. [32]
Hymenoptera sting	■		■	■	BAT-SPT-sIgE concordance = 57.1%	Eberlein-König et al. [80]
Wasp sting		HLA-DR/ CD123	■	■	high correlation BAT, SPT, sIgE (sensitivity 90%)	Sturm et al. [142]
Wasp and bee venom	■		■		cross-reactive allergens	Eberlein-König et al. [86]
Insect sting	■		■		CD203c BAT more sensitive (97 vs. 89%)	Eberlein-König et al. [34]
Wasp venom	■		■		BAT sensitivity 83.8%, specificity 100%	Ebo et al. [143]
Hymenoptera venom	■		■		higher performance of BAT than SPT, sIgE	Peternejl et al. [144]
Insect sting	■		■	■	CD63 BAT more sensitive than SPT	Korosec et al. [145]
<i>Latex allergy</i>						
Latex	■		■		high correlation of CD63+ and HRT	Sainte-Laudy et al. [21]
Latex	■		■		high BAT sensitivity (93.1%), specificity (91.7%)	Ebo et al. [43]
Latex	■		■		high BAT sensitivity (93%), specificity (100%)	Sanz et al. [44]
Latex	■			■	BAT sensitivity 75 vs. 50%	Boumiza et al. [39]
Latex (children)	■		■		BAT for specific allergens	Sanz et al. [84]
Latex	■		■		BAT detection of CD63	Nettis et al. [146]
Latex	■		■		BAT positive in latex workers	Lopata et al. [147]
<i>Inhalants, contrast media and others</i>						
Cypress pollen	■		■		BAT sensitivity (91.2%) vs. sIgE (76%)	Paris-Kohler et al. [148]
Grass pollen	■		■		BAT depends on pollen season	Saporta et al. [14]
Dermatophagoides and Lolium	■		■		BAT correlates with sIgE, HRT, LPT, SPT	Sanz et al. [149]
Birch and grass pollen	■			■	BAT sensitivity 93.3%, specificity 98.4%	Hauswirth et al. [33]
Cat allergy	■	CD45	■	■	62 BAT: correlation with CD63, r = 0.76	Ocmant et al. [35]
Occupational fungal allergy	■		■		CD63 and CD203c BAT comparable	Caballero et al. [150]
House dust mite	■		■		CD63 BAT positive in feed industries	González-Muñoz et al. [85]
Grass pollen	■			■	BAT sensitivity 83%	Potapińska et al. [68]
Dermatophagoides	■			■	BAT sensitivity 92.3%, specificity 100%	Potapińska et al. [68]
Carboxymethylcellulose	■		■		BAT sensitivity 92.3%, specificity 100%	Dumond et al. [151]
					BAT strategic in this anaphylaxis	Dumond et al. [151]

m. rel. = Muscle relaxants; NSAIDs = non-steroidal anti-inflammatory drugs; NMBA = neuromuscular blocking agent; LRT = LTC4 release test.

has recently been considered a good phenotyping marker to gate basophils in whole blood; CCR3 (CD193), the basophil receptor for eotaxin, has been shown to be a stable, reliable and highly expressed basophil selection marker, independent of the atopic background or basophil activation state, which should allow accurate identification of basophils without need of a second marker [93]. However, other authors have raised criticism about the effective reliability of this marker, mainly due to its possible downregulation following activation and its wide expression on other leukocytes [38, 89]. Few newly introduced markers in flow cytometry have been suggested for BAT improvement. In any case, even the use of CD203c, as can be observed from table 1, is less frequently considered compared to CD63, although it showed a performance similar to other routine allergy tests [94]. Probably, for most researchers the use of CD63-based BAT proved more feasible for diagnosing hypersensitivity reactions in a routine fashion. Patterns by which CD63 respond to external stimuli mean that researchers prefer this marker in cellular investigations. The movement of CD63-associated granules was studied some years ago in rat mast cell models; upon activation, granules reach the membrane in the very early minutes following stimulation, with a mean velocity of 100 nm/s [95], a trend that has been confirmed also in basophils [96]. Most of the CD63 is associated with membrane integrins, and its recycling turnover, probably using a synaptotagmin-mediated mechanism [97], occurs in the first 30–60 min after ‘bursting’ stimulation [98, 99]. Activation could also be elicited by many pre-analytical factors [9]. As a matter of fact, aside from downregulation mechanisms to be considered, the intracellular CD63 pool might be limiting to address CD63 upregulation in a BAT, simply due to its close relationship with basophil degranulation. However, this is a possible pitfall in using CD63 as a single diagnostic marker.

The percentage of CD63-expressing cells has to be considered as a raw average of all basophils in the sample that are upregulating the tetraspanin to the membrane while the stimulus is still present; the calculation is rather arbitrary and depends on the setting of the non-CD63-expressing cell population based on mean fluorescence and ultimately on the ability of basophils to respond to an external stimulus. In this context, a critical point is also the cutoff of basophil expression of CD63 upon activation. All these set points are variable and often depend on the initial experimental conditions. Moreover, CD63 membrane displacement can be elicited by mechanisms quite separate from histamine release [100, 101]. This mechanism is one of the main issues in the debate about non-IgE-mediated

allergic responses. IgE-mediated and non-IgE-mediated cellular responses are critical issues in BAT application [102]. For example, administration of a drug can classically result in an immunoglobulin E (IgE)-type sensitization but can also result in more complex activation of the immune system such as the drug-induced hypersensitivity syndrome, although the exact mechanisms of drug allergy are not well understood for most clinical manifestations. A complex interaction between individual characteristics, environmental factors and the drug itself is usually responsible for adverse reactions to drugs [103]. Basophil evaluation in these circumstances appears to be one possible way to discriminate between different types of hypersensitivity, as for instance in food allergy, where non-IgE-mediated allergy is frequently present [104]. A non-IgE-mediated response able to upregulate CD63 and degranulation also involves other receptors of the innate immune response. Formylated peptides such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) are commonly used in commercial BATs, but the mechanism by which fMLP induces CD63 translocation to the cell surface seems to be quite different from that elicited by an IgE-mediated event [101]. fMLP can be used as a good positive control to verify if basophils are viable and able to express CD203c and translocate CD63 molecules to the membrane, but its mechanism of action should not share any features of an IgE-mediated response.

Although quite expensive, some authors have suggested the introduction of intracellular flow cytometry to enrich the evidence obtained by evaluating the basophil response in allergy [105]. Flow cytometry actually allows a more integrated evaluation of immunophenotyping and the intracellular mechanisms underlying receptor signaling in allergy. In this context, phosphorylation of p38-MAP kinase (p38MAPK) represents a crucial step in IgE receptor signaling in basophils. The relationship between p38MAPK and the well-validated diagnostic cell surface marker CD63 has recently been evaluated in a clinical allergy model [106], although criticism of the use of intracellular p38MAPK in monitoring therapy has recently been raised [107].

Moreover, initial experimental conditions represent a fundamental issue. In a buffered medium or in whole blood, naturally not all the basophilic cells are resting cells. A first step should be to allow basophils to reach a ‘starting point’ at which most of the cells are prevented from spontaneously induced activation due to handling procedures, buffers and temperature. This starting point is ideally constituted by a prompt to upregulate the CD63 intracellular pool. Basophils should be used within very

few hours of venous withdrawal, and the use of anti-IgE to target cells for flow cytometry identification should be avoided, as the phenotyping marker might elicit a stimulatory response in nonactivated basophils [9]. In this context, the collection of basophils is another critical issue; samples should be collected and treated within the first 3 h following venous withdrawal in acid citrate dextrose or heparinized vials (selected in order not to deprive the test of calcium by chelating agents such as EDTA) and should not be stored at +4°C for more than this time, but not all authors are in agreement with these assumptions [108]. A good suggestion for performance of a reliable BAT might be to separate basophils from other blood components rather than to extremely purify them. A simple approach would be to remove most of the CD63-expressing platelets, although they should not affect BAT [8], and plasma-interfering components, such as immunoglobulins, complement and coagulation factors, able to interact with IgE/FcεRI activation [109–111]. Furthermore, spontaneously induced activation should be arrested, for example by putting washed and buffer-reconstituted samples into an ice bath (+2°C/+4°C) [11].

Allergen doses, more than immunochemical properties, represent another critical issue in enhancing BAT performance. Dose-response curves of purified allergens may be a possible strategy to solve BAT sensitivity, but the overall quality of a BAT depends certainly not only on allergen type and dose but on the relationship between these features and sensitized basophils.

Despite these preanalytical issues, membrane upregulation of CD63 has become of great interest because of its relationship with histamine release, as intracellular CD63 has been associated with histamine-containing granules [3]. Very few differences may be highlighted whether whole blood or physically purified basophils are used; most of the diagnostic performance optimization is related to the proper choice of functional basophil markers. Purification of basophils represents a time-consuming and expensive approach; attempts to ameliorate and enrich the endowment of those molecules able to assess basophil function during an allergic response have been widely made. Some activation markers, such as CD164, CD107a or also CD13 and CD69, have been proposed, but their use in BAT as a widely used application in routine diagnosis is still far from being achieved [112, 113]. Why this gap in BAT technical improvement? A possible trivial reason is the availability of new affordable fluorochrome-associated markers, while other considerations arise from the many opinions about different gating methods, the weak relationship between activation mark-

er and basophil releasability (except for CD63) and the expression of those markers on other blood cells besides basophils (except for CD203c).

Focusing on BAT: Is It Possible to Optimize Flow Cytometry for Allergy Diagnosis?

Concerns about the Use of CD63 in BATs

In light of the existence of the many issues and suggestions about possible improvements thoroughly discussed above, why are IgE/CD63-based BATs so widespread? Much of the success related to CD63 concerns its upregulation response to a stimulus from a basal starting point and its relationship with intracellular granule migration to the membrane, hallmarks that CD203c and other markers do not exhibit. However, what is the real situation of basophil releasability that one might tackle when evaluating a cellular allergy test? A possible reasoning may be suggested, as follows.

The ideally 'normal' basophil state is a previously sensitized cell expressing specific IgE on the FcεRI receptor, not yet degranulated and able to exhibit all the cellular mechanisms responsive to an IgE-mediated stimulus when challenged with an allergen. We call this cellular state 'resting'. In figure 1, a schematic representation of basophils with an intracellular pool of CD63 molecules (fig. 1a–d) and fluorescence histograms of CD63 expression from flow cytometry (fig. 1e) are shown. In this figure, circles with squares within represent schematic pictures of basophils containing different levels of prompt to release intracellular CD63. The resting cell represents our analytical functional baseline. This cell is potentially able to degranulate, to translocate CD63 or other activation molecules to the membrane and to release granule mediators when stimulated. A spontaneously degranulated population (for example, 7% CD63) would account for approximately 93% of intracellular CD63 that could be upregulated onto the membrane upon the forthcoming stimulation (fig. 1e, row 1). When activated, in most cases basophils expressing CD63 amount to 20–30% of the whole population investigated (28.7% in the example shown in fig. 1e, row 2; intracellular CD63 is 71.3%) following an optimal IgE-mediated challenge, but this value may increase when basophils are primed with 1.0 ng/ml IL-3 (fig. 1e, row 3; 45% CD63-positive cells, thus leaving 55% intracellular CD63). All these values represent an average of the different basophils within the experimental population and can be described as the total CD63 intracellular pool (black squares inside circles and percentage

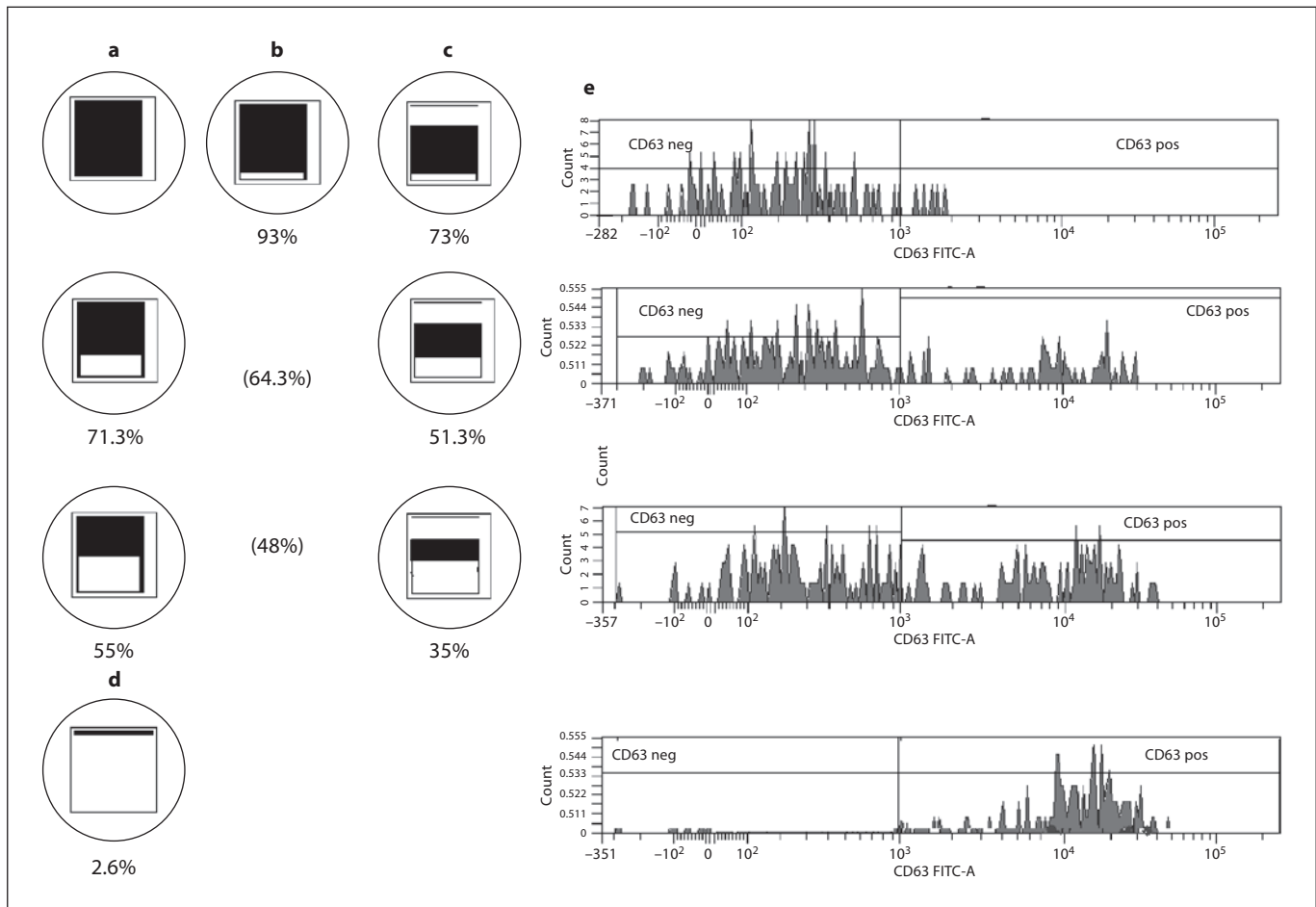


Fig. 1. Representation of the intracellular pool of CD63 (**a–d**) and its expression in a flow cytometry analysis (**e**). **a–d** Boxes illustrate cells with CD63 within (black boxes). **a** Repertory of intracellular CD63. **b** Resting condition. **c** Activated cell with depletion of the intracellular pool. **d** CD63 depleted intracellular pool. Values in parentheses correspond to the intracellular CD63. **e** Flow cytometry analysis. First row: resting, nonactivated cells; second row:

cells activated with 2.0 $\mu\text{g/ml}$ goat antihuman IgE (Invitrogen, Caltag Laboratories, UK) for 30 min at 37°C; third row: cells pre-treated with 1.0 ng/ml IL-3 (Invitrogen, Caltag) for 10 min and activated with 2.0 $\mu\text{g/ml}$ goat antihuman IgE (Invitrogen, Caltag) for 30 min at 37°C; fourth row: cells treated with saponin, according to Amano et al. [95].

values reported in fig. 1). The above-described situation is represented in figure 1a. Intracellular CD63, which is left in the bimodal pattern of CD63 expression [96], should be evaluated as the main counterpart of membrane CD63, which describes the basophil response to activation. Really, if we consider spontaneous degranulation, the CD63 intracellular pool should be lower (fig. 1b). In atopic subjects, this might be close to an exhausted or highly wasted pool due to continuous triggering, possibly leading to a scant upregulation event (fig. 1c; intracellular CD63 is reduced by 20%); in this case, activation might either not induce any significant shift in the right side of the fluorescence histogram (not shown) or even the translocation of

the residual intracellular pool leading to a CD63 upregulation in most of the basophils (fig. 1d; 4 $\mu\text{g/ml}$ anti-IgE in a birch pollen-atopic subject). What does this speculative reasoning suggest? It is conceivable that, while the activation response is quite regular, the scant pool of available CD63 may upregulate almost all or most of the intracellular storage, leading to a possible bias in the strength of the allergic response (fig. 1d). This late CD63 response may be due to intracellular pool availability or regulation mechanisms in intracellular trafficking of CD63-containing granules [101]. Possibly, it is important to evaluate how much available CD63 is ready to be translocated to the membrane upon activation. This investigation appears to

be quite rare in BAT application, but intracellular CD63 can be evaluated by using saponin, which even seems to ameliorate BAT performance [114]. The fusion process of CD63-containing granules with the basophil membrane is characterized by a rapid loss of granular contents, by the uploading of CD63 molecules on the membrane and by their subsequent recycling. This suggests the need for a reappraisal of CD63 evaluation in BATs. CD63 upregulation, as evaluated by calculating the percentage of basophils expressing the tetraspanin on the cell membrane, might be a parameter too weak for the evaluation of basophil response during allergy, if the mechanisms described above are not taken into account. Furthermore, CD63 can be expressed as a result of histamine release [101] and following non-IgE-mediated agonists. When an allergen is tested in BAT, the operator has no way of knowing if the upregulation of membrane markers is due to an IgE-mediated mechanism when using only an fMLP control; moreover, the use of anti-IgE to target basophils for their electronic capture in flow cytometry has been the subject of criticism because of the existence of IgE receptors (CD23) on other cells and the possibility of spontaneous activation [89, 96]. Besides, in addition to fMLP a good BAT should have at least one other positive control performed by challenging cells with an optimal dose of polyclonal anti-IgE. The response of basophils to any allergen is accompanied by multiple series of events that can reduce the ongoing stimulation by an allergen, as for instance downregulation of the high-affinity IgE receptor [115]; this is another issue to be considered. Actually, as with many other receptor-mediated mechanisms, granule release in basophils is a self-limited phenomenon; this fact may affect BAT performance, as previously discussed. About 10–20% of basophils within the population are nonreleasing cells due to a defect in receptor-mediated intracellular signaling [116]. Desensitization can involve mainly a modification of the intracellular signaling apparatus [117], but FcεR1s are also downregulated and recycled. FcεR1 cell surface expression decreases by 30% in the presence of IL-3, and downregulation lasts for the next 18 h following withdrawal, then the receptor, lost by a process of endocytosis, is partially recycled to the cell surface [118]. Anergic or desensitized basophils therefore represent a self-termination mechanism following all receptor-mediated stimulation [119]; at this point, the addition of an IgE-mediated agonist might not elicit any significant cellular response, while IL-3 may trigger different responses depending on the functional state in which most of the basophils stand, and even priming is a complex event in which other components could be involved [120].

The Search for a Diagnostic Algorithm

Really, it is very difficult to trace a possible algorithm encompassing these issues which would make BAT suitable for routine allergy diagnosis. Good diagnostic improvements to highlight the basophil response to an allergen or a group of allergens would be to:

- (1) check for preanalytical factors that may prime or activate basophils;
- (2) measure basophil membrane upregulation to at least 3 replicate points, and
- (3) use a reliable flow cytometry protocol.

While efforts to deal critically and constructively with optimization of BATs are still ongoing, this approach is affected by criticism that it is time-consuming and expensive to perform. Nonetheless, a diagnostic panel can be outlined here on the basis of some, albeit anecdotal, reasoning, which, however, begs assessment.

Actually, the interpretation of flow cytometry outputs should be reappraised. Mean fluorescence intensity (MFI) is represented by absolute numbers that may be affected by several technical issues; when it shows a wide variability it is useless for a reliable comparison. On the other hand, measurement of the percentage of CD63-expressing cells depends on the arbitrary setting made by the operator on resting, nonactivated cells; the same percentage of CD63-expressing basophils can possess a higher or lower MFI. Basically, what the MFI is supposed to measure is the shift in fluorescence intensity of a population of cells. In cases where the entire population of cells stains with the same saturating level of an antibody and all samples were run under the same preanalytical checking conditions and instrument settings, fluorescence parameters can be considered rather as a linear function of membrane molecule expression. For CD63, the median value does not always indicate a net shift in fluorescence units showing an activation event. The following proposal suggests that the complete numerical output of CD63 expression should also include MFI and event counts; the latter should be standardized according to the experimental setting, for example by evaluating the same number of cells per gate and using the same standardized methods and materials for basophil capture, and hence it can be excluded from entering the formula. This new parameter may be called 63_{index} :

$$63_{\text{index}} = [\text{MFI}^{\text{expr}} \times (\% \text{CD63}^{\text{expr}} / 100)]$$

Due to the inclusion of the MFI of CD63-expressing basophils, 63_{index} would be more reliable than a simple percentage. An allergen that is able to upregulate the same percentage of CD63 in two different allergic indi-

viduals but with two significantly different MFIs under the same experimental conditions might have a precise significance in the basophil response to the allergen, as MFI is proportional to the number of membrane molecules recognized by the fluorochrome-labeled antibody and hence to the amount of surface CD63. The parameter 63_{index} should indicate the percentage of basophils and how much CD63 they express on their membrane. Thus, an allergen may elicit two different responses as evaluated by the percentage of CD63 expressed but with MFIs that lead to the same 63_{index} :

example 1: sample No. 0087, %CD63^{expr} basophils = 21.6%, MFI = 8,457.76, 63_{index} = 1,826.88;

example 2: sample No. 0126, %CD63^{expr} basophils = 43.3%, MFI = 4,178.65, 63_{index} = 1,809.35.

The histograms of the first sample will show a marked separation of a few basophils with high expression of CD63. Reporting only the percentage of CD63 expression may introduce a bias in the evaluation of the allergy response (low in example 1 and high in example 2), while the two responses are qualitatively equivalent, though differing in their fluorescence distribution. In this case, the median would appear to be an alternative; however, while the 63_{index} includes only parameters of expressing cells, the median concerns the whole population of cells and it might not be so reliable in discriminating different situations, for example in the follow-up of allergy therapy.

What Meaning Should We Draw from These Arguments?

Differences in BAT performance when only %CD63^{expr} basophils are measured may depend on several issues, the main two being (1) allergen strength in inducing a CD63-upregulating response and (2) basophil releasability. To assess this issue, an anti-IgE control has to be introduced.

The index should allow comparison of a better basophil IgE-mediated response to an optimal dose of an allergen to that elicited by an optimal dose of anti-IgE used as activation control. For example, the operator can assess the following ratio

$$63_{\text{ratio}} = [(63_{\text{index}})^{\text{sample}} / (63_{\text{index}})^{\text{control}}]$$

which in turn may give a comparison of the allergen-triggered response and the anti-IgE control. The operator may also set a cutoff, better if calculated by the mean of three separate evaluations in the same experimental setting, to address a possible diagnostic implication. This

should become a new threshold that replaces the usual 10% cutoff for CD63 expression:

example 3: sample No. 0054, %CD63^{expr} basophils = 20.6%, MFI = 9,457.76, 63_{index} = 1,948.29
IgE control = 20.5%, MFI = 2,548.54, 63_{index} = 522.45,
 63_{ratio} = 3.73;

example 4: sample No. 0068, %CD63^{expr} basophils = 41.5%, MFI = 2,134.66, 63_{index} = 885.88
IgE control = 46.4%, MFI = 2,358.33, 63_{index} = 1,094.26,
 63_{ratio} = 0.81.

Considering that the basophil response to an optimal dose of anti-IgE should be at least comparable to that elicited by an antigen and that a cutoff of around 1.0 for 63_{ratio} may be set, activation in example 3 would require further investigation to clarify if this ratio depends on the concurrence of a non-IgE-mediated reaction or other biases. Example 4 involves the same anti-IgE batch as in example 3. An increase in the percentage of CD63 expression was observed but without a concurrent MFI increase, which would be expected. The basophil response was good (ratio around 1.0), but further investigations may clarify if CD63 upregulation on the membrane was hampered by possible other factors (e.g. CD63 intracellular depletion, downregulation mechanism).

In a second step, the operator should build up two CD63 expression controls which aim to evaluate the molecular pool available to the basophil. So, in addition to the resting, nonactivated control, a sample in which all CD63 is drawn out by a saponin-mediated lytic treatment is considered. Two outputs are hence available to focus on the mechanism by which basophils express CD63 on the membrane, i.e. CD63_{min} and CD63_{max}, corresponding to the 63_{index} of resting and extracted CD63, respectively. Furthermore, in the second-level step, at least three anti-IgE controls are considered, including a suboptimal, optimal and supraoptimal dose, with the purpose of probing potential priming or desensitization mechanisms. In the second step, each 63_{index} is compared to CD63_{max} (corrected for resting). The new parameter is called 63_{ON} :

$$63_{\text{ON}} = [(63_{\text{index}})^{\text{sample}} / (CD63_{\text{max}})] \times 100$$

Further insights into the mechanisms underlying CD63 storage, trafficking, membrane upregulation and recycling are undoubtedly required to comprehend the feasibility of this parameter. Saponin extraction of intracellular CD63 should give clues to possible blocking or depleting mechanisms, downregulation or inhibition of molecule trafficking and surface expression, molecular modifications leading to a decrease in fluorochrome-an-

tibody recognition and/or affinity binding. A possible reference range for the available CD63 intracellular pool in fresh, nonatopic, nonactivated basophils may be calculated, for example by enrolling a representative sample of screened healthy blood donors. What should occur in a gated basophil population when cells are challenged with an allergen? This issue was dealt with during the discussion of figure 1. Figure 1c indicates that the percentage of CD63 expression may be underestimated, i.e. 63_{ON} is underestimated, due to the lower $CD63_{max}$ observed compared to the reference range.

What Is Its Possible Functional Meaning?

A depletion in intracellular CD63 or a downregulation in its membrane uploading may come only from membrane expression of the molecule, which occurs when cells are activated; however, this fact may be misinterpreted as a decrease in the allergy response on the receptor level. Continuously triggered basophils tend to desensitize their membrane IgE receptors [101, 114, 118]. In this case, it is arguable that the optimal dose of anti-IgE may result in a lower 63_{index} in the control dose-response curve, a circumstance that may occur also for the supra-optimal dose included. On the other hand, suboptimal and optimal doses might work excellently, a condition that might indicate an active functional state or a primed cell. Signaling desensitization may be highlighted by introducing an intracellular marker into the second-level test; however, due to reasons of expense and time, the need for condition of receptor/signaling downregulation or anergy due to a nonreleaser state, should be addressed only when $CD63_{max}$ does not suggest exhaustion of the intracellular pool and is followed by a poor response of the IgE-mediated activation. This observation should be verified, keeping the same materials and experimental setting.

This series of arguments may appear too speculative and somewhat hindering. Nonetheless, this proposal contains at least three new possible advances: (1) the percentage of CD63-expressing basophils also accounts for the fluorescence indicator of the amount of molecules expressed on the cell membrane; (2) this parameter is compared with the same one calculated on an anti-IgE control, and (3) in order to focus on possible biases, a second-level investigation can be performed, in which calculation of an anti-IgE dose-response curve and evaluation of the intracellular CD63 pool are possible new pathways to enhance the diagnostic usefulness of BAT. As a matter of fact, while taking into consideration if a new algorithm can fulfill the criteria according to which a flow cytometry test has to be

applied in order to better evaluate cell function in allergy, any finding has necessarily to be endorsed and possibly supported by further clinical evidence. Although this cannot be the purpose of this work because the spectrum of allergen/BAT responses and related clinical manifestations of allergy that would have to be pointed out is too large, what follows has to be conceived as a proposal to reappraise CD63 evaluation in the light of flow cytometry and from a laboratory point of view.

Finally, the proposal aims at stressing some fundamental aspects of BAT use, as follows:

- (1) The application of the simple percentage of CD63-expressing basophils as a 'net' parameter to evaluate basophil response should be reviewed; other algorithms, such as those including fluorescence measurements, and possibly new added markers would be helpful to optimize diagnostic performance of the test.
- (2) The introduction of an anti-IgE control and/or a minimal dose-response curve of this control to compare allergen activation with the IgE-mediated response should be considered. A positive reaction of an allergen and of fMLP when basophils are unable to respond to optimal doses of anti-IgE may also indicate that the allergen solution contains substances able to activate basophils by non-IgE-mediated mechanisms.
- (3) The final judgment regarding whether the subject possesses the correct endowment to exert an allergic response to a defined allergen and, then, if the individual is allergic to that substance, cannot be based on the single calculation of the percentage of CD63 expression. Here, some possible further parameters are suggested.
- (4) Due to the high costs of BAT, a first-level analysis may be considered conclusive if other allergic parameters are suggestive or positive, ratio and index parameters of samples investigated in triplicate correspond to cut-off rules, the anti-IgE control is responsive at the optimal dose used and preanalytical criteria have been observed. The latter point has been discussed elsewhere in the text; in short, the minimum number of basophils within the gate has to be no less than 250, and tests have to be carried out in triplicate (a total of 750–800 basophils for each sample) [4, 96].

Conclusion

Many technical issues and diagnostic algorithms concerning BAT have been discussed in the past [121, 122]. BAT should be carried out as soon as possible after blood

withdrawal [9, 11, 123], and its performance may depend on several factors, such as the type of vial used for specimen collection [109]. BATs have proved to be useful tools to diagnose early onset of allergy and anaphylactic adverse reactions, such as with drugs [124], but also in monitoring immunotherapy [125–127] and to reintroduce food to allergic children [128]. Multicenter and pilot studies have also been carried out in order to achieve a consensus agreement and to outline possible guidelines for BAT optimization and application [129–131]. However, many critical issues still remain, and further investigation is needed. This review is an attempt to outline the state of the art of BAT and to address some of the issues concerning its performance. Stringent hallmarks are mostly constituted by (1) preanalytical factors, (2) the phenotyping and gating strategy and (3) analytical algorithms and parameters for making a diagnostic supposition. Cellular tests for allergy, of which BAT is the most well known, are functional assays that enable discrimination of passive sensitization to an IgE-mediated allergy. In drug allergy, by combining BAT with in vitro passive sensitization of donor basophils with patients' serum, one

can prove the IgE dependence of a drug reaction [13]. In allergy immunotherapy, BAT furthermore represents a powerful tool to follow up patients with allergy manifestations [51, 102, 107, 127]. Improvements in BAT methodology to achieve the best performance have also been made [107], but follow-up of allergy still represents a major concern, for which BAT seems to be a promising approach.

The introduction of anti-IgE controls and an alternative phenotyping strategy are two important aspects which expand the debate. Compared with the determination of sIgE, BAT offers the opportunity to be able to demonstrate functional responses and to prevent side effects due to SPT. This may be a very good reason to combine efforts and to update this interesting tool to facilitate the diagnosis of allergy.

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