

Pros and Cons of Clinical Basophil Testing (BAT)

Hans Jürgen Hoffmann^{1,2} · Edward F. Knol^{3,4} · Martha Ferrer⁵ · Lina Mayorga⁶ · Vito Sabato⁷ · Alexandra F. Santos^{8,9} · Bernadette Eberlein¹⁰ · Anna Nopp¹¹ · Donald MacGlashan¹²

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Abstract

Purpose of Review We review basophil testing by flow cytometry with an emphasis on advantages and disadvantages.

Recent Findings There are many tools available to assess the presence and severity of allergic diseases in patients. For 50 years, peripheral blood basophils have been used as tools to study these diseases. It is a very accessible cell that binds IgE antibody and secretes the classical mediators responsible for the symptoms of allergic reactions. In the last decade, an even more accessible methodology, using flow cytometry, has been developed to enhance the ability to use basophils for both mechanistic and clinical diagnostics. Basophil testing has been included in diagnostics for different forms of allergies as well as to monitor disease status.

Summary A variety of studies have begun to establish both precise methods and their clinical relevance for disease diagnosis, but there remain some important questions

on how to take optimal advantage of the behaviours of basophils.

Keywords BAT · Mast cell · Basophil granulocyte · CD63 · Allergy · Chronic urticaria

Introduction

Most diagnostic testing is preceded by mechanistic studies that explain the biology underlying the activation measured in this test. When studying the mechanism of activation of basophils, one has to remember that CD63, the cell surface protein that is being used extensively today as a metric of basophil function in clinical studies, is a very focused marker for anaphylactic degranulation and may not reflect other methods of activation (e.g., secretion of other biologically

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✉ Hans Jürgen Hoffmann
hjh@clin.au.dk

¹ Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

² Department of Respiratory Diseases and Allergy, Aarhus University Hospital, Nørrebrogade 44, Aarhus 8000, Denmark

³ Departments of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

⁴ Dermatology/Allergology, University Medical Center Utrecht, Utrecht, The Netherlands

⁵ Department of Allergy and Clinical Immunology, Clínica Universidad de Navarra, Pamplona, Spain

⁶ Research Laboratory and Allergy Service, IBIMA-Regional University Hospital of Malaga, UMA, Malaga, Spain

⁷ Department of Immunology-Allergology-Rheumatology, University of Antwerp and Antwerp University Hospital, Antwerp, Belgium

⁸ Department of Paediatric Allergy, Division of Asthma, Allergy & Lung Biology, King's College London, London, UK

⁹ Children's Allergy Service, Guy's and St Thomas' Hospital NHS Foundation Trust, London, UK

¹⁰ Department of Dermatology and Allergy Biederstein, Technische Universität München, Munich, Germany

¹¹ Immunology and Allergy Unit, Department of Medicine Solna, Karolinska Institutet, and Karolinska University Hospital, Stockholm, Sweden

¹² Johns Hopkins, Baltimore, USA

relevant mediators such as cytokines or changes in basophil phenotype).

Biological Considerations

Relationship of Mast Cells and Basophil Granulocytes

Mast cells and basophil granulocytes differentiate along different pathways from a granulocyte progenitor cell along different pathways with very different kinetics from a granulocyte progenitor. Basophils are myeloid cells closely related to the other myeloid cell types [1]. Mast cells mature in tissue from CD34⁺CD133⁺ stem cells [2], whereas basophils mature in bone marrow and enter blood as mature granulocytes. In man, there is no evidence for a common precursor beyond the common CD34⁺CD133⁺ myeloid precursor, and there is no evidence that basophils can differentiate into mast cells [3].

Based on reactivity to allergen activation, skin mast cells are replaced over a period of months [4–7], whereas blood basophils have a half-life of 24–36 h [8–10]. In some patients (non-responders), blood basophils respond much less if at all, to allergen that stimulates mast cells from the same person [11].

Both mast cells and basophils express the tetrameric FcεRI receptor and degranulate upon crosslinking by allergen and IgE (Fig. 1). Degranulation results in release of histamine, proteoglycan located in the granule, synthesis of arachidonic acid metabolites at the cell surface and synthesis of Th2 type cytokines. FcεRI-mediated activation is a relatively fast event, requiring 10–60 min in basophils granulocytes [12, 13] and 2–20 min in mast cells [14]. Both cell types can be desensitised by low doses of allergen [15].

Anaphylactic Degranulation

Assessing basophil activation by examining ex vivo histamine release is probably the best-known functional diagnostic test for functional specific IgE required for allergic disease. However, the analytical requirements for measuring released histamine severely limit broad application of this test to clinical practice. In the last decade, the measurement of CD63 expression by flow cytometric methods has begun to replace histamine release measurements. Up-regulation of CD63 expression accurately reflects the anaphylactic degranulation that causes allergic disease [16, 17•, 18, 19, 20•, 21, 22, 23•]. Other flow cytometric readouts for basophil activation such as the up-regulation of CD203c expression and other surface markers [24, 25], or the diamine oxidase measurement of intracellular histamine [26, 27] will give a similar response, but experience with these metrics is not as extensive or there are additional issues. A major difference is that up-regulation of CD203c occurs in response to many non-degranulation stimuli (such as IL-3), in contrast to CD63 [17•, 19].

Survival of Basophils: The Requirement for Fresh Blood

Fresh blood is required for basophil activation testing, and expanding this time frame is a major challenge for the community. It is being addressed by weighing the positive predictive value of the test and by designing the test such that basophil activation including erythrocyte lysis is temporally separated from flow cytometric measurements. Current guidelines are to test blood that is less than 4 h old although several studies suggest that qualitative results may be valid within a 24-h window.

What Do We Measure in Basophil Testing?

When studying mechanisms of activation, it may be useful to separate extracellular events—the contribution of plasma or serum (immunoglobulins and their receptors [21, 28–30] and other modulatory molecules like the histamine receptors [31, 32]) from the mechanisms of activation within the basophil granulocyte or mast cell.

Response to a Complex Environment

It is possible to manipulate the cell surface IgE and/or the environment of the isolated basophil granulocyte as reactivity depends on IgE affinity for allergen, FcεRI clustering on the cell membrane and on the integrated signal transduced into the cell from a number of receptor pathways. Commonly, only allergen concentration is varied to obtain reactivity, sensitivity or AUC (area-under-the-curve). The complexity of IgE composition (epitope specificity, affinity and concentration of individual clones) and their contribution to effector cell activation is subject to intense research [21, 33–35] (Hjort et al 2016, in preparation). Here, the basophil activation test serves as a useful workhorse to decipher contribution of epitopes and molecules to the allergic response [36].

Without repeated stimulation, IgE specific for drugs appears to be detectable in circulation with a half-life of less than 18 months depending on the responsible drug [37–39]. This sets a limit to the time frame in which it is possible to use the basophil to detect production of IgE that results from an acute exposure (such as exposure to penicillin). In passive sensitisation, the serum of one subject can be used to sensitise basophils of another subject [40, 41]. This allows testing of an allergic subject's IgE status with a functional readout that does not depend on immediate access to the serum-subject's basophils. There are technical issues that must be considered when performing this type of experiment [41]. Blood or enriched basophils can also be treated to enhance their response, offering greater sensitivity [42–46]. Lastly, blood basophils can be activated in autologous plasma from a previous visit to illustrate a change in plasma inhibitory components resulting from an intervention such as allergen immunotherapy [21].

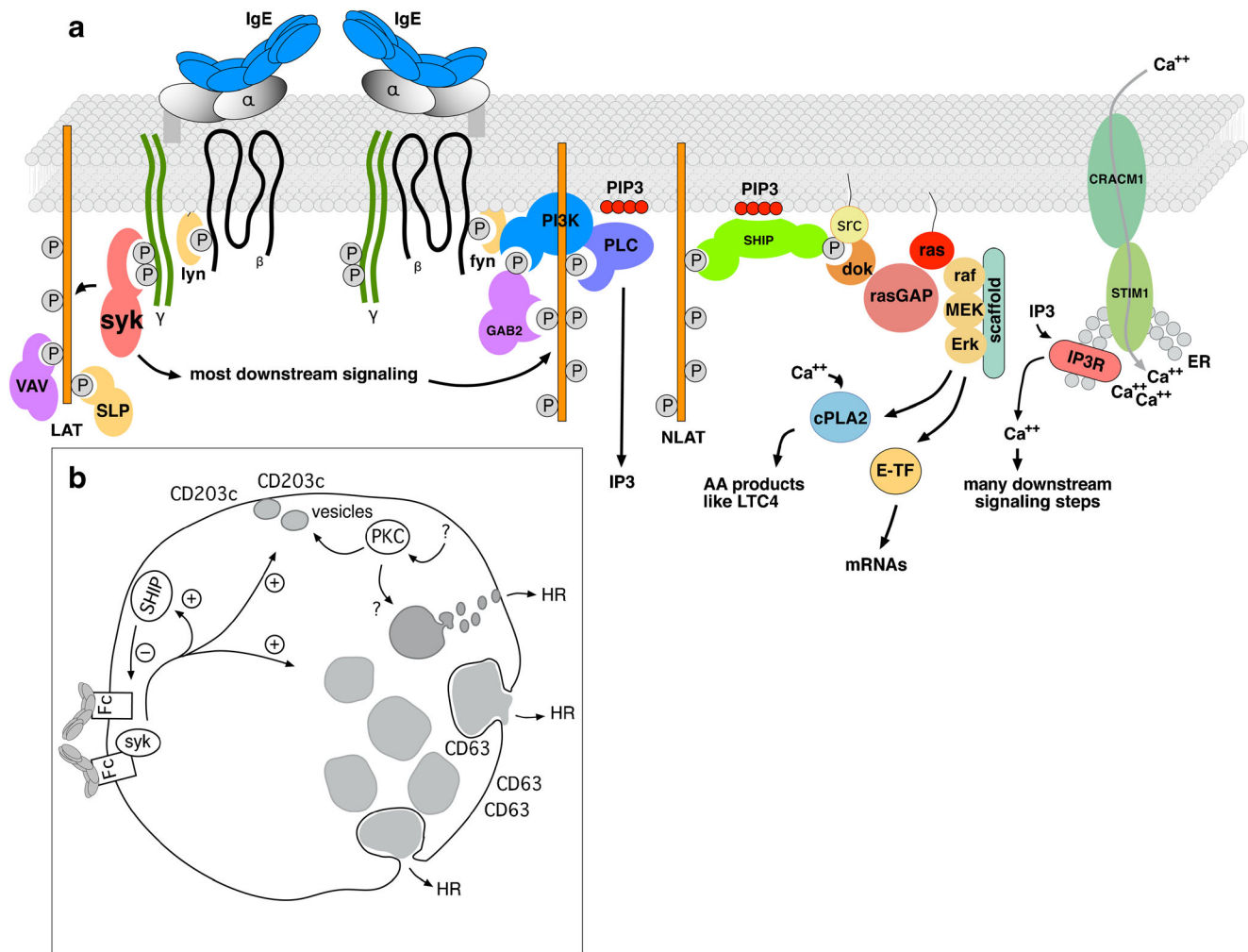


Fig. 1 Simplified cartoon of early IgE-mediated signal transduction as it applies to human basophils and mast cells. **a** Although no major differences in signalling have been found between human basophils and mast cells, there are behaviours that indicate that differences must exist. Studies of basophils have demonstrated that Syk expression levels are a unique regulator of secretion, while this is not the case for human mast cells. In addition, there are indications that some basophil phenotypes are associated with SHIP expression (a negative regulator) with the difference that SHIP functional studies have been done while Syk functional studies have been shown to correlate with its expression. Beyond the early signalling, studies have shown that the rise in cytosolic calcium tightly

correlates with downstream functioning and it controls most functional endpoints with the interesting exception of the expression of CD203c. **b** Evidence suggests that CD203c and CD11c reside on small vesicles that are also under the control of a PKC-dependent pathway, which is interesting since IgE-mediated signalling in human basophils generally does not appear to depend on PKC enzymes. There are two pathways to secrete granule-associated histamine, one which includes fusion of the granules with the plasma membrane and leading to both histamine release and CD63 expression and one which probably does not lead to CD63 expression and may be also influenced by a PKC-like process

Allergen extracts or recombinant molecules used in basophil testing (as well as it is in skin testing and serology) remain an approximation of the sensitising environmental agent.

Signal Transduction Leading to Degranulation

The signal transduction apparatus within the cell is complex, and there is clinically significant biological variation in the activation cascade that also determines the behaviour of basophils and mast cells [47, 48]. There is a natural biological variation in the IgE-mediated basophil response. Non-

responders (5–20 % of the subjects tested) show little or no response through the IgE receptor no matter what IgE-mediated stimulus is used. The serum of these patients may contain adequate circulating allergen-specific IgE, and this IgE is bound at reasonable cell surface densities on the basophil, but since the basophil is unresponsive through the IgE-receptor, a basophil-activation test does not show a positive result. The condition is easily detected by also stimulating the cell with a pan-crosslinking reagent like anti-IgE antibody or anti-FcεRI antibody, but this does not resolve the question of how to use the test results. There are some possible technical

approaches to this issue (such as expressing results as a fraction of the pan-stimulus IgE-mediated response [23•]) that are not yet incorporated into routine basophil activation testing. One of the causes of non-responders is the differential expression of the tyrosine kinase *Syk* that associates with FcεRI during activation [48–50].

An aspect of any cell's response to extracellular stimuli is its ability to self-terminate the response. Desensitisation (the general term used to describe the self-termination mechanisms) has properties that distinguish its pathways from those of activation [51, 52]. Overexpression of the SH-inositol phosphatase SHIP may limit degranulation and hence documentation of a relevant allergy [53]. Most notably, desensitisation pathways are more readily engaged than the activation pathways [54] that lead to secretion so that it is possible to incrementally expose the cells to allergen to induce desensitisation without significant secretion [15, 55–59]. Alternatively, it is possible to block the activation pathways without affecting the desensitisation pathways [51, 60]. All forms of activation (secretion of mediators, expression of cell surface markers, etc.) are blunted by desensitisation protocols.

Basophil Testing in the Diagnosis of Allergy

Basophil testing is a complex but useful and manageable element of allergy diagnosis (Table 1). It should only be considered if (a) no serological or skin test is available, (b) considerable risk of systemic reactions after skin testing exists or (c) (most frequently) results of serological and skin tests are discordant with the clinical history. Basophil testing should always be considered as an alternative to provocation, as a carefully planned and performed basophil test may predict a severe response as outcome of a provocation and may replace some provocations [61•]. A negative basophil test should always be confirmed with a provocation test. As basophil testing is performed *ex vivo*, it can be done with recombinant allergens and novel drugs or other substances not produced for use in man [36, 62–64].

Chronic Urticaria

The mechanism underlying chronic spontaneous urticaria (CSU) is still incompletely understood [65] and there are no biomarkers. CSU serum can induce histamine release from normal basophils [66] and can contain autoantibodies [67] mainly to the IgE receptor [68]. However, this ability to activate normal basophils is found in 40 % of the patients, and the remaining 60 % are elusive. BAT may replace autologous serum skin test (ASST) in the diagnosis of CSU, but the correlation between basophil degranulation and skin test responses is not perfect [69, 70]. BAT is a reliable, safe and accurate method where there is no need to inject serum into

Table 1 Pros and cons of Basophil Testing

Pros

- Basophil granulocytes and mast cells are related cells that respond similarly to FcεRI cross-linking.
- CD63 upregulation on basophil granulocytes and mast cells corresponds to anaphylactic degranulation
- Basophil activation is a quick functional test of response to allergen
- BAT is a flexible testing module that can address many difficult issues of allergy testing.
- It is possible to separate intrinsic and extrinsic factors affecting basophil activation
- Many drugs can be used as allergen in BAT, and alternative drugs can be found.
- Occupational allergens can easily be tested in BAT.
- BAT has a high positive predictive value in food allergy diagnosis, and can reduce the number of food challenges required for diagnosis.
- Basophil sensitivity is a quantitative measure of a patient's allergy.
- BAT can identify primary sensitising allergens in insect venom allergy.
- BAT can be used to monitor progression of natural resolution or treatment of allergy, and basophil testing may be used as companion diagnostic for AIT.

Cons

- Drug BAT (and other allergy tests) should be performed within a year of the most recent allergic response that elicits the diagnostic visit.
- BAT should be done on fresh blood (preferably less than 4 hours old)
- Non-responders cannot be tested with a BAT.
- BAT is complex and requires an experienced laboratory and interpretation.
- The testing setup and interpretation of BAT is complex and lacks standardisation
- BAT and ASST do not correspond exactly in the diagnosis of CSU.

patients. CD63 up-regulation is a sensitive and specific activation marker for CU [71–73].

Immediate Drug Hypersensitivity

The diagnostic work-up of immediate drugs hypersensitivity reactions (IDHR) aims at identifying the culprit medication and identifying safe alternative(s) for future treatment [74]. Although *in vivo* tests are the most widely used by the clinicians [75], they present some limitations: Drug provocation tests (DPT) and to a lesser degree skin tests [76] are not exempt of risk especially in patients with severe reactions *i.e.* anaphylaxis. For some drugs, skin tests do not have optimal sensitivity and may produce false positive results [77, 78].

BAT shows complementary results to those obtained in skin testing [79]. It is possible to obtain positive results in BAT in a percentage of patients with negative skin test and sIgE determination [38, 80]. Importantly, this test has demonstrated an excellent negative predictive value as the case of fluoroquinolones [81•, 82], where false positive skin tests may occur.

In contrast with available IgE assays, BAT can be performed with a wide range of drugs without need of synthesising the drug-carrier conjugates [83].

Principal applications of BAT have included IDHR to betalactams [80, 84, 85] especially with the new ones, as clavulanic acid, where no other in vitro method can be used [86], muscle relaxant [87–89], fluoroquinolones [81, 90, 91], radiocontrast media [92] and to single-NSAID-induced urticaria/angioedema or anaphylaxis [93] where an IgE mechanism is involved [38, 94, 95]. It may be useful to confirm the involvement of FcεRI in the response [13, 90]. BAT has also been used for diagnosis of many other individual drugs, where no other in vivo/in vitro tests are available [82, 96]. There are some specific points that have to take into account when using BAT for evaluating IDHR:

1. Drugs induce less basophil activation than protein allergens. In addition, significant higher concentrations of allergen are required. This implies that cytotoxicity has to be checked. It is more difficult to calculate a diagnostic threshold.
2. Drugs can be degraded under different conditions as temperature, pH and light, producing metabolites that can interfere with or prevent BAT results [91, 97]. Like other allergens, drugs must be prepared fresh for each test and avoiding the factors that influence their degradation [82].
3. As for other tests used for evaluation of IDHR, BAT sensitivity declines with time passed between reaction and testing. Therefore, it is recommended to perform BAT within a year after of last reaction to the drug [38, 98].

Food Allergy

Basophils are involved in food-induced acute allergic reactions and anaphylaxis [16, 20]; thus, basophil testing can be used to diagnose IgE-mediated food allergy [99, 100]. The main advantages of BAT to protein food allergens compared to sensitisation tests (i.e. skin prick test and specific IgE) are its high specificity (reaching 100 % in some studies) and its high positive predictive value, which allow to confirm the diagnosis of food allergy in the case of a positive BAT with a high degree of certainty [61, 101–103]. BAT can be performed using crude extracts [61, 101, 104] or single allergens [105–107]. Due to the technical and logistical aspects involved in its performance, BAT is often used as a second step in the diagnostic process, only in selected patients in whom the combination of the clinical history and the results of skin prick test and/or specific IgE could not confirm or exclude the diagnosis of food allergy [61]. This approach can lead to a significant reduction in the number of oral food challenges, in particular positive challenges, where patients suffer acute allergic reactions of varying degrees of severity. Interestingly,

basophil reactivity reflects severity and basophil sensitivity reflects threshold of allergic reactions to foods during challenges [22, 23, 108, 109]. This may provide useful information for the management of patients.

Respiratory Allergies

Measurements of sIgE or skin testing in combination with the clinical history are usually sufficient to diagnose respiratory allergies. Basophil sensitivity provides a quantitative measure that gives additional information of the patients' allergic condition e.g. during treatment. Measurements of basophil sensitivity correlate with the nasal provocation titre in allergic rhinitis [110], the allergen-specific bronchial provocation threshold in allergic asthma [18] and the asthma control test [111]. When using an allergen titration, the correlation of the outcome between basophil allergen sensitivity and bronchial allergen sensitivity was statistically significant. This indicates that basophil allergen threshold sensitivity (CD-sens or EC50) may quantitatively reflect clinical allergen sensitivity [18].

Occupational allergies are a frequently overlooked area in which basophil testing can play a major role [112–114]. Convincing documentation is required for sensitisation to a workplace allergen before compensation can be obtained and before the patient considers a change of career to move away from a daily or regular exposure to an offending allergen. The route of exposure is often through the airways, and a standardised approach for testing exists [82].

Hymenoptera Allergy

The clinical history in combination with measurements of sIgE or skin testing may be sufficient to diagnose insect venom allergies, but up to 60 % of insect venom patients may react with more than one insect in first-line tests [115]. This complicates the decision for immunotherapy with the correct allergen. Introduction of recombinant molecular allergens, especially for wasp venom allergens, can solve many of these cases. However, more cases are solved with BAT than with molecular serologic tests. The diagnostic sensitivity and specificity of BAT with insect venom extracts referred to history are good (83 to 100 %) [116–118]. Furthermore, BAT allows the identification of about two-thirds of patients with a clear history of systemic reactions after Hymenoptera stings with negative venom-specific IgE and negative skin test to bee and wasp venom [119, 120].

In patients with a double positive basophil activation test, the venom which the patient is markedly more sensitive (10-fold is arbitrarily set) might represent the clinically relevant trigger allergen [121], but this not yet proven, because diagnostic sting challenges are not recommended or performed.

Basophil Testing to Monitor Therapy

Great expectations to basophil testing are that it may be able to supplement and predict the outcome of provocations, and eventually reduce the number of provocations, and that it thus can be used to repeatedly test patients for their allergy without exposing them to allergen.

This is true for both allergen immunotherapy for protein allergens [21, 24, 61•, 122–125], as well as for treatment effect of anti-IgE or other similar treatments for chronic urticaria [126, 127•, 128], food [129] and respiratory allergies [110, 130–132].

Food Allergy

BAT can be used to monitor IgE-mediated food allergy over time, namely to assess spontaneous resolution and to evaluate the response to immunomodulatory treatments. For example, in patients with cow's milk allergy, BAT can help identifying different clinical phenotypes and can determine when to perform oral food challenge to assess the possible development of tolerance [104, 133]. In many oral or sublingual immunotherapy studies to foods, decreased allergen-induced basophil activation particularly at low concentrations of the allergen accompanied clinical improvement [123, 134–136]. BAT can also be used to monitor response to Chinese herbal formula for food allergy [137].

BAT can have additional applications in food allergy different from diagnostics, for instance, to assess the allergenicity of foods, to detect low amounts of allergen in food matrices and other complex mixtures, and to confirm the biological activity of food allergens [138–143].

Hymenoptera Allergy

Side effects during the build-up phase of venom immunotherapy are associated with a higher basophil sensitivity compared to well-tolerated immunotherapy [144]. Furthermore, the decrease in basophil sensitivity over years in patients submitted to venom immunotherapy is associated with the induction of tolerance [24, 122, 145]. Therefore, the parameter basophil sensitivity seems to reflect more closely the clinical situation with regard to anaphylactic reactions [17••] to insect stings than other known parameters.

Respiratory Allergies

One major advantage with measurements of basophil sensitivity is that it is an objective and reproducible method [106, 146, 147] and can be used to monitor treatment efficacy. During the last decade, several articles described the advantage of using basophil sensitivity during allergen-specific immunotherapy (AIT) to aeroallergens. It has been used to monitor patients

treated with AIT for birch [125, 148] and timothy grass [15, 21, 124], and showed reduced allergen sensitivity already during the up-dosing stage. Basophil sensitivity has successfully been used to objectively identify patients who respond to this anti-IgE treatment [130] and to assess treatment efficacy [110, 130–132] without the need of performing more complex bronchial allergen challenges.

Presentation and Interpretation of Basophil Testing

The precise clinical implications of a measurable basophil activation test are that the patient is sensitised as basophils react to allergen through IgE. It is well understood that reactive basophils are not always concordant with the clinical expression of allergic disease. However, this problem is similar to allergic testing that relies on serology or skin testing. It is possible to research this response in mechanistic studies and elaborate on the diagnostic implications of an ex vivo basophil response to allergen.

Diverse methods are used to evaluate the response in a basophil activation test, i.e. the commercial assay and even research methods have yet to settle on the best practice for evaluation of results. Reactivity is the basic metric for basophil testing; to determine reactivity, one sets a baseline at 1, 2, 5 or 10 % of resting basophils and obtains the fraction of all basophils responding to allergen—typically more than in the resting population. Reactivity is required to calculate the stimulation index (where the fraction of cells stimulated with allergen is expressed as a multiple of the fraction set arbitrarily as positive in the non-stimulated sample), for determination of basophil sensitivity (where the allergen concentration required to activate a given fraction of all cells that can be activated) and for calculation of the area under the curve obtained with different allergen concentrations. Median fluorescence intensity of CD63 on the individual basophil granulocyte may prove to be an alternate measure for activation. CD63 MFI sensitively reflects desensitisation [15]. A graphically compelling but statistically weak approach is to set the threshold for basophil activation midway between the peaks of resting basophils in the negative control and activated basophils in the positive control [149].

CD63 reactivity to 200 ng/ml of food allergen was the best predictor of clinical reactivity in a food challenge (area of 0.904 under a ROC curve) and strongly correlated with symptom severity in food allergen challenges [22]. Similarly, the severity of food allergen challenges could be predicted by the relationship of reactivity at 100 ng/ml peanut allergen divided by the reactivity to a constant response to an antibody activating basophils by crosslinking FcεRI [23•]. The mechanistic framework underpinning this approach has yet to be developed.

Table 2 Research needs in basophil activation

- Mechanisms of non-protein allergic reactions and non-IgE-mediated basophil activation need to be elucidated.
- The diagnostic utility of BAT needs to be validated for specific protein allergens (food, insect venom, respiratory) and in different populations.
- Changes in the basophil response during AIT and anti-IgE treatment in food allergy should be investigated.
- The minimal difference in sensitivity between primary sensitising and cross-reacting allergens needs to be defined.
- Basophil sensitivity tests could be considered as a supplement, and eventually possibly as replacement for allergen challenge tests.
- The predictive value of basophil suppression for treatment outcome has to be established.
- BAT should be established as a biomarker and eventually as a companion diagnostic for subcutaneous allergen immunotherapy

Activation by drugs and other small molecules may depend on non-covalent [150] or covalent modification of patient protein. Typically, much higher molar concentrations of drugs (2 mg/ml penicillin correspond to 6 μ mol/ml) are used than of protein allergen (100 ng/ml of Der p 2 corresponds to 10 pmol/ml). In spite of this million-fold difference in concentration, ex vivo activation of basophil granulocytes by a drug may be marginal and may not achieve complete anaphylactic degranulation. In order to optimise the use of basophil testing in the diagnosis of drug allergy, results are considered as positive when the reactivity is >5 % over spontaneous activation observed for the negative control (set at 2 %) [89, 151], or the stimulation index, calculated as the ratio between the percentage of degranulated basophils with the drug and the spontaneous basophil activation (set at 2 %), is >2 [38, 80, 84, 91, 152]. It is always better to establish a precise cut-off with a ROC analysis for each drug [89]. Either reactivity or stimulation index, based on a stated threshold for resting basophils, may be sufficient for establishing the result of basophil testing.

Clinically, a Bayesian approach to diagnosis that considers the merits of history, first-line tests and basophil tests sequentially to calculate the likelihood of sensitisation is a very strong approach that will provide allergy diagnoses of many patients without a provocation.

Conclusion

Basophil testing is a safe approach and of great interest in the evaluation of those allergens where specific IgE detection tests are not available; current clinical research questions regarding application of basophil testing are listed in Table 2. Basophil activation tests make a variety of allergen molecules accessible as diagnostic reagents, as they can successfully be performed with drugs [38, 80, 84–94] as well as recombinant venom allergen [153], food allergen [105–107] and aeroallergens [33, 154–156]. BAT is an important tool for

research of allergen molecules inducing activation of basophils by IgE-bridging on these cells.

As read out of basophil activation reactivity with CD63 is the most widely used parameter, as it in food allergy also correlates with symptom severity. The sensitivity measurement of basophils demonstrates that basophils were exposed to the relevant allergen concentrations and correlates closely with clinical sensitivity. Basophil sensitivity is important not only in diagnosis but also in monitoring of allergic disease.

Compliance with ethical standards

Conflict of Interest Dr. Santos reports grants from Medical Research Council (UK), grants from NIAID/Immune Tolerance Network, other from National Institute for Health Research (NIHR). Dr. MacGlashan reports grants from National Institutes of Health. Dr. Nopp report a grant from Novartis. Drs. Knol, Eberlein, Hoffmann, Ferrer, Sabato, and Mayorga declare no conflicts of interest relevant to this manuscript.

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- Of importance
- Of major importance

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