REVIEW

Diagnostic Tests Based on Human Basophils: More Potentials and Perspectives Than Pitfalls. II. Technical Issues

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Abstract

Cellular basophil activation tests (BAT) such as histamine or sulfidoleukotriene-release tests for allergy diagnosis have been available for some time, but expression of basophil-activation markers such as CD63 and CD203c detected by flow cytometry has attracted particular attention in recent years. Not only the potential but also the possible pitfalls of flow-cytometric BAT have been stressed recently. Some authors have suggested that the technical problems are still such that BAT should only be performed in specialist laboratories.
In an earlier review based on our clinical experience obtained over several years, we showed that, even using different protocols, reproducible and meaningful clinical results can be obtained. In this paper, we review the current knowledge in relation to several technical issues and show that flow-cytometric BAT already represents a major advance in the field of in vitro allergy diagnosis. We conclude that there are no serious technical justifications for depriving allergic patients of clinically indicated BAT tests, which can be performed reliably by any laboratory with the appropriate experience in allergy diagnosis and flow cytometry.

Key words: Basophil activation test. Allergy diagnosis. CD63. CD203c. Technical issues.

Resumen

El test de activación de basófilos (TAB), como las pruebas de la histamina o de liberación de sulfidoleucotrienos, se han utilizado durante mucho tiempo para el diagnóstico de alergias, aunque últimamente se ha dirigido una atención especial a la expresión de los marcadores activadores de basófilos, como el CD63 y el CD203c, que se detectan mediante citometría de flujo. Pero recientemente el acento no sólo se ha puesto en el potencial de estas técnicas, sino también en los posibles obstáculos que puede presentar la citometría de flujo TAB. Algunos autores han sugerido que los problemas técnicos aun son tales que el TAB sólo deberían llevarse a cabo en laboratorios especializados.
En una revisión previa basada en nuestra experiencia clínica, obtenida a lo largo de varios años, demostramos que se pueden obtener resultados clínicos reproducibles y significativos, incluso utilizando diferentes protocolos. En este trabajo, revisamos el conocimiento actual en relación con diversas cuestiones técnicas y mostramos que la citometría de flujo TAB ya representa un gran avance en el campo del diagnóstico de alergias in vitro. Concluimos que no existen justificaciones técnicas importantes para privar a los pacientes alérgicos de la práctica de pruebas TAB indicadas clínicamente, que se pueden realizar de un modo fiable por cualquier laboratorio con una experiencia adecuada en el diagnóstico de alergia y en citometría de flujo.

Palabras clave: Test de activación de basófilos. Diagnóstico de alergia. CD63. CD203c.Cuestiones técnicas.

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An opinion paper devoted to flow-cytometric basophil activation tests (BAT) in allergy diagnosis has recently been published [1]. The contention of the authors was that in the recent literature concerning BAT many of the basic ideas regarding basophil function have been overlooked. Accordingly, the authors attempted to reappraise some of these forgotten aspects in order to minimize interpretation errors. The overall picture presented was strongly influenced by experience gained with antibodies against immunoglobulin (Ig) E and allergen-induced histamine release. The general impression was that the diagnostic use of BAT is still plagued with many unresolved questions and that the use of such tests should be restricted for the time being to specialist laboratories.

In the first of 2 articles, we presented a comprehensive review of the clinical studies available on allergy to aeroallergens, insect venoms, latex, food allergens, and drugs such as muscle relaxants, ß-lactams, pyrazolones, and nonsteroidal anti-inflammatory drugs (NSAIDs) [2]. This collective review should give allergy specialists the message that these technologies have more potential than pitfalls and that despite using different laboratory protocols, the clinical results in suitable situations may already be useful. There remain, however, some issues that are mainly of interest to laboratories performing BAT and using either in-house protocols or commercially available kits. We therefore felt it necessary to address some contentious technical issues to complete the discussion. Readers specifically interested in flow-cytometric BAT are also referred to some recent reviews [3-10]

Mechanisms of Basophil Activation

The mechanisms of IgE-receptor-mediated activation and subsequent signaling leading to the release of various mediators such as histamine, leukotriene (LT) C4, and lymphokines, such as interleukin (IL)-4 and IL-13 have been extensively described [1]. However, the mechanisms of expression of various membrane proteins, which are the basis of diagnostic BAT, as well as the various mechanisms of non-IgE-mediated activation have not yet been extensively described. It has been shown recently that upregulation of CD63 expression is closely accompanied by and dependent on p38MAPK phosphorylation [11]. The discussion led to the impression that the same quantitative and qualitative rules may apply to all of the outcomes of basophil activation (eg, CD63/CD203c expression, histamine release, and LTC4 and lymphokine production). Indeed, it has been stated that "the parameters comprehensively studied during histamine release form the basis of the test outcome, independently of the final test readout. Promoting cellular tests for the evaluation of IgE-mediated sensitization has the caveat of introducing a number of interrelated variables" [1]. In fact, the various test readouts do not follow strictly and in all respects the rules established for histamine release: a number of differences in their activation cascades and regulation according to the mode of activation and the outcome examined have been well documented [12-20] (Table 1). These differences probably explain why in clinical practice, although often correlated, the various outcomes may occur independently of each other in some individuals and CD63 expression, LTC4 production, and histamine release may be entirely dissociated ([2] and see below). One should therefore be quite careful before applying general conclusions drawn from experience gained with a single BAT such as histamine release to other basophil activation assays.

Variations of IgE-Mediated Responses: Complex Interdependence

Essentially based on studies of IgE-mediated histamine release, we are rightly reminded by Kleine-Tebbe et al [1] of a number of variables that will determine individual basophil outcomes, such as (a) the total IgE receptor cell surface density; (b) the proportion of membrane-bound allergen-specific IgE antibodies versus total IgE; (c) the intrinsic cellular sensitivity of the basophils, ie assessed by the number of IgE molecules required for 50% of maximal cellular responses; (d) the cellular reactivity defined as the maximal cellular response after optimal stimulation; (e) the structural features of the allergen determining the number and respective distances of epitopes able to bind to IgE on a single allergen molecule and in a mixture of allergenic molecules; (f) the nature of the complexes formed by allergens and IgE (dimers, trimers, oligomers); (g) the duration of contact between allergen and membrane-bound IgE; and (h) the presence of specific IgG competing with IgE for allergen binding. Some of these parameters are interdependent or connected with serological parameters, while others are not.

While these "rules of the game" for immediate allergic reactions, already defined many years ago and firmly established [12-20], may be essentially valid for protein allergens, they may not be the end of the story, particularly for allergenic small molecules, haptens, and drugs. The need for an allergen with at least 2 epitopes to bridge 2 antibody molecules (the "bridging" hypothesis) was established long ago, even before the discovery of IgE and the development of in vitro BAT [21-23]. We should not forget, however, that apparent exceptions do exist, such as the elicitation of immediate reactions and of IgE-mediated basophil activation by apparently univalent haptens and drugs [23,24]. Despite numerous studies, the precise molecular mechanisms of such apparently "monomeric" reactions, which have been repeatedly reported [16,25-27], have not yet been fully clarified [16]. It may be postulated that even small molecules contain 2 or more functional epitopes. This should also lead to some caution in believing that the same rules apply strictly to all outcomes of basophil activation. Even if the initial molecular trigger may be the same, there are enough complex regulation steps along the various different activation cascades to explain different outcomes, as has been well documented [12-19]. For example, in a number of cases and according to the mode of activation, expression of CD63, LTC4 production, and histamine release may be entirely dissociated [2]. As another example, for in vitro IgE-mediated reactions, the expression of CD63 is much more sensitive to the external Ca^{2+} concentration than LTC4 production, and this relative Ca^{2+} sensitivity varies from one individual to another. This has led in some circumstances to the paradoxical finding of a

Table 1. Differences in Basophil Activation Pathways According to Outcome and Mode of Activation[a]

Protein Kinase Cascade		Agent	Activation Marker Expression		Histamine Release	Sulfidoleukotriene Production
			CD63	CD203c		
Receptor	Aggregation	a-IgE, All SHIP induction	Yes[b], no[c], ?[d]	Yes[b], no[c], ?[d]	Yes[b], no[c], ?[d]	Yes[b], no[c], ?[d]
	Disaggregation				Yes[b], no[c], ?[d]	
Lyn	Activation				Yes[b], no[c]	
	Inhibition					
Syk	Activation	Iatrunculin			Yes[b], no[c]	Yes[b]
	Inhibition	F-actin piceatannol			Weak inhibition[b]	Strong inhibition[b]
PI3K	Activation				Yes[b], no[b]	Inhibition[b]
	Inhibition	Wortmannin	Inhibition[b]	Little inhibition[b]	Inhibits[b], no inhibition[c]	Inhibition[b]
P38MAPK	Activation		Yes[b]		Yes[b]	
	Inhibition	SB203580	Inhibition[b]		Inhibition[b]	
PLC	Activation				No[b], yes[c]	
	Inhibition					
PKC	Activation	PMA			Enhances[b,c]	Blocks[d]
	Inhibition	Stauroporin			Inhibition[b], no inhibition	No inhibition[d]
IP3	Activation					
	Inhibition					
cAMP	Activation	Forksholin			Inhibition[b,c], no effect[d]	No effect[c]
	Inhibition					
Ca2+	High concentration		Enhances[b]			Enhances[b]
	Low concentration					
ERK	Activation				Not required[b]	Required[b,d]
	Inhibition	PD98059			Little inhibition[b]	Inhibition[b]
Effects of:						
IL-3	Direct induction		No	Yes	No	No
PGD2	Induction		No	Yes	No	
PGE2			Inhibits[b]		Inhibits[b]	
Glucocorticoids						

Abbreviations: ERK, extracellular signal-related kinase; IL-3, interleukin 3; IP3, inositol-3-phosphate; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PLC, phospholipase C; P38MAPK, P38 mitogen-activated protein kinase.

[a] Data from references 11-20

[b] Induction by anti-immunoglobulin E/allergen

[c] Induction by fMLP

[d] Induction by C5a

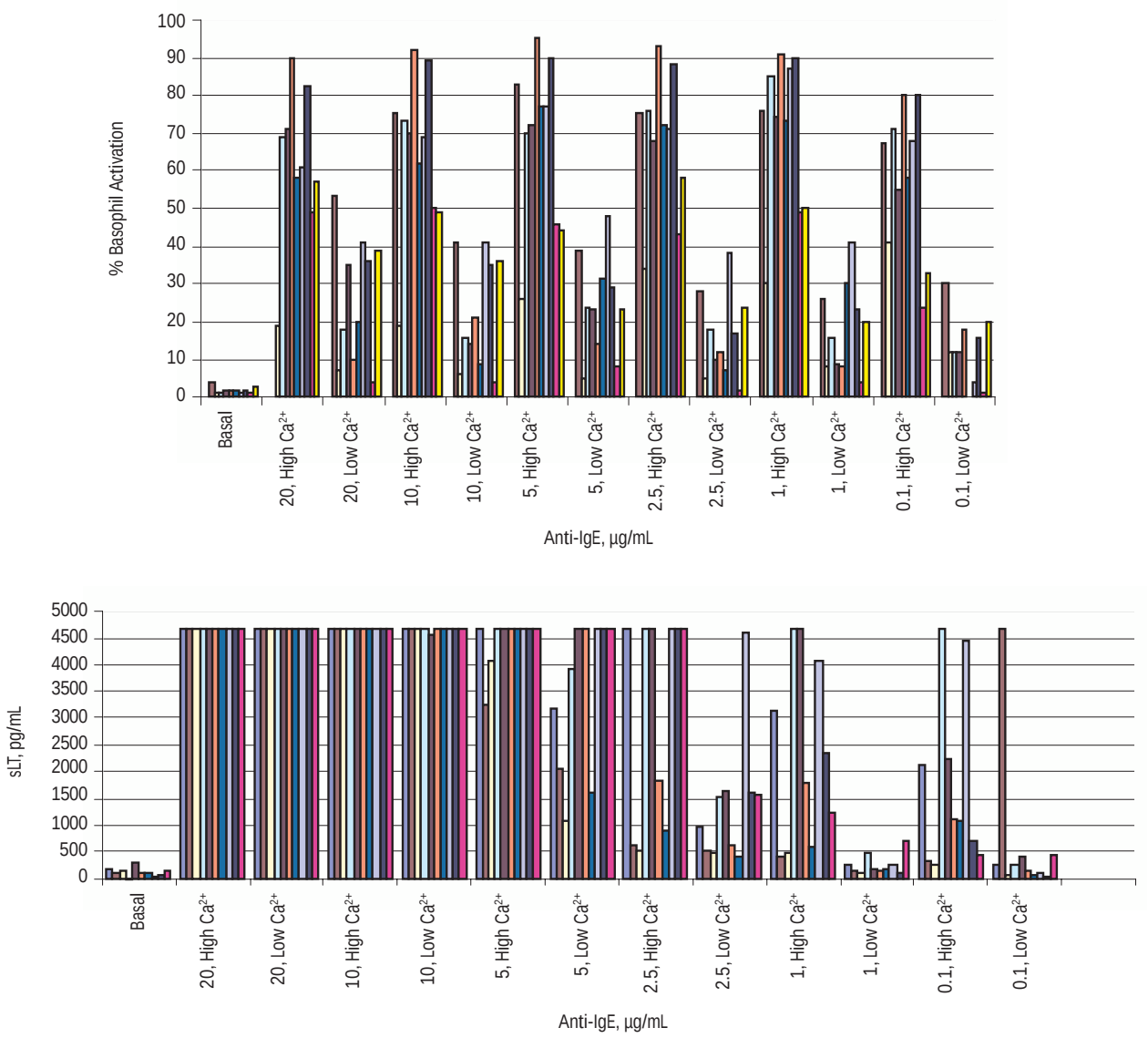

Figure 1. Comparison of calcium dependency of CD63 expression and sulfidoleukotriene (sLT) production in basophil activation tests (BAT). A dose-response curve was constructed for 10 healthy subjects according to the concentration of anti-immunoglobulin E receptor (anti-IgER1) antibody (0.1-20µg/mL). The response was measured as percentage basophil activation based on CD63 expression (upper panel) or production of the sLT leukotriene C4 (lower panel) either at optimal (high Ca^{2+}) or suboptimal (low Ca^{2+}) concentration. It can be seen that CD63 expression is more sensitive to low extracellular Ca^{2+} than is sLT production. This may lead to paradoxical BAT-negative but cellular antigen stimulation test-positive results in individual patients (false BAT non-responders). (M.L.S., unpublished data, 2008.)

negative control BAT but positive cellular antigen stimulation test (CAST) (Figure 1). Even the expression of 2 different membrane markers, CD63 and CD203c, obeys different rules (Table 2).

In practical terms, use of all 3 major tests of basophil activation—CD63/CD203c expression, sulfidoleukotriene (sLT) release, and histamine release—in clinical situations reveals that although the outcomes often correlate they may also be completely dissociated in individual patients [2]. Some of the rules found for histamine release—eg, the correlation between anti-IgE-mediated basophil activation, IgE receptor density, and serum IgE concentration [1,20]—do not apply so strictly to CD63 expression. For example, a study involving a large number of patients showed that the percentage of CD63-expressing basophils upon activation with polyclonal anti-IgE did not correlate with the serum IgE concentration [28]. Since only a limited number of aggregated IgE receptors is required to induce basophil activation [16], it is understandable that, above a certain threshold, basophil activation becomes independent of the extracellular IgE concentration.

Table 2. Differences in Regulation of CD63 and CD203c Expression

	CD63	CD203c
Synonym	Gp43: lysozyme-associated membrane protein	E-NNP3 enzyme
On resting basophils	Barely detectable	Constitively present
Present on upregulation	Other blood cells, platelets Maximum within 25 to 30 min Associated with mediator release	Basophil specific Maximum within 10 to 20 min Not associated with mediator release
Induction by interleukin-3	No	Yes
Induction by PGD2	No	Yes
Effect of wortmannin (PI3K inhibitor)	Total inhibition	Slight inhibition
Fluorescence intensity	Higher[a]	Lower[a]
Spontaneous occurrence in vivo[b]	Low	Higher
Clinical diagnostic efficiency	Better[a]	Better[a]
Clinical validation	Extensive (>35 studies)	Still restricted (<10 studies)

Abbreviations: E-NNP3, ectonucleotide pyrophosphatase/phosphodiesterase 3; PGD2, prostaglandin D2; PI3K, phosphatidylinositol-3-kinase.
[a] There is no clear agreement in the literature on this point.
[b] In food allergy and atopic dermatitis.

Some Technical Aspects of BAT in Clinical Diagnosis

Allergen Concentrations: The Legend of the Bell-Shaped Dose-Response Curve

Kleine-Tebbe et al [1] pointed out that the dose-response curve of basophil activation by various allergens is bell shaped and may vary among sensitized individuals by as much as 5 logarithms. They drew from that assumption the practical implication that "multiple allergen concentrations, ie spanning a range of at least 4 logs, are required to cover various individual responses; one single allergen concentration is not sufficient to analyze basophil responses, even if only qualitative results will be reported." This statement is again based on experience with histamine release but certainly not on clinical experience with CD63 expression. In fact, studies based on CD63 or CD203c expression have clearly shown that in most instances both the group curve and individual dose-response curves have a sigmoid shape, the percentage of activated basophil remaining stable or only minimally decreasing at supraoptimal antigen concentrations [29-33]. The reason for the bell-shaped curves often encountered in histamine release tests, like in the precipitin reaction, has been sometimes thought to be a decrease in the density of aggregated IgE receptors, an assumption confirmed by the fact that the activation decrease with excess antigen is more marked with monoclonal than with polyclonal anti-IgE antibodies and with bivalent rather than polyvalent antigens [10,16,24]. Even with histamine release, when the basophil response is due to IgE antibodies of several specificities and to allergens with multiple epitopes, which is most frequently the case in clinical situations, the bell-shaped curve is usually only rather slight. An alternative explanation for a bell shaped curve, however, is

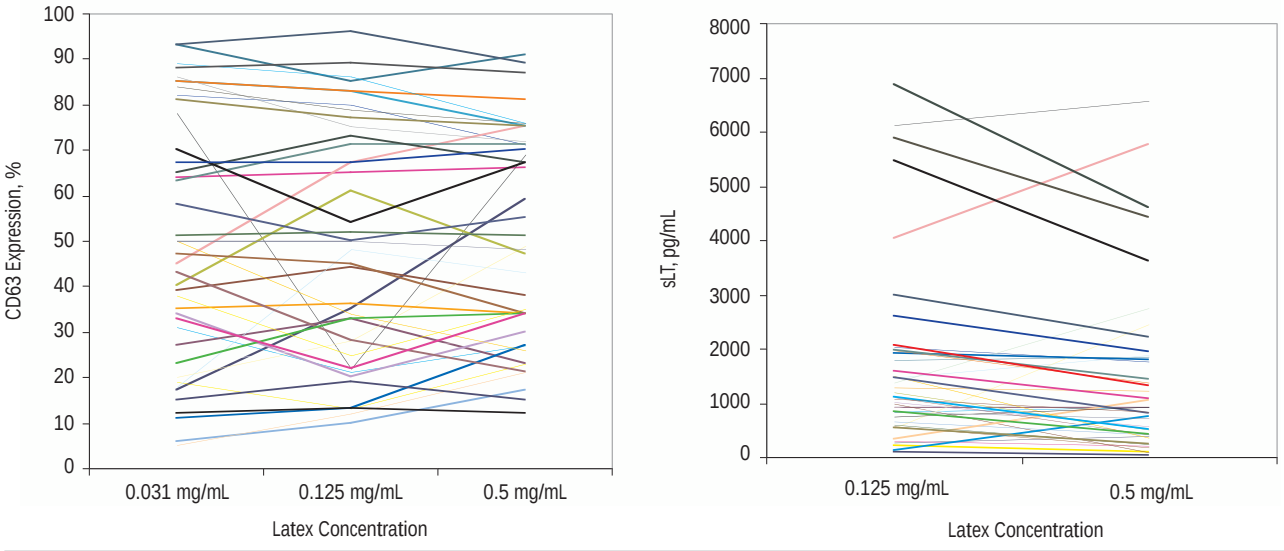

Figure 2. Dose response curves to latex. With a previously calibrated natural rubber latex allergen, individual response curves for the basophil activation test (left panel) and the cellular antigen stimulation test (right panel) show similar results in most cases. sLT indicates sulfidoleukotriene. (Data from Sanz et al [32].)

the induction of downregulating molecules such as SHIP under conditions of supraoptimal stimulation [16, 34].

In most reported clinical studies showing individual dose responses, allergen concentrations for maximal CD63 response do not differ by more than 1 to 2 logarithms (Figure 2), which, provided the allergen lot used has been calibrated beforehand, allows reliable routine qualitative diagnosis (positive/negative) to be obtained with 1 or 2 allergen concentrations. This point is quite important in practice for economic reasons, in terms of work and reagents involved. The activation peak is broader for protein allergens than for drugs; as a practical rule, 2 allergen concentrations are sufficient for proteins and 3 for drugs, with a dilution ratio of 1:5 (J. S. L., unpublished data, 2008).

For quantitative comparisons, on the other hand, either among individuals or in the follow-up of the same individual (eg, immunotherapy), dose-response curves encompassing the whole range of reactivity will obviously continue to be required. One may also determine the threshold concentration for response [35]. Admittedly, some authors have reported bell-shaped curves with CD63 expression [36-38]; whether this is due to the absence of IL-3 in these instances is still an open question. In 1 case at least [36], the reason for the bell-shaped curve is quite trivial: the authors used as allergen commercial prick test solutions containing additives that are cytotoxic at the highest concentrations used (M. L. S, unpublished data, 2008). Glycerinated allergen extracts (< 1% glycerin) may be used but only at less than 1:100 dilutions (J. S. L., unpublished data, 2008). This should remind us of an important pitfall not discussed by Kleine-Tebbe et al [1]: allergens used for flow-cytometric tests must be calibrated and standardized not only for reactivity with specific IgE but also for use in cellular tests. They must show absence of cytotoxicity (additives, preservatives) and of nonspecific stimulation (endotoxins, lectins), and effectiveness in specific cell stimulation (cellular standardization). Recombinant allergens might well help in this respect.

Flow-Cytometric Capture of Basophils

The originally described flow-cytometric technique used for identifying basophils in a complex mixture of blood cells involved the use of a fluorescently labeled polyclonal anti-IgE antibody. Monoclonal anti-IgE capture antibodies are less efficient [8]. The objection has often been raised that, since IgE is also found on other cells such as monocytes or eosinophils [39,40], the use of anti-IgE antibodies will capture cells other than basophils, and routine hematologic methods to count basophils have recently been criticized [41]. However, proper cytometric gating allows basophils to be selected easily, and as a result, this theoretical objection has no effect in practice. Some capture strategies (eg, anti-IgE/CD203c) enable capture of 90% to 95% pure basophils (J. S. L., unpublished data, 2008).

Much has been made of the contention that CD63-mediated fluorescence may in fact be due to platelets bound to activated basophils and not to the basophils themselves [1], a phenomenon extensively studied by Knol et al [42]. This objection, however, may also be more theoretical than practical. Firstly, in several clinical studies where the possible presence of platelets in the cluster of CD63-positive cells has been analyzed, for example by the use of platelet-specific CD41 antibodies, it has been found that the contribution of platelets is negligible [43,44]. Secondly, even if the percentage or total fluorescence of CD63-positive cells were augmented by platelets following activation, it would only magnify the difference between the negative (unstimulated) and the positive tests and would therefore remain a consequence of allergen-induced activation, with no essential consequence on qualitative (negative vs positive) results. Although microscopic examination confirms some platelet adhesion, this is also found in negative controls. Furthermore, blood sampling in EDTA rather than heparin eliminates platelet contamination (unpublished data, 2008).

A potentially more serious objection to the use of anti-IgE for basophil capture is the fact that the density of IgE and IgE receptors may vary considerably among individuals and also among basophils in the same individual [9,16]. This may play a negative role, particularly in nonatopic individuals and when the percentage of activated basophils is low (eg, due to drug treatment). On the other hand, it has been clearly shown that the percentage of CD63-positive basophils detected by flow cytometry varies very little between 100 and 1000 recovered basophils per test, a number which is almost always reached in practice [4,44]. Nevertheless, alternative means of capturing basophils have been identified, such as the CD203c marker, which is exclusively present on basophils among blood cells, the combination of anti-CD123 (interleukin [IL] 3 receptor) and anti-HLA-DR (exclusion of antigen-presenting cells/monocytes), of anti-CCR3 and anti-CD45 [45], and of anti-CRTH2 (D2 receptor) and anti-CD3 (exclusion of T cells) [9] have been more recently proposed. Only 1 study, however, has directly compared 2 different capture strategies in terms of overall clinical diagnostic efficiency [45].

Since most clinically validated studies have been performed with the anti-IgE protocol [2], we feel that this protocol remains for the time being the one with the widest clinical validation and may constitute essentially a first-generation BAT. It remains to be seen whether modifications of the basophil capture strategy—eg, by the combination of anti-CRTH2 with anti-CD3, as has been recently proposed [9], or with anti-CCR3—may lead to increased sensitivities, particularly in drug allergy.

Activation Markers: CD63 versus CD203c

Following the discovery and clinical use of CD63 as a basophil-activation marker in the early 1990s [46], the identification of CD203c as a more specific marker of basophils [47,48] has raised hopes in recent years of an improvement in the clinical diagnostic efficiency of BAT. Indeed, some authors have claimed an improvement of sensitivity with CD203c [49,50], but this has not been confirmed by others [51-53]. In the meantime, additional studies of the kinetics, mode of regulation, and activation of these 2 membrane markers have revealed a number of striking differences (Table 2). Expression of CD63 is closely related to the phenomenon of basophil degranulation [48], while expression of CD203c has different kinetics [48], partially different enzymatic regulation [54],

different activation by prostaglandin D2 [55] or IL-3 [43,55], and also seems to be more easily activated in a nonspecific manner (eg, by gradient centrifugation of blood [43,56] or by clinical conditions such as severe atopic dermatitis or food allergy [43]). Although it has been claimed that CD203c yields a 3-fold to 8-fold higher fluorescent signal than CD63 [9], others report exactly the opposite [8,52]. This apparent contradiction will possibly find its explanation in a direct comparison of the different CD63 conjugates used by the various groups. It has also been objected that basophil activation results in a continuous increase in fluorescent CD203c cells and intensity, in which the gating limits between nonactivated (also CD203c positive) and activated basophils are somewhat subjective, while expression of CD63 is an all-or-nothing phenomenon [52]. A main disadvantage of CD203c is its weak labeling of resting basophils, as this leads to a gating overlap between the selected basophils and the autofluorescent cell population (J. S. L., unpublished data, 2008)

Whatever the pros and cons may be, BAT with CD63 is at the present time a widely confirmed and clinically validated test [2], while BAT with CD203c, due to its more recent introduction, still requires more extensive validation in various clinical situations. Whether, in addition to CD203c, newly identified basophil activation markers such as CD107a (CD63-like) or CD13 and CD164 (CD203c-like) [54] will enable more reliable and sensitive second-generation BATs to be developed remains to be determined.

BAT in Whole Blood Versus Isolated Leukocytes

Among the groups using BAT for clinical diagnosis, 2 schools appear to have developed over the years. Many have preferred using whole blood, usually heparinized, due to its simpler and faster manipulation and also based on the conviction that leaving the basophils in their natural environment without manipulations ensures a better functionality. Others, mostly at first for historical reasons (need to isolate leukocytes in order to perform concomitant CAST with enzyme-linked immunosorbent assays) have preferred to isolate the cells first, either as buffy coat (first centrifugation at 500 *g* for 10 minutes) or as plasma leukocytes (first centrifugation at 200 *g* for 5 minutes).

The main advantage of using heparinized whole blood is one of speed and ease of manipulation. However, some disadvantages have been pointed out: heparinized whole blood is markedly less stable and functional than EDTA blood, and this considerably reduces its range of use (M. Schneider, oral communication, 2007; J. S. L., M. L. S., unpublished data, 2008). In some instances, the presence of allergen-specific (eg, IgG antibodies) or nonspecific (eg, complement) plasma factors may influence the results. Finally, in the few instances where a direct comparison has been made, a technique in whole blood (BASOTEST) appears to be less sensitive than a technique with isolated leukocytes (FLOW CAST) for anti-IgE and allergen-induced basophil CD63 activation [57]. In direct comparison with the same

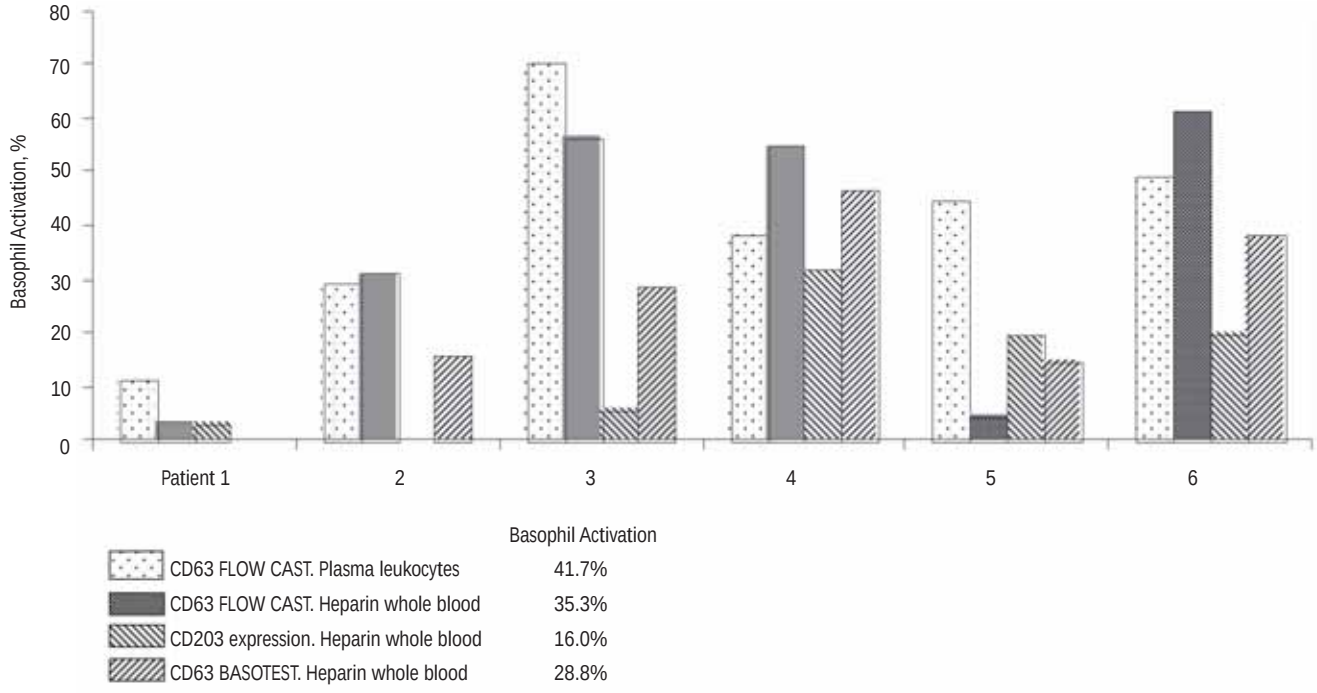

Figure 3. CD63 or CD203c expression in isolated leukocytes versus whole blood. The effect of anti-immunoglobulin E receptor antibody on CD63 and CD203c expression was compared using isolated plasma leukocytes or heparinized whole blood from 6 healthy individuals. In addition to overall mean differences, sizable individual differences are observed. (M. Schneider, oral communication, 2007.)

protocol, whole blood indeed seems less sensitive than isolated leukocytes [5] (Figure 3). However, this does not appear to be clinically significant for qualitative diagnosis (positive/negative) of allergies to inhalant allergens, foods, insect venoms, or latex, since clinically validated studies for both techniques give very similar results in terms of sensitivity and specificity [2]. For drugs, however, with a markedly lower grade of activation, differences may become apparent. This has been particularly the case for ß-lactams and NSAIDs [2], in which results obtained with BASOTEST have sometimes been reported to be inferior but in 1 instance similar to those obtained by FLOW CAST. Recently, however, a very high sensitivity in whole blood has also been reported for muscle relaxants [33]. Whole-blood protocols, particularly those with EDTA, may not be equivalent to those with heparin (J. S. L., unpublished data, 2008).

The use of isolated leukocytes also has some drawbacks: it is relatively difficult to standardize and a recent multicenter study has clearly shown that the mode of leukocyte isolation influences BAT reactivity to NSAIDs [58,59], possibly due to interference of erythrocytes with the drug concentration required for pharmacological activity in vitro (see below). This point is definitely of great practical importance and will require further study in order to improve second-generation BATs.

Negative Controls

It is desirable to obtain a negative control as low as possible, 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 (in 504 cases from multicenter studies [58,60]: stimulation 0%-5%, 79.9%; 5%-10%, 13.6 %; > 10%, 6.5%). Natural exposure in vivo to the allergen tested may cause high basal activation, for example in a pollen-allergic patient studied during the pollen season [61], although some authors disagree [30]. In the latter case, the use of whole blood with the corresponding increase of 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 [43,62] and in patients with venom allergy undergoing immunotherapy [63].

There are also several other causes which might be responsible in vitro for a high BAT basal value, particularly pyrogens and endotoxins that could contaminate the water used for reconstituting or diluting the reagents or other agents such as heparin, various preservatives, or even some plastic tubes or microtiter plates. It is therefore important to use ultrapure water and cell culture-grade plastic tubes and materials [5]. Since negative controls are most often below 5%, it is unlikely that the mild centrifugation used to select plasma leukocytes (eg, FLOW CAST) may by itself cause stimulation in vitro.

Positive Controls: The Problem of Nonresponders

Most studies use monoclonal or polyclonal anti-IgE antibody in the assay, but it is known that some patients, so-called nonresponders, do no react to anti-IgE, either by histamine release [64] or sLT production [4,65]. Polyclonal anti-IgE is recommended since many monoclonal anti-IgE antibodies are poor basophil activators, but there are exceptions (J. S. L., unpublished data, 2008). The percentage of nonresponders ranges between 15% and 25% depending on the study [62,66-68], but this seems to apply essentially to histamine release. For BAT, the percentage of nonresponders reported is usually markedly lower, near or below 10% [8]. A group of nonresponders for histamine release has been identified as deficient in the syk tyrosine kinase [69-72].

The sensitivity of the positive control can be improved using a monoclonal anti-IgE Fc receptor (FcεR1) antibody (eg, E22.7) instead of anti-IgE; this increases the activation percentage and the number of responders [5]. However, not all anti-FcεR1 monoclonal antibodies are equally efficient; some (eg, CRA) appear to yield much lower rates of BAT response [73]. In multicenter studies involving the largest series of patients investigated so far (n = 504) with the same reagents (FLOW CAST), the percentage of true nonresponders (negative for BAT and CAST) was 3.2% for CAST and 2.8% for BAT [58,60]. An additional 10.5% were found to be negative for BAT but positive for CAST; they were therefore not true nonresponders. It was later found that this dichotomy was due to reconstitution of the anti-IgE receptor antibody in buffer not containing Ca^{2+} and Mg^{2+}, ending up with a 15% lower Ca^{2+} and Mg^{2+} concentration than required for optimal stimulation. It was then confirmed that in some individuals BAT is more sensitive than CAST to low external Ca^{2+}/Mg^{2+} concentration (Figure 1).

If the positive control with anti-IgE or anti-Fcε receptor antibody is negative, a negative result with antigen cannot be interpreted properly. A negative control with anti-IgE seems to be more frequent in nonatopic patients [28]. 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 = not significant; results not shown), which reflects that there is no apparent relationship between the basophil reactivity determined by BAT and the IgE level. This finding contrasts with what has been reported for histamine release [16,20] and shows once more that both manifestations of basophil activation should not be summarily amalgamated.

Effect of Blood Storage

Blood sampling and storage for cellular tests, such as this one, requires some special conditions in order to obtain good cell 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 acid citrate dextrose blood samples kept at 4ºC maintain a suitable viability for at least 24 hours, while this is not the case for heparinized blood [4]. At room temperature, IgE-mediated reactions decrease markedly faster [61], possibly due to desorption of IgE from the ex vivo cells. At 48 hours and 4ºC, a sizable response can still be observed for numerous protein allergens but with lower sensitivities, as is the case with drugs, and a larger number of false negatives will occur [5].

Effect of Time of Incubation With Allergen

IgE-mediated activation is a relatively short process that reaches its peak within 15 to 20 minutes but varies

according to the marker chosen (Table 2). Therefore, maximum basophil activation with clinically relevant allergens can be expected within this time range. However, if BAT is performed concomitantly with sLT determination (CAST) in the supernatant, a longer incubation time has been recommended [68]. Some other manifestations of basophil activation, such as expression of CD203c [9,48,54] or activation by non-IgE-mediated mechanisms (eg, C5a, fMLP) [19] are faster and require only a few minutes to reach their peak. CD 203c expression already starts to decline after 15 to 20 minutes [54] and both CD63 and CD203c disappear after 4 to 5 hours incubation (J. S. L., unpublished data, 2008).

The Role of IL-3 in BAT

Preincubation or coincubation with IL-3 markedly increases sLT production (CAST) [19,69] and also increases histamine release [14,16,19] but there is no general consensus on its ability to improve the sensitivity of BAT. Some authors have reported that it increases CD63 expression, thereby increasing the assay's sensitivity [74,75], which may be relevant for allergens causing little specific stimulation, such as drugs [9]. In an early series of comparative experiments, we observed that addition of IL-3 to the stimulating buffer increased the BAT reactivity to ß-lactams, while marginally affecting the negative controls [76]. However, the enhancing effect of IL-3 on CD63 expression does not seem to apply to all circumstances [77,78]. It has also been argued that IL-3 may act as direct activator of basophils [79], not merely as priming agent. This, however, may be the case for expression of CD203c but not for CD63 at the commonly used concentrations of 2 to10 ng/mL [42].

In conclusion, the addition of IL-3 is definitely required if CAST has to be determined in the same cellular supernatant. The need for IL-3 when performing BAT alone is possibly not absolute for strong stimulations by protein allergens; its requirement in drug allergy should be more systematically reassessed but its addition for the time being does not seem to be deleterious

Evaluation of Results

For appropriate evaluation of the results, 2 values should be taken into account:

(1) The absolute number of basophils evaluated, which should be over 150. It has been shown that between 150 and 1000 the percentage of CD63-expressing basophils varies little [5,44,77]. However, one must take into account that the population of basophils may be heterogeneous in its reactivity; hence the theoretical desirability to recover as many basophils as possible

(2) The percentage of activated basophils. In the negative (nonstimulated) control, the percentage of activated basophils is usually below 5%. The positive control after activation of the cells with anti-IgE or anti-IgE-receptor antibody has been discussed above. Some authors also describe a decreased mean fluorescence of the anti-IgE-labeled population activated in vitro by allergen [80-82], a phenomenon which does not seem to affect the percentage of basophils expressing CD63. The practical use of an index based on this phenomenon has been recently proposed [83].

BAT Positivity Criteria

To establish cutoff points for each allergen, it is necessary to set up receiver operating characteristic (ROC) curves to establish optimal sensitivity and specificity [84-86]. The recommendation to determine cutoffs by this method [8,9] had in fact already been followed for a number of years by the majority of groups having published BAT clinical studies (eg, Sanz et al [87]). In the experience of the Pamplona group, the cutoff points offering the highest specificity and sensitivity values determined by ROC curves are the following [4,10]: for inhalant allergens >15%; food allergens >15%; latex >10%, hymenoptera venoms >10%; ß-lactam antibiotics >5% and stimulation index (SI) >2; metamizol >5% and SI >5; aspirin and NSAIDs >5% and SI >2. For most other groups, cutoff points have been very similar but usually not adding SI as an additional requirement [8,9].

Comparison With Other In Vitro Diagnostic Techniques

Comparisons among various in vitro tests have been made for several allergens, and BAT usually shows a good correlation with histamine release or sLT determinations (CAST) [4,5,28,62,87-90], while in general appearing more sensitive and specific than the other tests [5,62]. A detailed comparison of several studies for histamine release and BAT confirms that general impression for inhalants, venoms, food and latex allergies, and particularly for drug allergies

For diagnosis of immediate reactions to ß-lactams, the combined use of BAT and sLT assay (eg, CAST) improves results and allows detection of up to 80% of the cases [60]. The same is true for pyrazolones [90]. Although the 3 major basophil activation outcomes are correlated as a group, they may be totally discordant in some individual patients [2]. This explains why the combined use of 2 tests, BAT and CAST, may yield a higher diagnostic efficiency than 1 test alone. This effect is minimal with protein allergens causing high basophil activation [78] but becomes more relevant with drugs.

Indications, Performance, and Interpretation of BAT Tests

BAT tests are not primary diagnostic measures; they are essentially complementary to skin tests and allergen-specific IgE determinations, particularly when these cannot be performed or have given doubtful results in comparison with a patient's clinical history. They are particularly indicated in insect venom allergy [91,92], food allergy [43], latex allergy, and the major immediate-type drug allergies [2]. The proper performance of the test requires some precise attention to detail, particularly in the conservation of the blood samples, the preparation and quality of the reagents used, and the flow-cytometric gating processes established. Most important is the quality and standardization of the allergens used, which must be submitted to special quality control measures for use in flow cytometry. If these flow-cytometric quality control measures are not provided by the commercial allergen source, the laboratory will have to

perform them by itself. In fact, experience with allergens is probably more important for quality BAT tests than experience with basophil handling, which is easily acquired by any flow-cytometry laboratory. Some of these technical details have been discussed above and in various reviews [5,10].

Conclusions

BAT tests are rapidly becoming an important addition to the tools available for the diagnosis of allergy. However, the number of European groups with varied and sustained practical BAT experience in routine diagnosis is still rather limited. The most recent opinion paper on this matter may give the impression of a requirement to possess many skills before engaging in clinical BAT diagnosis, "which should be restricted to selected cases and to experienced laboratories" [1]. In fact, the common positive scientific and clinical experience with BAT obtained by several groups and with various available protocols and commercial kits over a number of years [2] is more significant than the technical issues that remain to be resolved. A special effort is underway to standardize procedures and evaluation at a European level, as highlighted by the First International Allergy Flow Cytometry Workshop recently held in Pamplona, Spain in 2007. In our opinion, there are no rational or scientific reasons why BAT should not be offered in appropriate clinical contexts by any allergy group wishing to provide optimal diagnostic techniques to its patients.

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