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Basophil activation tests: time for a reconsideration

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Summary

Challenges in *in vitro* allergy diagnostics lie in the development of accessible and reliable assays allowing identification of all offending allergens and cross-reactive structures. Flow-assisted analysis and quantification of *in vitro* activated basophils serves as a diagnostic instrument with increasing applications developed over the years. From the earliest days it was clear that the test could constitute a diagnostic asset in basophil-mediated hypersensitivity. However, utility of the basophil activation test (BAT) should be reassessed regarding difficulties with preparation, characterization and validation of allergen extracts; availability, and the potential of more accessible diagnostics. Today, the added value mainly lies in diagnosis of immediate drug hypersensitivity. Other potential indications are monitoring venom-immunotherapy and follow-up of natural history of food allergies. However, results in these non-diagnostic applications are preliminary.

We review the most relevant clinical applications of BAT. Some personal comments and views about perspectives and challenges about flow-assisted allergy diagnosis are made.

Key words: basophil activation test, allergy, drug hypersensitivity, food allergy, Hymenoptera venom, diagnosis, CD63, flow-cytometry

The basophil

Basophils are granulocytes that develop from CD34+ pluripotent progenitor stem cells and represent less than 1% of the peripheral blood leukocytes. They exhibit a segmented nucleus and are identifiable by means of metachromatic staining with basic dyes such as toluidine blue. Together with tissue resident mast cells, basophils can be activated in various ways, which may be differentiated whether or not cross-linking of the membrane-bound high-affinity receptor for IgE (FcεRI) is involved. IgE/FcεRI-dependent activation mainly occurs via (glyco)protein allergens, chemical allergens and auto-antibodies (mostly IgG isotype) directed against the FcεRI receptor or membrane-bound IgE. FcεRI-independent activation mainly results from engagement of receptors for endogenous (e.g. cytokines, anaphylatoxins, chemokines, IgG, complement, neuropeptides) or exogenous (e.g. pathogen associated molecular patterns) substances. However, it should be kept in mind that the degranulation of basophils/mast cells might also result from alternative largely unknown means (e.g. direct mast cell degranulation as seen with drugs and related compounds such as opiates, iodinated contrast, vancomycin and quinolones). Upon activation, basophils will release and synthesize a broad variety of mediators that are responsible for the early and late phase manifestations of the allergic reaction.

Basophil activation tests: from past to present

For about half a century, *in vitro* basophil activation studies have mainly focused on histamine and leukotriene release in supernatants of activated cells [for review: [1]] Although for many these techniques still remain the “reference test” for effector cell activation and degranulation, mediator release assays have never really entered mainstream application, mainly because of their time- and cost-consuming two-step approach, i.e. (a) cell isolation and incubation and (b) quantification of mediators.

The foundations of modern flow cytometric basophil activation tests were laid almost 25 years ago [2] and in the meantime BAT has largely supplanted mediator release tests. The principles and requirements have repeatedly and extensively been described elsewhere [3]. Briefly, ~~the~~ traditional BAT relies on a flow cytometric identification and quantification of various specific activation markers on the surface membrane, or activated proteins inside the cells. These changes can be detected and quantified on a single-cell level using specific monoclonal antibodies coupled to fluorochromes. In most studies, basophils are identified by specific surface markers such as IgE/CD203c, followed by CCR3/CD3 or CD123/HLA-DR. Of these markers, only CD203c is lineage specific. Subsequently, after activation with an allergen, the up-regulation or appearance of specific surface activation markers, such as CD63 and/or CD203c, can be measured. Alternative methods to measure *in vitro* basophil activation and degranulation imply quantification of expression of inhibitory receptors such as CD300a [4, 5], phosphorylation of intracellular signaling molecules such as p38 mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT)5 [6-8]. Last but not least, quantification of the intracellular histamine content by an enzyme affinity method using the histaminase diamine oxidase in a technique called HistaFlow® can be established [9, 10].

Figure 1 displays the principles of a traditional BAT, whereas figure 2 shows a HistaFlow® dotplot of a patient with an allergy to Hymenoptera venom.

Basophil activation tests in immediate drug hypersensitivity

The applications of BAT in immediate drug hypersensitivity reactions (IDHR) have been reviewed recently [11-13]. It appears that BAT is being rapidly incorporated in the diagnosis of IDHR and has mainly been applied to document allergy to neuromuscular blocking agents (NMBA) (table 1), antibiotics (β -lactam antibiotics and quinolones) (table 2), aspirin and other non-steroidal anti-inflammatory antibodies.

The diagnostic approach for IDHR to β -lactam antibiotics generally relies on skin testing and quantification of specific IgE (sIgE). However, as addressed by Blanca *et al.* [14] in particular cases, the BAT has gained importance. At present 9 studies (table 2, top) have investigated the BAT in the diagnosis of IDHR to β -lactam antibiotics, mainly amoxicillin. Compared with the measurement of sIgE in the diagnosis of allergy to β -lactam antibiotics, BAT shows a higher sensitivity (about 50%) and specificity (approximately 90%).

Correct diagnosis of IDHR to fluoroquinolones can pose a significant challenge. Although several attempts were undertaken to prepare fluoroquinolone conjugates [15, 16], there are still no sensitive and well-validated drug-sIgE assays readily available. Moreover, Baldo *et al.* [17], who were the first to develop such conjugates, recently rose significant controversy about the specificity of some of these solid-phase IgE immunoassays. Although some authors observed skin prick testing (SPT) and intradermal testing (IDT) valuable diagnostics for fluoroquinolone hypersensitivity [18, 19], others have shown considerable numbers of positive results in healthy control individuals without overt fluoroquinolone hypersensitivity [20-23]. With regard to BAT, literature shows divergent findings with respect to the sensitivity

of the test. As revealed by table 2 (bottom), it seems that fluoroquinolones might induce basophil degranulation that is difficult to depict in traditional CD63-based assays. Using this readout only Aranda *et al* [16], unlike 2 other groups [19, 22], managed to demonstrate *in vitro* basophil responses measured with CD63 to fluoroquinolones. Alternatively, the more consistent and promising results with CD203c [24, 25] could indicate mediator release in response to fluoroquinolones to result from an alternative degranulation pathway [26]. Anyhow, further studies with dual labelling for CD63 and CD203c are warranted in elucidating these issues.

Immediate hypersensitivity reactions to NSAIDs are very common and it is commonly accepted that the majority of these reactions are not IgE-mediated but correspond to a pharmacological mechanism caused by the inhibition of cyclo-oxygenase (COX)-1, which results in depletion of prostaglandin E2 with unstrained synthesis of cysteinyl leukotrienes and mediator release from basophils, mast cells and eosinophils. Consequently, BAT generally displays a too low sensitivity to diagnose IDHR to NSAID hypersensitivity (table 3). From table 3 it emerges specificity of BAT for NSAID can also pose a problem with significant numbers of clinically irrelevant positive results in apparently healthy control individuals [27, 28]. However, although without firm proof, a minority of IDHR to NSAIDs have been supposed to be 'real' IgE-mediated reactions in patients reacting exclusively to a single NSAID family. So far, five studies have been published on selective NSAID reactors to pyrazolones [29-33]. In these cases, BAT yielded a sensitivity between 42 and 70% with a specificity of 86-100%. However, one study which evaluated the potential of the BAT in 6 patients with a suspected selected metimazol hypersensitivity showed a very low sensitivity of the BAT, contradicting the other studies [33].

Immediate drug hypersensitivity reactions to iodinated radiocontrast materials (RCM) have been described, but their prevalence is low and estimated between 0.02% for nonionic RCM and 0.4% for ionic RCM. Direct histamine release by means of nonspecific RCM binding to surface receptors on mast cells or basophils and indirect cell activation by means of the complement or kinin cascade outweigh the genuine IgE-mediated reactions that might be overlooked by skin testing [34, 35]. These reactions are believed to account for approximately 4% of the IDHR to RCM [36, 37]. Up to now, three studies reported on the value of BAT in the diagnosis of IDHR to iodinated contrast media [34, 38, 39]. These studies demonstrate a sensitivity of 46-63% depending on the chosen threshold, and a specificity of 89-100% (table 4). Furthermore, it seems that the results of BAT and skin testing are complementary [34] .

In addition, BAT has recently been applied to diagnose allergy and sensitization to illicit drugs such as *Cannabis sativa* [40]. Actually, it has been shown that *C. sativa* allergy can result in an allergy-syndrome towards a large amount of plant-food products [41]. Basophil activation test with the *Cannabis sativa* extract can help in establishing this diagnosis.

Basophil activation in Hymenoptera venom allergy

For people suffering from IgE-mediated Hymenoptera venom allergy, a re-sting can elicit life-threatening allergic reactions. In such patients correct diagnosis is absolutely mandatory for effective treatment, i.e. venom-specific immunotherapy (VIT). In clinical practice, identification of the culprit insect involves a detailed history complemented with quantification of venom sIgE antibodies and venom skin tests. However, correct diagnosis of Hymenoptera venom allergy can be fraught with difficulties, mainly because of clinically irrelevant IgE results [42]. As revealed by table 5, and recently addressed and reviewed in this

Journal [43], in such cases BAT has proven to be a reliable adjunct diagnostic tool. However, anno 2014 it is evident that utility of BAT should be viewed in the context of recent discoveries in the field of venomics research and the developments in molecular diagnostics in Hymenoptera venom allergy. For example, in an own series combined use of sIgE antibodies to recombinant (r) rVes v 1 and rVes v 5 from wasp (*Vespula vulgaris*) enabled a correct diagnosis of wasp venom allergy in 139 out of 148 (94%) of the patients [44]. Similar observations have been made for a combination of rApi m 1, rApi m 2, rApi m 3, native Api m4, rApi m 5 and rApi m 10 from honey bee (*Apis mellifera*) in honeybee venom allergic patients [45]. By consequence, in our opinion BAT should be reserved to take the sting out of the most difficult cases that yield equivocal or false-negative results in skin testing and component resolved diagnosis. For example, in our series of 148 patients 10 patients showed negative results in skin testing en sIgE. In 6 patients it was possible to establish the diagnosis based on the CRD results but in the remaining 4 patients, additional BAT was needed for correct diagnosis [44]. Besides this, the BAT/HistaFlow® can still be useful as an instrument to monitor venom immunotherapy (VIT) and to study the effects of treatment on effector cells [10, 46]. However, additional large scale studies are mandatory whether BAT might predict effectiveness of VIT.

Basophil activation tests in food allergy

The applications of BAT in food allergy are numerous and involve diagnosis, evaluation of cross-reactivity, follow-up and risk stratification, monitoring of immunotherapy and oral tolerance induction and detection of trace allergens [for review: [47]].

In our opinion, like for Hymenoptera venom allergy, today these applications should be reconsidered carefully in view of the immense evolution of single- and multiplexed molecular

diagnostics. As a matter of fact, as revealed by table 6, a 5-year review of the literature reveals no new diagnostic applications of BAT in food allergy.

In our view, concerning the diagnosis of food allergy, the BAT can be of help when the traditionally used tests, such as skin testing, sIgE testing and component resolved diagnosis are unavailable or when they yield negative or equivocal results. For this purpose, the sensitivity and specificity of BAT varies between 75 and 95%. In addition to diagnosing food allergies the BAT can sometimes discriminate between sensitization and clinical relevant allergy [47]. The BAT can also be performed using recombinant allergens. So far, only a limited number of studies used recombinant allergens in the BAT [48-50]. In birch pollen allergic patients, sensitivity of the BAT for Mal d 1, Dau c 1 and Api g 1 has been shown to vary between 65-75% whereas specificities ranged from 68% to 100% [48]. This provides a valuable new *in vitro* method for the detection of sensitization of food that can be added to the routine tests for food allergy.

Furthermore, the BAT is also used to study the natural evolution of food allergies. Moreover, it can be applied to determine the occurrence of tolerance to milk or eggs [51]. Lastly, there is evidence that the BAT can be used to detect traces of allergens in food, thereby studying the allergenicity of food [52].

Expert commentary

From the earliest days of flow-assisted analysis and quantification of *in vitro* basophil activation it was clear that the technique would mostly be applied for diagnostic purposes and to monitor therapy. However, for the time being, utility and usefulness of BAT should be carefully reassessed in view of difficulties inherent to the correct preparation, characterization and validation of allergen extracts and availability and potential of alternative more easily accessible diagnostic instruments such as component resolved diagnosis in food and

Hymenoptera venom. Nowadays, the remaining diagnostic applications of the BAT are mainly to be sought in the field of allergy to medications that are manufactured according to GMP standards and that are readily available. However, additional collaborative studies are mandatory to verify whether the technique keeps promise, to optimize and harmonize the protocols, to avoid instigation of skepticism and cynicism, and allow and guarantee its entrance in mainstream diagnostic use.

Five-year view

In the next five-years we predict the expansion and further entrance in mainstream application of BAT/HistaFlow® technology in the *in vitro* diagnosis of IgE-mediated and probably also non-IgE-mediated IDHR that result from activation and degranulation of effector cells such as basophils and mast cells.

Thus the fact that the technique does not allow to discriminate between IgE-dependent and IgE-independent basophil activation should not be considered as a shortcoming. As a matter of fact, using multicolour flow cytometry with simultaneous analyses of surface activation markers and inhibitory receptors, signalling molecules and intracellular histamine content on a single cell level might capture data that are currently inaccessible and might help to unveil the pathomechanism(s) of certain IDHR. Finally, as the technique parallels the *in vivo* pathway leading to symptoms, it seems justified to anticipate that the technique will also further contribute in the discrimination between clinically relevant and irrelevant IgE serology.

Key issues

- In vitro activation of basophils (BAT) constitutes a useful and safe diagnostic that can be applied for IgE-dependent and probably also for IgE-independent basophil- and mast cell-mediated hypersensitivity reactions.
- Nowadays, application of the BAT in food and hymenoptera venom allergy needs to be reconsidered in view of the new, more accessible diagnostic tools, e.g. component resolved diagnosis.
- Today the usefulness of BAT has mainly to be sought in the domain of drug hypersensitivity diagnosis.
- Sufficient numbers of well identified patients and (exposed) control individuals are needed in order to study and validate the BAT in drug allergy.
- Studies regarding BAT in drug allergy should adopt standardized (clinical and laboratory) protocols on which inclusion of individuals can be based.
- BAT with simultaneous analyses of intracellular signalling and histamine release might not only improve diagnosis of drug hypersensitivity but also help to elucidate drug-mediated basophil and mast cell activation pathways.
- Other applications of BAT involve monitoring of allergen-specific immunotherapy and occurrence of natural tolerance.

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Figure 1

Figure 1: Basophil selection and activation. Panel A: aggregates are avoided by selecting unique cells based on FCS (forward scatter) area and FCS height plot. Plot B: IgE high positive cells are gated out. Panel C: Gated cells are IgE+ and CD203c+ (without stimulation). Panel D: activated basophil in control setting: R0 are CD203c+CD63- , R3 are CD203chi+CD63-, R4 are CD203chi+CD63low+, R5 are CD203chi+CD63hi+. Panel E and F are equal to C and D but after activation with anti-IgE as positive control.

Figure 2:

HistaFlow analysis: Basophils are selected as described in figure 1. See also legend figure 1 for R0-R5 definition. The left column displays results of buffer (negative control), The second column shows results of positive control, the right column displays results of activation with wasp antigen in a wasp allergic patient. Diamine oxidase (DAO) results (intracellular histamine content) show histamine release in 61% of the basophils after activation with wasp antigen.

Reference annotations

[1] Demoly, 2003: *Describes in detail the available allergen-induced mediator release assays.

[2] Knol EF, 1991: ** First paper on CD63 as a marker of activation of the human basophil.

[47] Faber M 2014:**Complete overview of the diagnostic tools, utility and application, in food allergy

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Stimulus	Reference Test	Activation marker	Sensitivity (%)	Specificity (%)	N	Ref.
Various NMBA	H	CD63	64	81	26	[53]
		CD45	43	96		
Various NMBA	H ± ST	CD63	54	100	56	[54]
Various NMBA	H	CD63	79	100	31	[55]
		CD203c	36	100		
Various NMBA	H ± ST	CD63	36-86 ¹	93	92	[56]
Rocuronium	H ± ST	CD63	92 ²	100	22	[57]
Various NMBA	H ± ST ± slgE	CD63	60	100	49	[58]
Rocuronium	H	CD63	80	96	104	[59]
Various NMBA	H+ST	CD63	68	100	56	[60]
Atracurium	H ± ST	CD63	71 ³	100	75	[61]
NMBA: neuromuscular blocking agent, H: history, ST: skin test, slgE: specific IgE, N: number of patients and control individuals						

¹ Increasing sensitivity when only the reactions that occurred during the 3 years were taken into account, ² taking into account the non-responders sensitivity is 76%, ³ taking into account the non-responders sensitivity is 63%.

Table 2: BAT in immediate β -lactam and quinolone hypersensitivity (adapted from Leysen et.al. [11])

Stimulus	Reference test	Activation marker	Sensitivity (%)	Specificity (%)	N	Ref.
<i>β-lactam antibiotics</i>						
β-lactam	H	CD63	50	93	88	[62]
β-lactam	H + DPT ¹	CD63	39	93	53	[63]
β-lactam	H ± ST ± slgE ± DPT	CD63	49	91	110	[64]
Amoxicillin	H ± ST	CD203c	52	100	41	[65]
		CD63	22	79		
β-lactam	H	CD63	50	89-97	262	[66]
β-lactam	H ± ST ± slgE	CD63-CCR3*	55	100	39	[67]
		CD63-IgE*	53			
Amoxicillin	H	CD63	29	/	14 patients, no controls	[68]
Amoxicillin	H ± ST ± DPT	CD63	50	/	61 patients, number of controls not mentioned	[69]
Amoxicillin	H ± ST	CD63	50	/	30 patients	[70]
<i>Quinolones</i>						
Various quinolones	H + DPT	CD63	0**	/	4	[22]
Various quinolones	H + ST ± DPT	CD63	0**	100	18	[19]
Various quinolones	H	CD203c	100	100	5	[24]
Various quinolones	H ± DPT	CD63	71	/	73	[16]
Various quinolones	H ± DPT	CD203c	NA	100	34	[25]

For legend see table 1.
¹ Drug provocation test (DPT)
² DPT with moxifloxacin was only performed in control individuals to establish tolerance status.
 *Selection marker
 **Note that all CD63-based assays yield entirely negative results, except the Aranda series.

Table 3: BAT in immediate NSAID hypersensitivity (adapted from Leysen et.al. [11])

Stimulus	Reference test	Activation marker	Sensitivity (%)	Specificity (%)	N	Ref.
Metamizol	H $\pm$ DPT ¹	CD63	42	100	56	[29]
Various NSAID	H $\pm$ DPT	CD63	15-55	74-100	90	[71]
Diclofenac	H	CD63	No significant difference in CD63 expression between patients and controls, (IgE-independent basophil degranulation)		26	[72]
Various NSAID	H	CD63	43	100	72	[73]
Pyrazolones	H $\pm$ IDT ² $\pm$ DPT	CD63	55	86	107	[30]
ASA	H $\pm$ DPT	CD63	34	79	42	[74]
		CD203c	17	100		
Diclofenac		CD63	17	92		
		CD203c	22	100		
ASA	H $\pm$ DPT	CD63	30	40	20	[27]
		CD203c	70	45		
Diclofenac	H $\pm$ DPT	CD63	0	/	22	[75]
Aspirin	H + DPT	CD63	80* 78**	83* 50**	59	[76]
Various NSAID	H+DPT	CD63	61	91	29	[77]
Various NSAID	H	CD63	37	90	80	[78]
Metamizol	H $\pm$ ST	CD63	70	100	30	[31]
Various NSAID	H $\pm$ DPT	CD63	100	20	91	[28]
Metimazol	H	CD63	0	/	6 patients, no conrols	[33]
Metimazol	H	CD63	42.3	100	56	[32]

For legend see table 1

ASA= aspirin salicyl acid, ¹Drug provocation test, ²Intradermal skin test

* for anaphylaxis, ** for asthma/rhinoconjunctivitis

Table 4: BAT in immediate hypersensitivity to iodinated radiocontrast media (adapted from Song WJ et. al. [13])

Stimulus	Reference test	Activation marker	Sensitivity (%)	Specificity (%)	N	Ref.
Various RCM	H+ST	CD63	100	100	3 patients, unknown number of controls	[39]
Various RCM	H ± ST	CD63	46-62%*	89-100%*	40	[34]
Various RCM	H±ST±DPT ¹	CD63	63	100	28	[38]
For legend: see tabel 1 ¹ drug provocation test *Depending on the cut-off value						

Table 5: BAT in Hymenoptera venom allergy (adapted from Ebo et. al. Exp rev 2014 [43])

Stimulus	Reference test	Activation marker	Sensitivity (%)	Specificity (%)	N	Ref.
Hymenoptera	H	CD63	100	100	55 ¹	[79]
Hymenoptera	H	CD203c	91	100	35	[80]
Wasp	H	CD63	92	80	70	[81]
Wasp	H	CD63	85	83	87	[82]
Bee			91	90		
Hymenoptera	H	CD63	91	100	34	[83]
Hymenoptera	H	CD63	89	100	68	[84]
		CD203c	97	89		
Wasp	H	CD63	97	100	61	[85]
Bee			100	100		
Wasp	H	CD63	99 ²	100	94	[46]
Wasp	H	CD63	87	97	84	[86]
Bee			58	90		
Wasp	H	CD63	100	100	32	[87]
		pP38 MAPK*	100	86		
Wasp and Bee	H	CD63	67-75%	/	15 patients, no controls	[88]