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Flow-assisted basophil activation tests in immediate drug hypersensitivity: 2 decades of Antwerp experience.

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Running title: *In vitro* diagnosis of immediate drug hypersensitivity reactions

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29 **Abstract**

30 The last 2 decades have witnessed that flow-assisted analysis of *in vitro* activated basophils
31 can constitute a valuable adjunct in the *in vitro* diagnostic approach of immediate drug
32 hypersensitivity reactions (IDHR). This article summarizes the current experience with the
33 basophil activation test (BAT) in the diagnosis of IDHR, with particular focus on allergy to
34 curarizing neuromuscular blocking agents, antibiotics (β -lactams and fluoroquinolones),
35 iodinated radiocontrast media and opiates.

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Introduction

Immediate drug hypersensitivity reactions (IDHR) constitute an important health issue with serious consequences of diagnostic error (1, 2). Unfortunately, correct diagnosis of IDHR is not always straightforward and (too) easily overlooked. As a matter of fact, one of the major challenges and unmet needs in the diagnostic approach of IDHR, lies in the development and validation of reliable *in vitro* assays enabling easy, safe and correct identification of all incriminated agents and potential alternatives for the future. Indeed, although drug provocation tests (DPT) are generally considered as the “gold standard” for diagnosis of IDHR, in mainstream use DPT are severely hampered and can simply be contra-indicated because of obvious practical and mostly ethical reasons. Moreover, even DPT have proven not to be absolutely predictive (3). Therefore, diagnosis of IDHR usually starts with an evocative history that is complemented with skin testing and quantification of commercially available specific IgE (sIgE) antibodies when an underlying IgE-dependent pathomechanism with activation of basophils and mast cells is suspected (4).

However, as recently reviewed by Uyttebroek et al (5), only a very limited number of drug-specific IgE assays are currently available and most of them have not been thoroughly validated (yet). For example, we recently demonstrated sIgE to poppy seed (*Papaver somniferum*) and morphine to be useless in the diagnosis of opiate hypersensitivity (6). In addition, IDHR with degranulation of mast cells and basophils do not always imply involvement of IgE antibodies with cross-linking of IgE/FcεRI, but might also result from alternative mechanisms that cannot be captured by traditional sIgE assays, reinforcing the requirement for the development and validation of cellular tests that can be informative for IDHR independent from sIgE. Although for many, the “reference test” for basophil activation

remains mediator release tests, the technique has never really entered routine clinical application (7). The foundations of modern BAT were laid about quarter of a century ago (8), and in the meantime, BAT has largely supplanted mediator release tests, particularly in IDHR. Traditional BAT relies on flow cytometric identification and quantification of alterations (appearance and/or up-regulation) of specific activation such as CD203c or degranulation makers such as CD63 on the surface of the cells (figure 1) (9). Alternative flow-assisted methods to assess antigen-specific *in vitro* basophil activation imply the study of particular inhibitory receptors (10), phosphorylation of intracellular signalling molecules (11, 12), and last but not least quantification of the intracellular histamine content in a technique we have named HistaFlow® (figure 2) (13). However, except for the latter (14), none of these alternative methods have already be implied to study IDHR. The objective of this article is, in a non-exhaustive way, to review the main clinical applications of the BAT starting from our clinical priorities and experience. Undoubtedly, as with a subject still beset by many questions and issues, alternative interpretations, hypotheses, or explanations expressed here might not find universal acceptance. In this respect, we did not address the polemics around BAT in IDHR to non-steroidal anti-inflammatory drugs (see (5)).

Curarizing neuromuscular blocking agents

In our regions, curarizing neuromuscular blocking agents (NMBA) remain the most common cause of IDHR during general anaesthesia (15). Most of these reactions are IDHR and are triggered by an IgE-dependent mechanism or non-specific release of mediators. Although some NMBA-specific IgE tests have recently been developed and to some extent clinically evaluated (e.g. for rocuronium (16) and atracurium (17)), skin tests remain the primary instrument to document NMBA-mediated IDHR (18). The BAT has proven to be particularly

relevant in difficult cases where skin tests yield equivocal or negative results (19). Table 1 displays the most relevant studies of BAT in NMBA hypersensitivity, including our experiments with rocuronium (20, 21) and atracurium (22). From this table it is clear that the BAT with curarizing NMBA, like for most drugs, appears highly specific but less sensitive. Alternatively, the BAT can be extremely useful and complement skin testing in the identification of cross-reactivity between NMBA and the identification of safe alternatives for future anaesthesia (19, 20). It is unlikely that the newer, more laborious, HistaFlow® technique might add to the diagnosis of NMBA hypersensitivity (14), but it seems theoretically justified that the technique might unveil alternative basophil degranulation patterns accounting for particular IDHR (13).

Antibiotics: β -lactams and quinolones

Diagnosis of IDHR to β -lactam antibiotics generally rely upon skin testing and quantification of sIgE antibodies (23). However, as addressed by Blanca et al (24), in particular cases the BAT might add to the diagnosis. As displayed in table 2, there are currently nine studies that have evaluated the BAT in the diagnosis of IDHR from β -lactam antibiotics, mainly amoxycillin. Hitherto, we were unable to thoroughly validate the BAT in β -lactam antibiotics. Preliminary results, mainly obtained in cefazolin and aminopenicillin hypersensitivity, confirm the test to be rather insensitive as compared to skin testing.

Quinolones are synthetic antibiotics based on the 4-quinolone and 1,8-naphthyridine nuclei. Quinolones can elicit severe IDHR, even after a first exposure, with moxifloxacin being an important culprit (25). Unfortunately, correct diagnosis of quinolone IDHR is not easy to establish, mainly because of the absence of drug-specific sIgE immunoassays and uncertainties associated with skin testing with potentially nonspecific histamine releasers

(26-28). Table 2 summarizes the current literature published on BAT in quinolone hypersensitivity. It appears that the BAT generates rather conflicting data in quinolone IDHR, particularly with CD63-based assays that appear only positive in a Spanish series (29). In our hands, the appearance of CD63 with significant release of histamine is only seldom observed in patients with an evocative history of moxifloxacin IDHR, whereas up-regulation of CD203c is to some extent also observed in control individuals uneventfully exposed to this fourth generation quinolone (manuscript in preparation). Collectively, these findings call into question the value of BAT in quinolone IDHR and could indicate release of mediators from basophils and mast cells to originate from alternative (nonspecific) cell activation that goes generally undetected in traditional CD63-based BAT.

Iodinated radiocontrast media

IDHR to iodinated radiocontrast media (RCM) have been described, but their prevalence is low and estimated between 0.02% for nonionic RCM and 0.4% for ionic RCM. Direct mediator release following binding of nonspecific RCM to surface receptors on mast cells or basophils and indirect cell activation by means of the complement or kinin cascade outnumber the genuine IgE-mediated reactions and might be overlooked by skin tests (30). Hitherto, three studies reported on the value of BAT in the diagnosis of IDHR to RCM (31-33). These studies demonstrate a sensitivity of 46-63% depending on the chosen threshold, and a specificity of 89-100%. Furthermore, it seems that the results of BAT and skin testing can be complementary.

Opiates

As recently reviewed, despite their frequent and ubiquitous use, genuine IgE-mediated hypersensitivity reactions to opiates remain exceedingly rare and correct diagnosis of opiate

allergy is not always obvious (34, 35). Actually, the major challenge in the diagnosis of IgE-mediated opiate hypersensitivity lies in the correct interpretation of positive skin test (34) and sIgE (6) results. In contrast, opiates such as morphine and diamorphine (heroin) seem not to trigger histamine release from human basophils (6, 36, 37). Recently, we have described 3 patients with an IDHR to the opiate antitussive pholcodine in whom measurement of pholcodine-sIgE antibodies together with HistaFlow® leads to the diagnosis of genuine IgE-mediated pholcodine hypersensitivity (figure 3) (38). Moreover, basophil experiments with the structurally almost identical compounds morphine and codeine were negative and correctly predicted the uneventful challenge with codeine. Obviously, larger studies are needed to confirm the diagnostic potential of BAT/HistaFlow® in IgE-mediated opiate hypersensitivity.

Miscellaneous

Tables 4 and 5 display a non-exhaustive alphabetical list of application of flow-assisted BAT in the diagnostic management of IDHR to drugs and related compounds. In most of these studies experiments are limited to small groups (table 4) or individual cases (table 5). In these publications BAT proved particularly useful when no other diagnostics are available or when skin testing and other *in vitro* tests yielded equivocal results. Because it is unlikely that the BAT/HistaFlow® will become validated in these rather exceptional allergies, we advocate to perform the experiments in the patient and to compare the data with findings in 3 healthy control individuals who, preferentially, were uneventfully exposed or challenged with the alleged culprit.

Conclusion and perspectives

It seems justified to state that the BAT provides the physician and the laboratory with an interesting tool that can add in the diagnosis of IDHR that involves IgE-dependent and probably also IgE-independent activation of basophils and/or mast cells. Actually, BAT reveals to be particularly helpful when other diagnostics are lacking or yield equivocal results and when DPT's are not possible. One of its most important limitations is that the technique needs fresh basophils that cannot easily be transported and cannot be stored. Whether passive sensitization experiments using donor basophils as described in (39) or one of the recently developed human mast cell cultures (40, 41) might broaden the application of flow-assisted diagnosis of IDHR remains to be investigated.

Figure 1: Principles of flow-assisted analysis of in vitro activated basophils (traditional basophil activation test; BAT). The allergen of interest is added to a fresh blood sample of the patient. When the allergen is able to cross-link at least two specific IgE/FcεRI complexes

bound on the surface of the quiescent basophils (mid panel), activation of the basophil occurs (right panel). This activation leads to an up-regulation of activation marker CD203c and can culminate in anaphylactic degranulation with the appearance of CD63. FcεRI is the high affinity receptor for IgE antibodies. CD203c is also known the ectonucleotide pyrophosphatase/phosphodiesterase family member 3 enzyme (E-NPP3), and is lineage specific for basophils. CD63 or lysosomal-associated glycoprotein-3 (LAMP-3). For description of BAT technique: (9).

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175 *Figure 2: Principles of the HistaFlow®. In contrast to traditional CD63- and CD203c-based*
 176 *BAT, the HistaFlow® does not rely upon analysing phenotypic alterations using conjugated*
 177 *monoclonal antibodies. Actually, in HistaFlow® intracellular content and its release is*
 178 *quantified using an enzyme affinity method based upon the affinity of diamine oxidase*
 179 *(DAO). To allow flow cytometric analysis DAO is conjugated with a LASER-excitable*
 180 *fluorochrome. The left panels show resting cells, the right panels activated cells with*
 181 *emptying of their intracellular granules. For a description of the HistaFlow: (13).*

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Figure 3: representative plot of CD63 appearance and histamine release in response to buffer, anti-IgE as a positive control, the opiate antitussive pholcodine 10 µg/mL (top), and the structurally almost similar opiates codeine 100 µg/mL and morphine 100 µg/mL (both bottom) (from (38)). Intracellular histamine content and its release is analysed flow cytometrically using the histaminase diamine oxidase (DAO) conjugated to LASER-excitable fluorochromes (13). For a description of the structure formula of pholcodine, codeine and morphine: (35).

Table 1: BAT in immediate neuromuscular blocking agent (NMBA) hypersensitivity (updated from (42))						
Stimulus	Reference test	Activation marker	Sensitivity (%)	Specificity (%)	Number of patients and controls	Reference
Various NMBA	H	CD63	64	81	26	(43)
		CD45	43	96		
Various NMBA	H $\pm$ ST	CD63	54	100	56	(44)
Various NMBA	H	CD63	79	100	31	(45)
		CD203c	36	100		
Various NMBA	H $\pm$ ST	CD63	36-86 ¹	93	92	(46)
Rocuronium	H $\pm$ ST	CD63	92 ²	100	22	(20)
Various NMBA	H $\pm$ ST $\pm$ IgE	CD63	60	100	49	(47)
Rocuronium	H	CD63	80	96	104	(21)
Various NMBA	H+ST	CD63	68	100	56	(48)
Atracurium	H $\pm$ ST	CD63	71 ³	100	75	(22)
NMBA: neuromuscular blocking agent, H: history, ST: skin test, 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 to positive control stimulation and drug, the sensitivity is 76%, ³ taking into account the non-responders to positive control stimulation and drug, the sensitivity is 63%.						

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Table 2: BAT in immediate β -lactam and quinolone hypersensitivity (updated from (42))						
Stimulus	Reference test	Activation marker	Sensitivity (%)	Specificity (%)	Number of patients and controls	Ref.
<i>β-lactam antibiotics</i>						
β -lactam	H	CD63	50	93	88	(49)
β -lactam	H + DPT ¹	CD63	39	93	53	(50)
β -lactam	H $\pm$ ST $\pm$ IgE $\pm$ DPT	CD63	49	91	110	(51)
Amoxicillin	H $\pm$ ST	CD203c	52	100	41	(52)
		CD63	22	79		
β -lactam	H	CD63	50	89-97	262	(53)
β -lactam	H $\pm$ ST $\pm$ IgE	CD63-CCR3*	55	100	39	(54)
		CD63-IgE*	53			
Amoxicillin	H	CD63	29	/	14 patients, no control individuals	(55)
Amoxicillin	H $\pm$ ST $\pm$ DPT	CD63	50	/	61 patients, number of controls not mentioned	(56)
Amoxicillin	H $\pm$ ST	CD63	50	/	30 patients	(57)
<i>Quinolones</i>						
Various quinolones	H + DPT	CD63	0*	/	4	(58)
Various quinolones	H + ST $\pm$ DPT	CD63	0*	100	18	(59)
Various quinolones	H	CD203c	100	100	5	(60)
Various quinolones	H $\pm$ DPT	CD63	71	/	73	(29)
Various quinolones	H $\pm$ DPT	CD203c	NA	100	34	(61)
For legend see table 1. ¹ Drug provocation test (DPT) ² DPT with moxifloxacin was only performed in control individuals to establish tolerance status. *Note that all CD63-based assays yield negative results, except the Aranda series (29).						

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Table 3: BAT in immediate hypersensitivity to iodinated radiocontrast media (RCM)						
Stimulus	Reference test	Activation marker	Sensitivity (%)	Specificity (%)	Number of patients and controls	Reference
Various RCM	H+ST	CD63	100	100	3 patients, unknown number of controls	(31)
Various RCM	H ± ST	CD63	46-62%*	89-100%*	40	(32)
Various RCM	H+ST+DPT	CD63	63	100	28	(33)
For legend: see table 1. *Depending on the cut-off value						

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Table 4: BAT in immediate hypersensitivity to various drugs and related compounds (alphabetical order)						
Stimulus	Reference test	Activation marker	Sensitivity (%)	Specificity (%)	Number of patients and controls	Reference
chlorhexidin	H	CD63	100	100	12	(62)
gadobutrol	H	No info	100	100	8	(63)
IV gelatin* (succinylated)	H	CD63	100	87.5	11	(64)
glatiramer	H	CD63	66.7	83.3	9	(65)
L-asparaginase	H	CD203c	75	82	32	(66)
methyl-prednisolone	H + ST + DPT	CD63	75	100	14	(67)
rituximab	H	CD63	NA	NA	18	(68)
For legend: see table 1. N.A.: not available. *Note that severe intravenous (IV) bovine gelatin IDHR can occur from a sensitization to the α -gal moieties that is not despicable by sIgE to gelatin (69).						

Table 5: BAT in immediate hypersensitivity to various drugs and related compounds (not exhaustive and in alphabetical order)		
bovine serum albumin (70)	carboxymethylcellulose (71)	cremophor EL (72)
cisplatinum (73)	H1-receptor antagonists (74, 75)	heparin and low molecular weight heparins (76)
hyaluronidase (77)	hydroxyl ethyl starch (78)	infliximab (79)
(methyl)prednisolone (80, 81)	ophthalmologic atropine (82)	patent blue (83)
vaccines (e.g. recombinant hepatitis B) (84)		

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