

# In Vitro Diagnosis of Immediate IgE-Mediated Drug Hypersensitivity Warnings and (Unmet) Needs

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## KEYWORDS

- Basophil activation • Drug provocation • Flow cytometry • IgE
- Immediate drug allergy

## KEY POINTS

- Immediate drug hypersensitivity reaction (DHR) constitutes an important health condition, with serious consequences of inadequate diagnosis. In this article, some of the most important, but certainly not all, issues related to in vitro diagnosis of IgE-mediated allergies are discussed.
- Correct development and validation of novel tests need sufficient numbers of appropriately identified patients and (exposed) control individuals. It is likely that collaborative studies are necessary to meet these requirements.
- These studies should adopt standardized and harmonized clinical and laboratory protocols, to avoid erroneous inclusions that might impede appropriate clinical validation of the assays.
- Although basophil activation testing in drug allergy might seem to be still in its infancy, with increasing original applications over the last years and with increasing employment, we can expect the assay to enter mainstream clinical use, especially for DHR that cannot readily or correctly be diagnosed by other means.
- In vitro diagnostics will benefit from expanded and novel insights and understandings in drug chemical reactivity, protein binding, biotransformation, degradation, identification of (cross-reactive) drug antigenic determinants, and deeper understanding of sensitization routes. DHR remains a conundrum, because knowledge in these areas remains sparse and ignorance great. Collective efforts should be undertaken to activate fundamental and clinical investigations.

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## INTRODUCTION

Challenges in *in vitro* immediate drug hypersensitivity reaction (DHR) diagnosis lie in the development and validation of reliable assays enabling safe and correct identification of all incriminated agents and potential alternatives for the future. Although drug provocation tests (DPT) are considered as the gold standard for drug hypersensitivity diagnosis, DPT are hampered by the risk of severe, life-threatening reactions and might be contraindicated (ie, patients taking  $\beta$ -blockers or angiotensin-converting enzyme inhibitors) or not possible for obvious reasons (ie, hypersensitivity to neuromuscular blocking agents [NMBA]). Besides, even DPT do not show absolute predictive values.<sup>1</sup> Hitherto, diagnostic DPT have not entered mainstream clinical practice.

Therefore, diagnosis of immediate DHR usually starts with a thorough history complemented with skin tests or *in vitro* quantification of (commercially available) specific IgE (sIgE) antibodies when an IgE-mediated mechanism with activation of mast cells and basophils is suspected.<sup>2</sup>

However, only a few drug-sIgE assays are available, and most of them have not been thoroughly clinically validated. In addition, immediate DHR with release of mediators by mast cells and basophils does not per se always involve crosslinking of the high affinity IgE receptor (Fc $\epsilon$ RI), but might also result from various alternative mechanisms that cannot be depicted by traditional sIgE assays, reinforcing the requirement for the development and validation of additional cellular tests such as basophil activation assays, because these tests can also be informative for immediate DHR independent from IgE. Starting from our clinical priorities and experience, the objective of this article is to identify some misconceptions, shortcomings, and unmet needs in *in vitro* immediate DHR diagnosis and to emphasize different obstacles that might hamper correct validation and harmonization of sIgE quantification and cellular tests. Undoubtedly, as with any subject still beset by many questions, alternative interpretations, hypotheses, or explanations expressed here may not find universal acceptance.

## QUANTIFICATION OF SIGE ANTIBODIES

There are different automated *in vitro* techniques to quantify serum sIgE antibodies. However, unlike protein allergens, for routine application of immunoassays to drug-sIgE antibodies, the current situation is characterized by a limited availability of well-validated drug-specific tests. The only drug-specific IgE assays that are readily accessible comprise penicilloyl G (c1), penicilloyl V (c2), ampicilloyl (c5) and amoxicilloyl (c6) determinants, cephaclor (c7), chlorhexidine (c8, antiseptic), chymopapain (c209), (bovine) gelatin (c74), human (c73), bovine (c71) and porcine (c70) insulin, morphine (c260, marker for sensitization to tertiary and quaternary substituted ammonium determinants), pholcodine (c261) and suxamethonium (c202), which are commercially available from Thermo Fisher Scientific, Uppsala, Sweden. In addition, and for research purposes only, adrenocorticotrophic hormone, atracurium, bacitracin, carboplatin, cefamandole, cefoxitin, cefotaxime, cefuroxime, cisplatin, mepivacaine, methylprednisolone-21-succinate, nafamostat (4-guanidinobenzoic acid), oxaliplatin, penicillin minor determinants (eg, penicillanyl), propyphenazone, protamine, rocuronium, and tetanus toxoid are offered via the Thermo Fisher Scientific special allergen service. However, as exemplified in the introductory paragraph, most of these assays have not been sufficiently clinically validated, mainly as a result of the unavailability of sufficient numbers of accurately phenotyped patients and exposed control individuals.

Probably the most studied sIgE tests are those for  $\beta$ -lactam antibiotics. The most used antibiotics of this class in clinical practice consist of monocyclic molecules,

such as monobactams, and bicyclic molecules, such as penicillins and clavams, cephalosporin, and carbapenems. The European Network for Drug Allergy recommends for safety reasons to perform serum sIgE assays in patients with a history of immediate penicillin hypersensitivity in addition to skin test in the allergologic workup.<sup>3</sup>

Although IgE assays show a generally low sensitivity (12.50%–50%), several cases of positive sIgE results in immediate reactions with negative skin test have been described.<sup>4–7</sup> However, not only sensitivity but also specificity of commercial available tests are far from ideal, for reasons such as increased total IgE and immunologic cross-reactivity.<sup>8,9</sup> For example, Fontaine and colleagues,<sup>4</sup> who compared DPT and skin testing, observed sIgE to  $\beta$ -lactam antibiotics to yield a specificity between 83.30% and 100%.

Because patients with positive sIgE results are generally precluded from DPT, improvement of these techniques is mandatory to avoid erroneous overdiagnosis of penicillin allergy. Another important issue in the diagnostic approach of IgE-mediated  $\beta$ -lactam allergy is the assessment of cross-reactivity and identification of safe alternatives. However, the currently available drug-sIgE assays do not contribute significantly in the prediction of the clinical outcome.

Another representative example showing the potential and limitations of drug-specific IgE immunoassays is the morphine solid-phase assay. Morphine, by virtue of its overtly accessible single tertiary *N*-methyl ammonium group, is readily recognized by sera from most, but certainly not all, patients who reacted to curarizing NMBA. Moreover, morphine solid-phase immunoassays have been reported to be superior to NMBA-specific assays for the detection of NMBA-reactive sIgE antibodies.<sup>10–12</sup> One of these immunoassays is available as the ImmunoCAP Allergen c260 Morphine (Thermo Fisher Scientific); the package leaflet indicates that the test is an alternative quaternary ammonium marker to suxamethonium and is intended for use as a diagnostic tool in NMBA-induced anaphylaxis. However, several issues should be addressed here. First, the morphine solid phase might not identify all patients allergic to NMBA, particularly to benzyloquinolines, such as atracurium, cis-atracurium, and mivacurium.<sup>10,12</sup> The excellent sensitivity that applies to suxamethonium and rocuronium allergy cannot simply be extrapolated to benzyloquinolines. Second, attention needs to be drawn to a prevalence of IgE reactivity to morphine between 1% and 10% in individuals without NMBA allergy.<sup>11–13</sup> Although the morphine solid-phase test might be a valuable backup to equivocal and negative skin tests in highly suggestive cases, caution is advised on too loose application of these assays and thoughtless interpretation of positive sIgE results. This situation could not only result in overestimation of NMBA allergy with unnecessary avoidance measures but, more seriously, could also entail dramatic consequences by overlooking alternative diagnoses. From the earliest days of application, it was obvious that NMBA-reactive and substituted ammonium-reactive sIgE antibody tests should not be used in isolation to diagnose curare allergy, but as a supplement to skin tests,<sup>14,15</sup> and should never be used to predict NMBA allergy in primary prevention. *Mutatis mutandis*, this recommendation applies to opiate allergy, because patients showing sIgE reactivity in the morphine solid-phase assay generally have negative basophil activation tests (BATs) and provocation tests for morphine or structurally closely related compounds such as codeine (Fig. 1).<sup>16</sup> Although frequently found in sera from allergic patients, especially with rocuronium and suxamethonium allergy, there is no compelling evidence that these morphine sIgE antibodies underlie allergic reactions to this opiate. Nevertheless, it seems that in the ninth French survey (2005–2007),<sup>17</sup> 12 patients had their diagnosis of NMBA allergy established by an isolated positive sIgE result to a quaternary ammonium, and 13 patients were identified as morphine allergic.

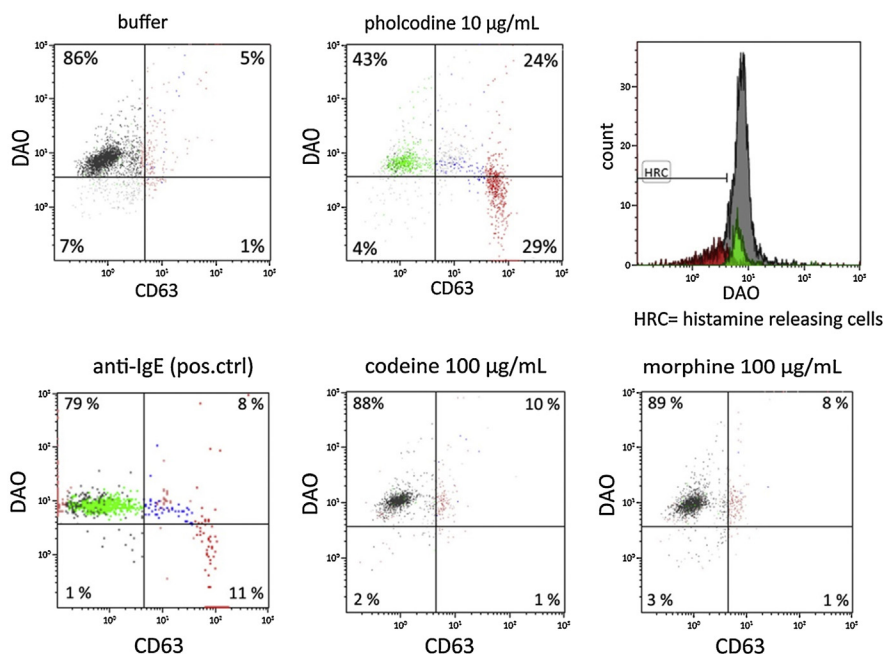


**Fig. 1.** BAT/HistaFlow plot in patient with pholcodine allergy. Representative plot CD63 appearance and histamine release in response to buffer, anti-IgE as a positive control, pholcodine 10 µg/mL, and the structurally almost similar opiates codeine 100 mg/mL and morphine 100 µg/mL. Di-aminooxidase (DAO) (Sigma Aldrich, Saint Louis, MO, USA); pos.ctrl, positive control. (From Leyssen J, De WL, Sabato V, et al. IgE-mediated allergy to pholcodine and cross-reactivity to neuromuscular blocking agents: lessons from flow cytometry. *Cytometry B Clin Cytom* 2013;84:69; with permission.)

Generally, in the absence of clinical validation, manufacturers have traditionally been recommending a positive cutoff of 0.35 kUA/L for serologic assessment of allergen-specific IgE. However, this threshold might not be interchangeable between different techniques and may not be appropriate for correct diagnosis. Different studies indicate that predefined and usually arbitrarily chosen decision thresholds for determination of sensitivity, specificity, and predictive values should be abandoned.<sup>11,18</sup> All possible combinations that can be achieved by changing the cutoff value of the test can be summarized using a single parameter, the area under the receiver operating characteristic (ROC) curve. In addition, the ROC technique enables comparison of different tests and can be used to optimize threshold values with regard to a given prevalence in the target population and cost ratio of false-negative and false-positive results.<sup>19,20</sup> Obviously, it is recommended to calculate drug-specific decision thresholds for other diagnostic methods, such as the BAT.

sIgE results can also be affected by the preparation of the drug solid-phase conjugates. For example, it has been shown that solid-phase assays prepared by alkali treatment of some cephalosporins might lack the potential allergenic R2 side chain, because of the lability of their dihydrothiazine ring.<sup>21</sup> Such tests not only could potentially put patients at risk of anaphylaxis if allergy was falsely excluded but could also fail to correctly predict cross-reactivity with other  $\beta$ -lactam antibiotics. Therefore, it is mandatory to detail the preparation and conjugation processes when reporting on a new drug-specific solid-phase assay.

## CELLULAR TESTS

Before considering the potentials and shortcomings of cellular tests, it should be remembered that the signs and symptoms of an IgE-mediated reaction are a direct consequence of cross-linking of adjacent IgE/Fc $\epsilon$ RI complexes present on the membrane of basophils and mast cells with triggering of almost immediate release of various preformed mediators such as histamine, tryptase, and platelet activating factor. In addition, newly synthesized mediators such as different leukotrienes and cytokines are also released. However, release of these quantifiable mediators does not necessarily reflect an IgE/Fc $\epsilon$ RI-mediated mechanism but might also result from various alternative means of mast cell activation (eg, via anaphylatoxins [C5a, C3a]) or direct mast cell degranulation, as seen with opioids, NMBAs, iodinated contrast media, quinolones, and vancomycin. Although for many the reference test for effector cell activation remains the basophilic histamine release test, the technique has never really entered mainstream application.<sup>22</sup> The foundations of modern BAT research and practice were laid about 2 decades ago,<sup>23</sup> and in the meantime, BAT has largely supplanted histamine release tests. The principles and applications of BAT are detailed elsewhere.<sup>24</sup> BAT relies on flow cytometric identification and quantification of alterations of specific activation or degranulation markers on the surface membrane or inside the basophils. These changes can be detected and quantified on a single-cell level using specific monoclonal antibodies conjugated to particular laser-excitable fluorochromes. Alternative methods to assess antigen-specific *in vitro* basophil activation imply quantification of phosphorylation of intracellular signaling molecules<sup>25,26</sup> and quantification of the intracellular histamine content by an enzyme affinity method.<sup>27</sup> During the last decade, an increasing number of studies have shown that flow cytometric analysis and quantification of *in vitro* activated basophils can be a reliable instrument in the diagnostic management of immediate DHR (for review, see Refs.<sup>28,29</sup>). BAT not only allows safe simultaneous testing of different compounds, including active components as well as excipients and additives, but also enables tailoring safe alternatives for the future. BAT can be complementary to skin tests, and BAT results might have more clinical relevance than sIgE data.<sup>16,30–32</sup> Likely, this observation reflects the underlying processes in the different tests. SlgE tests simply measure the interaction between the epitope(s) of the drug (or related compound) and the paratope(s) of the antibody, whereas BAT and skin tests depict drug-induced cross-linking of membrane-bound slgE, with subsequent degranulation and release of mediators, thereby more closely mirroring the *in vivo* situation. Moreover, for drugs such as opioids, BAT might help to discriminate between genuine allergy and nonspecific release of mediators by mast cells.<sup>16</sup> Alternatively, because cross-linking of slgE/Fc $\epsilon$ RI is not an absolute prerequisite for basophil activation and degranulation, the technique might also be valuable to document IgE-independent immediate DHR.

Another example of the potential of BAT is allergy toward quinolones, a family of synthetic antibiotics based on a 4-quinolone and 1,8-naphthyridine nuclei. These drugs can cause severe allergies, often after first exposure; moxifloxacin is the most important culprit.<sup>33</sup> Diagnosis of quinolone allergy is not straightforward. Although several attempts have been undertaken to prepare different quinolone conjugates, there are still no well-validated drug-specific slgE assays readily available, and controversy has arisen about the specificity of some methods.<sup>34</sup> Quinolone skin testing is also associated with uncertainties, and positive skin responses to various quinolones have been reported in individuals without overt quinolone allergy. Because questions remain concerning the relevance and applicability of slgE and skin tests in quinolone allergy, several groups have pinned their hopes on BAT. Studies have reported

divergent findings with respect to the sensitivity of the test. The literature and our own preliminary experiments seem to indicate that (some) quinolones might induce basophil degranulation that goes completely undetected in traditional CD63-based assay. Using this readout, only Aranda and colleagues,<sup>35</sup> and not Seitz and colleagues<sup>36</sup> or Lobera and colleagues,<sup>37</sup> managed to show *in vitro* basophil activation. The reasons for this observation remain elusive. However, the more consistent results with CD203c<sup>38,39</sup> could indicate that basophil degranulation in response to quinolones results from an alternative (IgE-independent) pathway.

BAT provides the physician with a novel instrument in the diagnosis of DHR. However, additional trials are required to further optimize and validate the technique, to avoid skepticism and cynicism and to allow its entry in to mainstream diagnostic use. These studies should address several points. First, as with any study of this nature, a major difficulty could be to accurately identify sufficient numbers of well-phenotyped patients and to compose an appropriate exposed control group. In this context, medical history can be inaccurate and even misleading, because it relies on the patients' or relatives' recollection, appreciation, and description of events. For example, a maculopapular exanthema as seen in cell-mediated type IV reactions toward penicillins could easily be mistaken for an urticaria observed in the context of an IgE-mediated type I reaction. The same holds true for urticaria induced by an infectious agent. Pharmacologic adverse events erroneously described or interpreted as DHR can also profoundly affect the outcome. These validation studies, ideally, should enroll only patients with a well-established and unequivocal (doctor-based) diagnosis of DHR. In this context, not all apparently allergic reactions are truly immunologic in nature or even drug induced. For example, various drugs such as opioids, iodinated contrast media, vancomycin, and quinolones can elicit direct mast cell (and basophil) degranulation, whereas nonsteroidal antiinflammatory drugs can mimic drug allergy via a redirection of mediator synthesis and intake of angiotensin-converting enzyme inhibitors can trigger angioedema caused by accumulation of bradykinin. Moreover, because of the absolute necessity of collaborative inputs from clinic and laboratory, studies would need clearly harmonized practice parameters and standardized procedures and should be performed in specialized/dedicated allergy clinics/centers for drug allergy testing and management. From a recent World Allergy Organization survey on diagnostic procedures and therapies in drug allergy,<sup>40</sup> it seems that such centers are available in only about 60% of the participating countries. This harmonized collaborative approach should not only help to circumvent the issues of small numbers and inadequate diagnosis but also identify other confounders that might seriously affect the outcome. Perhaps one of the most relevant examples to show this point is the highly disputed proposal to apply BAT in hypersensitivity to nonsteroidal antiinflammatory drugs. A likely explanation for this dispute could be the occurrence of IgE-mediated reactions to the pyrazolone-derived nonsteroidal antiinflammatory drugs. Proponents of this proposal have generally been applying BAT for allergic reactions to metamizol<sup>41</sup> and dipyrrone,<sup>42</sup> whereas critics of the proposal studied pyrazolones,<sup>43</sup> and mostly other compounds such as diclofenac<sup>44,45</sup> and salicylic acid.<sup>45,46</sup>

Second, small chemicals, such as drugs, might elicit highly heterogeneous and less pronounced *in vitro* basophil responses, compared with large protein allergens. Therefore, it will be critical to identify optimal allergen-specific stimulation conditions, which cannot always be extrapolated from studies with histamine and leukotriene release tests or from tests with (structurally or pharmacologically) related compounds. For example, stimulation conditions for aminosteroid myorelaxants might not apply for benzylisoquinolines, and vice versa. These optimized conditions might differ according to the applied technique (whole blood vs purified leukocytes, CD63 vs CD203c).

Third, as previously exemplified for quantification of sIgE, arbitrarily chosen thresholds should be abandoned but drug-specific decision cutoffs should be applied. Moreover, the decision threshold might not be interchangeable for the different read-outs. For example, data obtained for the appearance of surface CD63 might not be extrapolated to the upregulation of CD203c.

Fourth, about 5% to 10% of patients are not responsive to BAT (ie, they do not react to positive control stimulation). In such patients, it becomes impossible to interpret negative allergen stimulation, and the test is valueless as a diagnostic tool. Whether repeating the test in such patients is merited requires to be established, but results and conclusions of studies that do not mention nonresponders should be interpreted carefully. Other reasons for false-negative results are inappropriate storage of cells, incorrect stimulation conditions (eg, with cytotoxic drugs or cytotoxic concentrations), concomitant drug intake,<sup>47</sup> or postponement of testing.<sup>42,48,49</sup> However, many of these issues remain poorly studied.

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