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Mast cell activation test in chlorhexidine allergy: a proof of concept

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Abstract

Background: Immediate drug hypersensitivity reactions are an increasing public health issue and a frequent cause of life-threatening anaphylaxis. Conventional confirmatory testing include skin tests and, for a few drugs, quantification of drug-specific immunoglobulin E (IgE) antibodies. However, none of these tests are absolutely predictive for the clinical outcome, and can yield false-negative and false-positive results. We performed a proof-of-concept study to assess whether a mast cell activation test could improve diagnosis of IgE-mediated chlorhexidine hypersensitivity, a common cause of perioperative anaphylaxis.

Methods: Human mast cells were generated from $CD34^+$ progenitor cells and sensitised with patients' sera to become IgE+ human mast cells (dMC^{IgE+}), and then incubated with chlorhexidine to assess degranulation. We compared the diagnostic performance of this mast cell activation test with serum from patients with and without positive skin test and basophil activation test to chlorhexidine.

Results: In dMC sensitised with sera from patients with a positive skin test and basophil activation test to chlorhexidine showed drug-specific and concentration-dependent degranulation upon stimulation with chlorhexidine, determined by surface upregulation of the degranulation marker CD63. In contrast, dMC sensitised with sera from patients with a negative skin test and basophil activation test to chlorhexidine were unresponsive in the mast cell activation test.

Conclusions: Our study suggests that the mast cell activation test can be used to diagnose IgE/FcεRI-dependent immediate drug hypersensitivity reactions. It also shows potential to assess the clinical relevance of drug-specific IgE antibodies in their ability to elicit mast cell degranulation, and therefore discriminate between allergy and sensitisation. Extended studies are required to verify whether this technique can be used in other causes of perioperative anaphylaxis.

Keywords: allergy; anaphylaxis; basophil activation test; CD63; chlorhexidine; flow cytometry; immediate drug hypersensitivity reaction; mast cell activation test

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Editor's key points

- Currently used tests for immediate drug hypersensitivity reactions have a high rate of false-negative and false-positive results.
- The authors performed a proof-of-concept study to assess whether a mast cell activation test could improve diagnosis of immunoglobulin E (IgE)-mediated chlorhexidine hypersensitivity, a common cause of perioperative anaphylaxis.
- Human mast cells sensitised with sera from patients with a positive skin test and basophil activation test to chlorhexidine showed specific degranulation upon stimulation with chlorhexidine.
- A mast cell activation test was able to diagnose IgE-dependent immediate drug hypersensitivity reactions by assessing the ability of drug-specific IgE antibodies to elicit mast cell degranulation.
- Further studies are required to verify whether this assay can be used in other causes of perioperative anaphylaxis.

Immediate drug hypersensitivity reactions (IDHRs) constitute a significant and increasing health burden with significant consequences of diagnostic error.[1,2] However, correct diagnosis of IDHRs is not always straightforward for many reasons. The gold standard for diagnosis of IDHRs is a controlled graded drug challenge, in which increasing doses of a drug or placebo are administered under strict medical supervision.[3] Unfortunately, this approach is hampered by different ethical (risk of anaphylaxis and fatalities) and practical (costly and time consuming) limitations that have hindered its entrance into mainstream practice. Moreover, full-dose drug challenge might not be possible (e.g. for anaesthetics and neuromuscular blocking agents [NMBAs]),[4] not predictive for clinical outcome,[5] or simply not possible because of absence of a validated protocol (e.g. for chlorhexidine [CHX]).[6–8] During anaesthesia, problems are certainly compounded as multiple drugs are administered simultaneously.

In clinical practice, confirmatory testing of IDHRs generally starts with skin tests[9] or *in vitro* tests, such as quantification of drug-specific immunoglobulin E (IgE) (sIgE) antibodies. However, skin testing is still associated with some diagnostic inaccuracy, especially for non-specific histamine releasers that might act via off-target MRGPRX2 receptors (e.g. opioids and quinolones),[9–13] whilst the few available drug-sIgE assays exhibit highly varying accuracy depending on the drug and clinical phenotype.[14–16] Consequently, many efforts have been undertaken to improve diagnosis of IDHRs. One of the strategies to develop more accurate tests has focused on *in vitro* activation of basophils. In the basophil activation test (BAT), allergen-specific activation of patient basophils is measured via flow cytometry of the upregulation of specific surface markers, such as CD63 and CD203c. The principles and utility of the BAT to diagnose IDHRs during anaesthesia have been assessed in multiple studies.[17,18] Overall, the BAT appears a promising diagnostic tool for IDHRs, especially for NMBAs and some β-lactam antibiotics. The key strength of the BAT is that it does not require coupling of drugs to a solid phase, a coupling that might be difficult and can mask relevant epitopes. The major weaknesses of the BAT are the requirement for fresh patient blood and the unpredictable non-responder status that is observed in about 5–15% of the population. In non-responders, basophils do not respond to IgE-mediated activation with the positive control anti-IgE.[19] Both these hurdles seem to be circumventable by mast cell activation tests (MATs), in which cultured human donor mast cells (dMCs) are passively sensitised with patient sera (henceforth called dMC^{IgE+}). To the best of our knowledge, exploration of the MAT using dMC^{IgE+} has sofar been limited to protein allergens (food, pollen and venom).[20–22]

We sought to take advantage of our experience with dMC cultures[20,23] and applications of the BAT in perioperative anaphylaxis[17,18] to study the utility of the MAT in IDHRs. We selected CHX allergy as a model, as CHX is a common cause of perioperative anaphylaxis[24,25] and the diagnosis of CHX allergy can be readily established using skin tests in combination with *in vitro* tests, such as quantification of sIgE in combination with BAT.[6–8] In addition, overdiagnosis of CHX allergy can occur, mainly because of unverified clinically irrelevant sIgE results, that is, CHX-reactive sIgE antibodies that do not trigger basophil and or dMC activation. Use of the MAT, a more functional test, could enable exploration of sensitisation and improve correct diagnosis. Utility of the MAT has only been used to assess allergies to protein allergens that are considered more potent cell activators than small molecules, such as small-molecule drugs.

Methods

Participants gave written informed consent, and the study was approved by the Ethical Committee of the University Hospital of Antwerp (Belgium B300201316408).

In vitro culture of human mast cells

Human mast cells were cultured as described.[20,23] Briefly, peripheral blood mononuclear cells were isolated from 50 ml of fresh peripheral blood from healthy volunteers, and $CD34^+$ progenitor cells were enriched using the EasySep™ Human CD34 Selection Kit (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Isolated $CD34^+$ progenitor cells were cultured in a serum-free methylcellulose-based medium (MethoCult™ SF H4236; STEMCELL Technologies) supplemented with penicillin (100 units ml^{-1}; Life Technologies, Waltham, MA, USA), streptomycin (100 μg ml^{-1}; Life Technologies), low-density lipoprotein (10 μg ml^{-1}; STEMCELL Technologies), 2-mercaptoethanol (55 μM; Life Technologies), stem cell factor (100 ng ml^{-1}; Miltenyi Biotec, Bergisch Gladbach, Germany), interleukin-3 (100 ng ml^{-1}; PeproTech, Rocky Hill, NJ, USA), and interleukin-6 (50 ng ml^{-1}; Miltenyi Biotec) for 4–5 weeks.

Sera from patients with perioperative anaphylaxis

Sera from 10 patients with witnessed perioperative anaphylaxis (predominantly Grades 3 and 4 according to the 6th National Audit Project [NAP6] classification[26]) and sIgE to CHX >0.35 kUA L^{-1} (ImmunoCAP® system fluorescence enzyme immunoassay; Phadia Thermo Fisher Scientific, Uppsala, Sweden) were selected (Table 1). In five of these patients, positive skin tests and positive BAT, as described,[27] confirmed the diagnosis of IgE-mediated CHX hypersensitivity.[6–8] All patients had positive skin prick test (SPT) (neat solution: 5 mg ml^{-1}), except one who tested positive only on intradermal

Table 1 Patient characteristics and results of confirmatory testing. Months, months between the reaction and performing of the tests; A, angio-oedema; B, bronchospasm; BAT, basophil activation test; CHX, chlorhexidine; F, female; H, hypotension; IgE, immunoglobulin E; M, male; MC, mucocutaneous lesions; NA, not available; NAP6, 6th National Audit Project; ND, not defined; NMBA, neuromuscular blocking agent; Rocu, rocuronium; S, shock; sIgE, specific IgE; SK, skin lesions; ST, skin test; TC, tachycardia.

Patient	Sex	Age (yr)	Total IgE (kUA L^{-1})	sIgE	Months	ST	BAT	NAP6	Signs	Culprit	Acute tryptase (μg L^{-1})	Basal tryptase (μg L^{-1})
1	M	41	68	10.3	3	+	+	2	B, SK	CHX	NA	6
2	M	68	65	1.2	2	+	+	4	H, TC	CHX	41	6
3	M	58	60	8.77	3	+	+	4	H, A, SK, MC	CHX	34	9.2
4	M	73	149	0.66	2	+	+	4	B, H, TC, SK	CHX	NA	4.8
5	M	64	195	3.28	1	+	+	3	H, TC, B, A, SK, MC	CHX	NA	7.7
6	M	78	4848	1.71	3	−	−	2	B, SK, MC	ND	23	8.7
7	F	54	815	6.8	4	−	−	4	H, TC	ND	NA	2.4
8	F	51	188	3.6	4	−	−	3	H, TC, SK, MC	NMBA (Rocu)	132	4.6
9	M	64	6079	24.8	3	−	−	4	S	NMBA (Rocu)	20	4.9
10	F	44	2483	2.17	2	−	−	4	H, B	NMBA (Rocu)	7.5	2.2

testing (IDT) (0.002 mg ml^{-1}). In the remaining five patients, both BAT and skin testing (SPT and IDT) to CHX were negative using the aforementioned concentrations, leaving uncertainties about the clinical significance of their isolated sIgE result. In three of these five patients, NMBAs were diagnosed as the culprit drug, and in the other two, no cause was identified.

Activation

Degranulation of dMC was measured by passively sensitising the cells (5×10^5 cell ml^{-1}) with serum in a 1:1 ratio at 37°C in a humidified CO_2 incubator overnight. The dMC^{IgE+} were then centrifuged (500×*g*; 5 min; 20°C) and the cell pellet suspended in pre-warmed Tyrode's buffer (Sigma-Aldrich, St Louis, MO, USA) to 5×10^5 cells ml^{-1}. Then, 100 μl of the cells was pre-incubated with interleukin-33 (IL-33) (100 ng ml^{-1}) (PeproTech, London, UK) for 20 min at 37°C, and the pre-incubated dMC^{IgE+} were stimulated with 100 μl Tyrode's buffer as a negative control or 100 μl CHX (Sigma-Aldrich) for 20 min at 37°C. Based on preliminary experiments, the final concentrations of CHX were 0.05, 5, 500, and 50 000 ng ml^{-1}. Reactions were stopped by placing the cells on ice and subsequently supernatants were removed after centrifugation (500×*g*; 5 min; 4°C). Cells were stained with monoclonal anti-human CD117-APC (clone 104D2; BD Biosciences, Erembodegem, Belgium), anti-human CD203c-PeCy7 (clone NP4D6; eBioscience, San Diego, CA, USA), and anti-human CD63-FITC (clone H5C6; BD Biosciences) for 20 min at 4°C. Finally, cells were washed and resuspended in phosphate-buffered saline (ingredients) with 0.1% w/v sodium azide and degranulation of dMCs measured as surface upregulation of the lysosomal degranulation marker CD63. Mast cell activation test was repeated on dMCs of two different volunteers.

Flow cytometry

Flow cytometry was performed on a FACSCanto II™ flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA) equipped with three lasers (405, 488, and 633 nm). Correct compensation settings for antibodies conjugated with fluorochromes were performed using BD™ CompBeads (BD Biosciences). Flow cytometric data were analysed using Kaluza Analysis 1.5 software (Beckman Coulter, Brea, CA, USA). Unstained samples were used to distinguish between positive and negative cells according to the 99th percentile. A fluorescence minus one was used to distinguish between positive and negative cells. Mast cells were gated out as CD117-and CD203c-positive cells. More than 1500 mast cells were counted per sample.

Statistical analysis

GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA, USA) was used for data analysis. Mann–Whitney test was performed, with *P*-value <0.05 considered significant. Results are expressed as median and 25th–75th percentile.

Results

As shown in Figure 1, mast cells were gated based on forward scatter and side scatter and double positivity for CD117 and CD203c. In resting dMC, there was (almost) no spontaneous expression of the lysosomal degranulation marker CD63. As shown in Figure 2, dMC^{IgE+} (cells passively sensitised with patient sera), CD63 was upregulated after activation with CHX, by 1% (1–20), 10% (5–66), 57% (15–72), and 31% (6–76) for the corresponding concentrations of 0.05, 5, 500, 50 000 ng ml^{-1} CHX. However, degranulation of dMC^{IgE+} was restricted to the five patients who also had a positive skin test and BAT to CHX. As shown in Table 1, the sIgE CHX in these patients varied between 0.66 and 10.3 kUA L^{-1}. In contrast, in patients with an isolated sIgE CHX (skin test and BAT both negative), no upregulation of CD63 was demonstrable. In these patients, sIgE varied between 2.17 and 24.8 kUA L^{-1}. Note that total IgE is numerically lower in patients with positive skin test and BAT (68 kUA L^{-1}; 63–172) compared with patients with an isolated sIgE CHX (2483 kUA L^{-1}; 502–5464) (*P*=0.02). As shown in Figure 2b, similar observations were made with mast cells obtained from a second donor, adding rigour to our results. Similar results of CD63 upregulation were obtained with the second donor: 2% (1–20), 7% (3–38), 30% (4–53), and 45% (5–69)

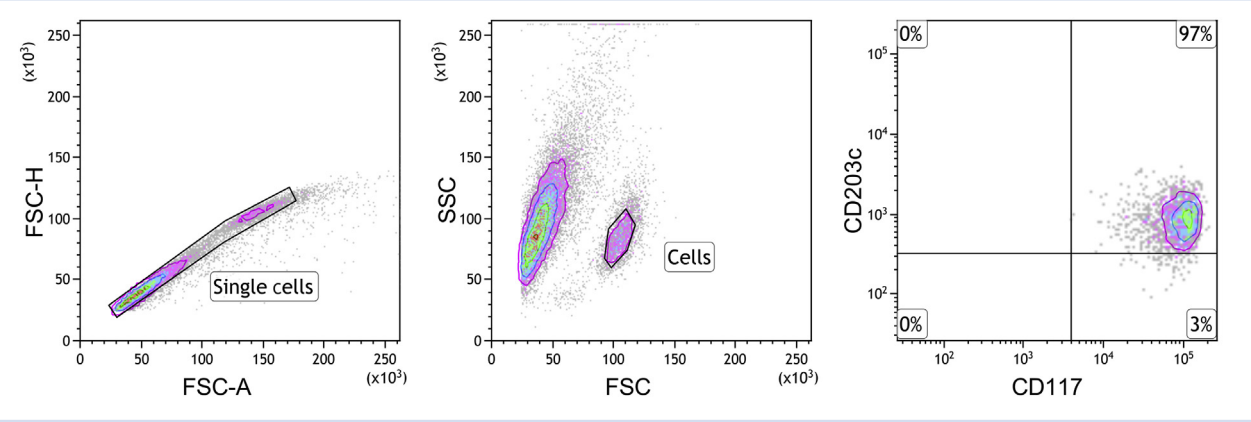

Fig 1. Gating strategy for mast cells. Single cells were gated based on the forward scatter (FSC)–H and FSC-A plot. Cells were gated based on FSC–side scatter (SSC). Mast cells were $CD117^{+}CD203c^{+}$. A fluorescence minus one sample was used to set the marker to the 99th percentile.

for the corresponding concentrations. A representative individual plot is shown in Figure 3. The dMC^{IgE-} did not respond to CHX (data not shown).

Discussion

Here, we provide novel proof-of-concept evidence that human-derived mast cells can be passively sensitised with CHX-reactive IgE antibodies and become responsive to this antigen. Moreover, our technique seems to have potential to determine the clinical significance of CHX-reactive sIgE.

Chlorhexidine (1:6-di(4-chlorophenyldiguanido)-hexane) is a synthetic cationic bisbiguanide with two biguanide groups both linked to a terminal 4-chlorophenyl group, with the resultant chloroguanide structures connected by a hexamethylene bridge. Chlorhexidine, usually a gluconate or acetate salt, has widespread application in various domestic and industrial products, and is the most effective disinfectant in the healthcare setting. In 1984, Nishioka and colleagues[28] first suspected an IgE/FcεRI-dependent mechanism in immediate CHX hypersensitivity, and Ohtoshi and colleagues[29] subsequently developed a radioallergosorbent test technique to measure CHX-reactive sIgE. In 2007, an sIgE assay became commercially available,[30] which has proved to have high sensitivity and specificity in the perioperative setting.[6] However, in the presence of elevated total IgE titres, CHX sIgE results should be interpreted cautiously.[31] More recently, CHX has proved to be one of the principal causes of perioperative anaphylaxis.[7,24] Efforts have been undertaken to identify the fine structural specificities of the CHX epitopes complementary to CHX-reactive sIgE antibodies.[32,33] In clinical practice, diagnosis of IgE/FcεRI-dependent CHX allergy generally rests upon an evocative story combined with two or more positive tests, including sIgE, skin testing (SPT or IDT), and a mediator release test (such as BAT).[6–8]

As with all proof-of-concept studies, appropriate inclusion of well-documented patients and control individuals is critical for robust analyses. We randomly selected sera from five

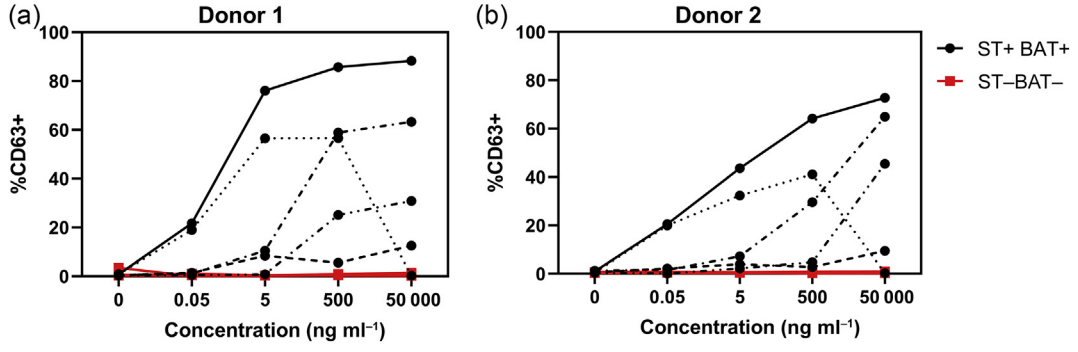

Fig 2. Mast cell activation with chlorhexidine. Cultured human-derived mast cells were activated with chlorhexidine after passive sensitisation of the cells with sera of patients with positive skin test and basophil activation test (SPT+BAT+) (black lines: round symbols), or patients with negative skin test and basophil activation test (SPT–BAT–) (red lines: square symbols). A and B reflect the two different donors used. The different types of lines reflect different patients' sera. n=5 in each group.

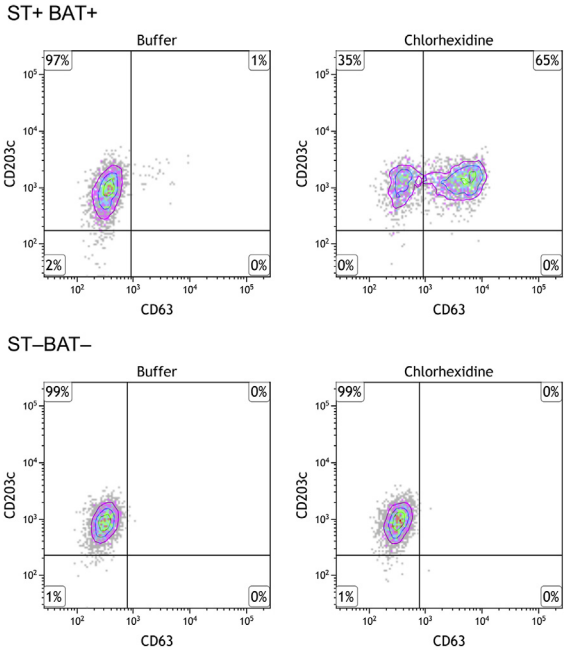

Fig 3. Representative plots of mast cell activation tests with chlorhexidine. Cultured human mast cells were activated with chlorhexidine (50 000 ng ml^{-1}) after passive sensitisation of the cells with serum of a patient with positive skin test and basophil activation test (ST+BAT+), or a patient with negative skin test and basophil activation test (ST−BAT−).

patients with an evocative and witnessed history of a perioperative hypersensitivity reaction combined with positive results for sIgE, skin testing, and a CD63-based BAT, a combination of tests considered diagnostic for IgE-mediated CHX allergy.[6–8] In addition, we analysed sera of patients with an evocative history and an isolated positive sIgE result to CHX, but negative skin tests and BAT, likely not allergic to the antiseptic. Our experiments show that dMC can effectively be sensitised with CHX-reactive sIgE antibodies from patients testing positive in skin tests and CD63-based BAT, and that these dMC^{IgE+} can be triggered to degranulate in response to CHX. Moreover, our MAT method has a high analytical sensitivity, as successful passive sensitisation was attained for titres of CHX-reactive sIgE as low as 0.66 kUA L^{-1} in the traditional ImmunoCAP assay. In contrast, when dMCs from the same donor are sensitised with CHX-reactive sIgE antibodies obtained from patients with negative skin test and CD63-based results, cells remain unresponsive to CHX. In other words, the MAT shows the potential to discriminate between genuine CHX allergy and CHX sensitisation, suggesting that an isolated positive drug-sIgE result may be false positive, with doubtful clinical relevance. One could argue that in the absence of a CHX challenge test, no absolute conclusions can be drawn. However, in accord with current recommendations about direct challenge,[3,34,35] we deemed it unethical to perform direct challenge in patients who had experienced life-threatening Grades 3–4 reactions according to the NAP6 classification and who had their diagnosis confirmed by both skin testing and BAT.[6–8] Besides, for the time being, there is no validated CHX challenge protocol available that could be applied in sensitised patients (sIgE positive, skin tests, and BAT negative). Collectively, our findings suggest not relying on sIgE antibodies to CHX in isolation to confirm IgE/FcεRI CHX allergy, especially when total IgE is elevated.[31,36] To avoid misdiagnosis, an elevated sIgE result should be confirmed by a positive result in either skin tests (SPT or IDT), BAT, or MAT.

Admittedly, the MAT is technically more difficult than the traditional BAT, but our proof of concept shows that the technique offers several advantages. Unlike the BAT, the MAT does not require fresh blood, it circumvents the non-responder issue observed in about 15% of BAT,[37] and allows deepening our insights in the molecular mechanisms and pathogenesis of IDHR.[38]

In conclusion, we show that application of the MAT extends beyond allergies towards protein allergens. We have shown that the technique can be used to diagnose IgE/FcεRI-dependent allergy to small drug molecules, such as CHX. However, larger collaborative studies are required to confirm these promising observations and to allow mainstream use.

Authors' contributions

Experimental design: JE, CHB, CM
Experimentation: JE
Coordination: VS, DGE
Supervision: CHB, CM, VS, DGE
Writing of paper: JE, VS, DGE
Proofreading/revising of final paper: all authors.

Declarations of interest

The authors declare that they have no conflicts of interest.

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