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

The mast cell activation test in chlorhexidine allergy: a proof of concept

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The authors declare no conflict of interest.

Word count abstract: 250

Word count manuscript: 2518

Short title: MAT in immediate drug hypersensitivity

Abbreviations

BAT: basophil activation test

CHX : chlorhexidine

DC: drug challenge

dMC: donor mast cells

dMC^{IgE+}: donor mast cells sensitized with patients' sera

FcεRI: high affinity receptor for sIgE

FMO: fluorescence minus one

IDHRs: immediate drug hypersensitivity reactions

MAT: mast cell activation test

NMBA: neuromuscular blocking agent

sIgE: specific IgE antibody

ST: skin test

Abstract

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

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

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.

Conclusion: Our study suggests that the MAT can be used to diagnose IgE/FcεRI-dependent IDHR. Besides, it shows potential to assess the clinical relevance of drug-sIgE antibodies in their ability to elicit MC degranulation and therefore discriminate between allergy, and merely sensitization. Extended studies are required to verify whether this technique can benefit in other causes of perioperative anaphylaxis.

Key words: CD63**,** chlorhexidine (CHX)**,** flow cytometry**,** Human mast cell**,** mast cell activation

Introduction

Immediate drug hypersensitivity reactions (IDHRs) constitute a significant and increasing health burden with sometimes dramatic consequences of diagnostic error. [1, 2] However, correct diagnosis of IDHRs is not always straightforward for many reasons. The gold standard for the diagnosis of IDHRs is a controlled graded drug challenge (DC), in which increasing doses of a drug or placebo are administered under strict medical supervision. [3] Unfortunately, DCs are hampered by different ethical (risk of anaphylaxis and fatalities) and practical (costly, time consuming) limitations that have hindered its entrance in mainstream practice. Moreover, full-dose DC might not be possible (e.g. for anaesthetics and neuromuscular blocking agents (NMBAs)), [4] not predictive for the clinical outcome, [5] or simply not possible because of absence of a validated DC protocol (e.g. for chlorhexidine). [6-8] During anaesthesia, problems are certainly compounded as multiple drugs need to be administered simultaneously. Therefore, 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 nonspecific histamine releasers that might act via off-target MRGPRX2 occupation (e.g. opiates 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 (BAT). In the BAT, allergen-specific activation of patients' basophils is measured via flow cytometric analysis 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 and was recently reviewed elsewhere. [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 basophilic non-responder status that is observed in about 5-15% of the population.

In non-responders, basophils do not respond to an 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 (dMC) are passively sensitized with patients' sera (henceforth called dMC^{IgE+}). At present, to the best of our knowledge, exploration of the MAT using dMC^{IgE+} has sofar been limited to protein allergens (food, pollen, venom). [20-22]

Here, we sought to take advantage of our experience with dMC cultures [20, 23] and applications 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]

Alternatively, prudence should be called upon over diagnosis of CHX allergy, mainly because of unverified clinically irrelevant sIgE results, that is, CHX-reactive sIgE antibodies that do not trigger basophil and or dMC. Therefore, it is attractive to speculate that the MAT, being a more functional test, could enable the exploration of sensitization and benefit correct diagnosis. To the best of our knowledge, this approach is innovative, as currently utility of the MAT has only been assessed allergies to proteinaceous allergens that which are considered more potent effector cell activators than small molecules such as drugs.

Materials and methods

In vitro culture of human MCs

Human MCs were cultured as described elsewhere. [20, 23] Briefly, peripheral blood mononuclear cells were isolated from 50 mL fresh peripheral blood from healthy volunteers. $CD34^+$ progenitor cells were enriched using the EasySep Human CD34 Selection Kit (Stemcell Technologies, Vancouver, 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, USA), streptomycin (100 µg mL^{-1}, Life Technologies), low-density lipoprotein (LDL, 10 µg mL^{-1}, Stemcell Technologies), 2-mercaptoethanol (55 µmol L^{-1}, Life Technologies), stem cell factor (SCF, 100 ng mL^{-1}, Miltenyi Biotec, Bergisch Gladbach, Germany), interleukin-3 (IL-3, 100 ng mL^{-1}, PeproTech, Rocky Hill, USA) and interleukin-6 (IL-6, 50 ng mL^{-1}, Miltenyi Biotec) for 4-5 weeks. Participants gave written informed consent and the study was approved by the Ethical Committee of the University Hospital of Antwerp (Belgium B300201316408).

Sera from patients with perioperative anaphylaxis

As shown in table 1, sera from 10 patients with a witnessed perioperative anaphylaxis (predominantly grade 3 and 4 according to the NAP6 classification published in this Journal [26]), and specific IgE (sIgE) to chlorhexidine (CHX) > 0.35 kUA L^{-1} (ImmunoCAP system fluorescence enzyme immunoassay (FEIA) (Phadia Thermo Fisher scientific, Uppsala, Sweden)), were selected. In five of these patients positive skin tests and positive basophil activation test (BAT) as described in Ebo et al., [27] confirmed the diagnosis of an IgE-mediated CHX hypersensitivity according to. [6-8] All patients had positive skin prick test (SPT) (neat solution: 5 mg mL^{-1}), except one who tested positive only on intradermal testing (IDT) (0.002 mg mL^{-1}). In the remaining five patients both BAT and skin testing (SPT and/or IDT) to CHX were negative using the concentrations mentioned above, leaving uncertainties about the clinical significance of their isolated sIgE result. In three of these 5 patients, NMBA's are diagnosed as the culprit drug, in the remainder 2 no cause could be identified.

Activation

Degranulation of dMC was measured by overnight passively sensitizing the cells, at a concentration of $5x10^5$ cell mL^{-1}, with serum, in a 1:1 ratio, at 37°C in a humidified CO_2-incubator. Next, dMC^{IgE+} were centrifuged (500g, 5 minutes, 20°C) and the cell pellet was resolved in pre-warmed Tyrode's buffer (Sigma-Aldrich, St. Louis, USA) at a concentration of $5x10^5$ cells mL^{-1}. Thereafter, 100 µL of the cells were pre-incubated with interleukin 33 (IL-33) (100 ng mL^{-1}) (Peprotech, London, UK) for 20 minutes at 37°C. Subsequently, the pre-incubated dMC^{IgE+} were stimulated with 100 µL Tyrode's buffer as a negative control or 100 µL of CHX (Sigma-Aldrich) for 20 minutes at 37°C. Based upon preliminary dose-finding experiments, the final concentrations of CHX were: 0.05, 5, 500, 50,000 ng mL^{-1}. Reactions were stopped by placing the cells on ice and subsequently the supernatants is removed after centrifugation (500 g, 5 minutes, 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, USA) and anti-human CD63-FITC (clone H5C6, BD Bioscience) for 20 minutes at 4°C. Finally, cells were washed and resolved in PBS with 0.1% sodium azide and measured. Degranulation of dMCs was measured as surface upregulation of the lysosomal degranulation marker CD63. The mast cell activation test was repeated on dMCs of two different volunteers.

Flow cytometric analysis

Flow cytometric analysis was performed on a FACSCanto II flow cytometer (BD Immunocytometry Systems, San Jose, CA) equipped with three lasers (405 nm, 488 nm 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, California, Brea, USA). Unstained samples were used to set a marker between positive and negative cells according to the 99^{th} percentile. A fluorescence minus one (FMO) was used to set a marker between positive and negative cells. Mast cells were gated out as CD117 and CD203c positive cells. Al least 1500 MCs were counted per sample.

Statistical analysis

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

Results

As shown in figure 1, MC were gated based on forward scatter (FSC) and side scatter (SSC) 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 sensitized with patients' sera), CD63 was upregulated after activation with CHX, for 1% (1-20), 10% (5-66), 57% (15-72), 31% (6-76) for the corresponding concentrations of 0.05, 5, 500, 50000 ng mL^{-1} CHX. However, this degranulation of dMC^{IgE+} was absolutely restricted to the five patients who also demonstrated 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) as compared to patients with an isolated sIgE CHX, 2483 kUA L^{-1} (502-5464) (p=0.02). As shown in panel B of figure 2, similar observations were made with MC obtained from a second donor, adding rigor to our results. Similar results of CD63 upregulation was obtained with the second donor 2% (1-20), 7% (3-38), 30% (4-53) and 45% (5-69) 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 the proof-of-concept that dMC can be passively sensitized with CHX-reactive IgE antibodies and become responsive to the antiseptic. Moreover, our technique seems to have potential to determine the clinical significance of CHX-reactive sIgE. To the best of our knowledge, these findings are innovative.

Chlorhexidine (1:6-di(4-chlorophenyldiguanido)-hexane) is a synthetic cationic bis-biguanide with two biguanide groups both linked to a terminal 4-chlorophenyl group, with the resultant chloroguanide structures connected via a hexamethylene bridge. CHX, usually a gluconate or acetate salt, has a widespread application in various domestic and industrial products and it is the most effective disinfectant in the health care setting. In 1984, Nishioka et al., [28] firstly suspected an IgE/FcεRI-dependent pathomechanism in immediate CHX hypersensitivity. Two years later, Ohtoshi et al., [29] developed a Radio-Allergo-Sorbent-Test (RAST) technique to depict CHX-reactive sIgE. In 2007 a specific IgE assay became commercially available [30] which has later proven to have a high sensitivity and specificity in the perioperative setting. [6] However, in the presence of elevated total IgE titers, chlorhexidine sIgE results should be interpreted cautiously. [31] More recently, CHX has proven to be one of the principal causes of perioperative anaphylaxis. [7, 24] Different 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, that is, sIgE, skin testing (SPT and/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. Therefore, we randomly selected the sera of five 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 sensitized with CHX-reactive sIgE antibodies from patients testing positive in skin tests and CD63-based BAT and that these dMC^{IgE+} can subsequently be triggered to degranulate in response to CHX. Moreover, our MAT method demonstrates 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 dMC, from the same donor, are sensitized with CHX-reactive sIgE antibodies obtained from patients with negative skin test and CD63-based results, cells remain completely unresponsive to CHX. In other words, the MAT shows the potential to discriminate between genuine CHX allergy and CHX sensitization, 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 DC, [3, 34, 35] we deemed unethical to perform DC in patients who had experienced life-threatening grade 3-4 reactions according to the NAP6 classification and who had their diagnosis already 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 sensitized patients (sIgE positive, skin tests and BAT negative). Therefore, we think that collectively, our findings should suggest to avoid 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 always be confirmed by a positive result in either skin tests (SPT or IDT), BAT or MAT.

Admittedly, the MAT is technically more difficult than traditional BAT, 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 as observed in about 15% of BAT, [37] and allows deepening our insights in the molecular mechanisms and pathogenesis of IDHR. [38]

In conclusion, we demonstrate for the first time that application of the MAT extends beyond allergies towards proteinaceous allergens. We have shown that the technique can be used to diagnose

IgE/FcεRI-dependent allergy to small drug molecules such as chlorhexidine. However, Larger collaborative studies are required to confirm these promising observations and to allow its entrance in mainstream use.

Author 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.

Acknowledgements

Vito Sabato is a Senior Clinical Researcher of the Research Foundation Flanders/Fonds Wetenschappelijk Onderzoek (FWO: 1804518N). Didier Ebo is a Senior Clinical Researcher of the Research Foundation Flanders/Fonds Wetenschappelijk Onderzoek (FWO: 1800614N). Athina Van Gasse is a fellow of the Fonds voor Wetenschappelijk Onderzoek - Vlaanderen (FWO) (1113617N).

Declaration of Interest

The authors declare that there are no conflicts of interest.

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TABLE 1: Patients characteristics and results of confirmatory testing

Patient	Sex	Age (y)	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

M, male; f, female; y, years; sIgE, specific IgE chlorhexidine; ST, skin test; Months, months between the reaction and performing of the tests; BAT, basophil activation test; NAP6, National audit project reaction grade; +, positive; -, negative; H, hypotension; TC, tachycardia; A, angio-edema; B, Bronchospasm; S, Shock; MC, mucocutaneous lesions; SK, skin lesions; CHX, chlorhexidine; NMBA, neuromuscular blocking agent; Rocu, rocuronium; ND, not defined; NA, not available.

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Figure 1: Gating strategy of MC

Single cells were gated based on FSC-H and FSC-A plot. Cells were gated based on FSC-SSC. MC were $CD117^{+}CD203c^{+}$. A fluorescence minus one sample is used to set the marker according to the 99^{th} percentile.

Figure 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.

Figure 3: Representative plot of mast cell activation test with chlorhexidine

Cultured human mast cells were activated with chlorhexidine (50,000 ng mL^{-1}) after passive sensitization 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-).