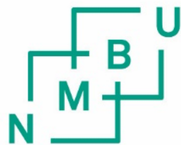

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Mast cell activation test as a diagnostic tool in chlorhexidine allergy and to study cross-reactivity

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Biotechnology

Preface

This thesis presents my work on the topic of drug hypersensitivity during the period from August 2020 to May 2021, conducted at the department of Immunology at the University of Antwerp (UA). The external supervisor from UA was Prof. Didier Ebo, the internal supervisor from NMBU was Prof. Dzung B. Diep.

This year has been educational for me, thanks to a great team that supported me along the way. I want to thank especially my mentors Jessy Elst and Christel Mertens for their incredible guidance throughout my project. Jessy's passion and dedication for research is something I very much admire. Her patience and natural feeling for mentoring have helped me tremendously this year during both the practical work and the writing of my thesis. I want to thank Christel for being a solid support during this time. She was always available to answer questions and provide great advice and suggestions regarding the lab work.

I want to thank Prof. Ebo for giving me the opportunity to conduct research in a field I was not yet well-known in. His enthusiasm and dedication were inspiring and gave me the motivation to bring my project to a successful conclusion. I also want to thank Prof. Ebo for his almost continuous availability and great communication throughout the year. This much appreciated aspect led to a very smooth process.

A special thanks to the staff of the department of Allergology at the Antwerp University Hospital (UZA) for taking blood samples as well as donating blood themselves. Because of the COVID-19 pandemic, I was dependent on them for my weekly blood donation. Finally, I also want to thank the rest of the staff of the department of Immunology at UA and the department of Allergology at UZA for making these ten months a pleasant experience.

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List of abbreviations

ALX	Alexidine
APC	Allophycocyanin
BAT	Basophil activation test
BMMC	Bone marrow derived mast cells
BSA	Bovine serum albumin
CCL5	Chemokine (C-C motif) ligand 5
CD	Cluster of differentiation
CHX	Chlorhexidine
COX	Cyclooxygenase
CRH	Corticotrophin-releasing hormone
CXCL8	Chemokine (C-X-C motif) ligand 8
DHR	Drug hypersensitivity reaction
FcεRI	High-affinity IgE receptor
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
HMC-1	Human mast cell-1
IDT	Intradermal test
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified dulbecco's medium
ITS	Insulin-Transferrin-Selenium
KIT	Tyrosine-protein kinase KIT
LAD	Laboratory of allergic diseases
LDL	Low-density lipoprotein
MRGPRX2	Mas-Related G-Protein-coupled Receptor X2
NMBA	Neuromuscular blocking agent
NRL	Natural rubber latex
OCT	Octenidine
PBCMC	Peripheral blood cultured human mast cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy7	PE-Cyanine7
pMAT	Passive mast cell activation test
POH	Perioperative hypersensitivity
PRR	Pattern recognition receptor
RBL	Rat basophilic leukemia
SCF	Stem cell factor
SD	Standard deviation
sIgE	Specific immunoglobulin E
SM	Systemic mastocytosis

SP	Substance P
SPT	Skin prick test
SSC	Side scatter
ST	Skin test
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

Summary

Background: Chlorhexidine (CHX) allergy has become a predominant cause of perioperative hypersensitivity (POH) including anaphylaxis. In general, confirmatory diagnosis of CHX-allergy starts with skin tests (STs) and quantification of CHX specific immunoglobulin E (sIgE). However, in some cases diagnosis remains challenging, mainly because of divert results between both methods. The passive mast cell activation test (pMAT) could improve diagnosis and possibly elicit cross-reactivity between CHX and structurally similar compounds such as alexidine (ALX) and octenidine (OCT).

Objective: Confirming the hypothesis that the pMAT, in which donor mast cells (MCs) are passively sensitised with CHX-sIgE reactive sera, might benefit diagnosis of IgE-mediated CHX allergy and to study whether pMAT enables to demonstrate cross-reactivity between CHX and ALX and/or OCT.

Methods: Human MCs were generated from peripheral blood $CD34^+$ progenitor cells and sensitised with sera from 30 patients with a documented CHX-allergy (CHX-$sIgE^+$ in addition to positive STs and/or positive basophil activation test [BAT]), 9 patients with CHX sensitisation (CHX-$sIgE^+$, ST^-, BAT^-) and 20 control individuals (CHX-$sIgE^-$, ST^-). MCs were then stimulated with CHX to assess degranulation. Additionally, cells were sensitised with sera from 10 CHX-allergic patients who tested positive in pMAT, 5 CHX-sensitised patients and 5 healthy controls and subsequently challenged with ALX and OCT. MC degranulation was measured via quantification of up-regulation of the lysosomal degranulation marker CD63.

Results: MCs showed activation for 12/13 CHX-allergic patients with positive ST and BAT, 7/11 CHX-allergic patients with positive ST and no BAT and 3/6 CHX-allergic patients with negative ST but positive BAT. ALX and OCT responsiveness was demonstrable with 4/10 and 3/10 of the sera of CHX-allergic patients, respectively. No reactivity to CHX, ALX nor OCT was demonstrable when using sera from CHX-sensitised patients or when using sera from control individuals. MCs incubated with CHX, OCT and ALX without prior passive sensitisation with patients' sera remained unresponsive to all three antiseptics.

Conclusion: The pMAT is a reliable diagnostic of which the application could be extended from traditional proteinaceous allergens to small molecules such as drugs. Unlike BAT, deferred and more standardized batch analyses are possible, making it easier to translate in a clinically valuable diagnostic. In addition, pMAT constitutes an attractive tool to explore cross-reactivity between structurally similar compounds, including (preclinical) assessment of molecules not approved for human use yet.

Sammendrag

Bakgrunn: Klorheksidin (CHX) allergi har blitt en ledende årsak til perioperativ overfølsomhet (POH) og anafylaksi. Hudtester (STs) og måling av CHX spesifikke immunglobulin E (sIgE) kan brukes for å diagnostisere CHX-allergi. Å diagnostisere CHX-allergi på denne måten har i noen tilfeller vist seg å være utfordrende, hovedsakelig grunnet ulike resultater fra de to metodene. Passiv mastcelle aktiveringstest (pMAT) kan forbedre diagnostiseringen av CHX-allergi og muligens vise kryssreaktivitet mellom CHX og andre strukturelt lignende forbindelser som alexidin (ALX) og octenidin (OCT).

Mål: Bekrefte hypotesen om at pMAT, hvor donor mastceller (MCs) er passivt sensibilisert med CHX-sIgE reaktivt sera, kan forbedre diagnostiseringen av IgE-mediert CHX-allergi samt undersøke om pMAT kan brukes til å demonstrere kryssreaktivitet mellom CHX og ALX og/eller OCT.

Metode: Humane mastceller ble generert fra perifere $CD34^+$ progenitorceller og sensibilisert med sera fra 30 pasienter med dokumentert CHX-allergi ($CHX\text{-}sIgE^+$ i tillegg til positiv hudtest og/eller positiv basofil aktiveringstest [BAT]), 9 pasienter med CHX sensibilisering ($CHX\text{-}sIgE^+$, ST^-, BAT^-) samt en kontrollgruppe på 20 individer ($CHX\text{-}sIgE^-$, ST^-). MCs ble så stimulert med CHX for å vurdere degranulering. MCs ble også sensibilisert med sera fra 10 CHX-allergiske pasienter som testet positivt i pMAT, 5 CHX-sensibiliserte pasienter, samt 5 friske kontroller, og deretter stimulert med ALX og OCT. MC degranulering ble målt ved en kvantifisering av oppregulering av den lysosomale degranuleringsmarkøren CD63.

Resultat: MCs viste aktivering for 12/13 CHX-allergiske pasienter med positiv ST og BAT, 7/11 CHX-allergiske pasienter med positiv ST og ingen BAT og 3/6 CHX-allergiske pasienter med negativ ST, men positiv BAT. MC aktivering av ALX og OCT ble demonstrert for henholdsvis 4/10 og 3/10 sera av CHX-allergiske pasienter. Ingen reaktivitet mot CHX, ALX og heller ikke OCT ble demonstrert når sera fra CHX-sensibiliserte pasienter eller kontroll individer ble brukt. MCs som ble inkubert med CHX, OCT eller ALX uten tidligere passiv sensibilisering med pasientens sera, viste ingen aktivering.

Konklusjon: pMAT er en pålitelig diagnostikk hvorav applikasjonen kan utvides fra proteinholdige allergener til små molekyler slik som legemidler. I motsetning til BAT, mer standardiserte batch analyser er mulig, som gjør det lettere å gjøre det til et klinisk verdifullt diagnoseverktøy. I tillegg kan pMAT brukes til å undersøke kryssreaktivitet mellom strukturelt lignende forbindelser, som inkluderer molekyler som ennå ikke er godkjent for human bruk.

Introduction

Drug hypersensitivity reactions

Approximately 80 % of adverse drug reactions are predictable, dose-depending reactions to drugs, related to the pharmacological properties of the drug. Dose-independent, unpredictable, and unintended reactions to a drug are categorised under the term drug hypersensitivity reactions (DHRs).[1, 2]

Clinically, DHRs can be divided into immediate and delayed type reactions depending on the time in which they occur after drug administration. This thesis will focus on immediate drug hypersensitivity. When the activation of mast cells and basophils is not specific, the reaction is non-allergic and mediated by the ligation of MRGPRX2 or other receptors, inhibition of the cyclooxygenase (COX) pathway or non-specific immediate histamine releasers such as opiates.[1, 3, 4] The reaction is allergic when there is specific activation of immune cells through IgE/FcεRI cross-linking.[1, 2]

Diagnosis

The correct diagnosis of drug hypersensitivity reactions is important to prevent future exposure to the culprit drug and asses possible cross-reactivity with structurally similar compounds. It includes the evaluation of clinical history, with additional *in vivo* and *in vitro/ex vivo* tests.

Evaluation of clinical history

Obtaining the clinical history of the patient is the first step in diagnosis of drug hypersensitivity reactions. It includes examination of the symptoms, the chronology of the symptoms, the medical background of the patient, and other medication taken.[1, 2]

Skin tests

The most readily available method to diagnose a DHR is skin tests. However, their sensitivity and predictive values are dependent on the culprit drug and clinical presentation of the reaction. In case a skin prick test (SPT) turns out negative, an intradermal test (IDT) could provide a better sensitivity for drug specific IgE. Skin tests are not suited to distinguish between IgE/FcεRI-dependent and -independent reactions.[1, 2, 5]

Quantification of specific IgE

When an IgE-mediated mechanism is suspected, the quantification of specific IgE with an immunoassay, such as ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden), can be useful. This technique is based on the ability of sIgE in patients' sera to bind to its substrate coupled to a solid phase. Unfortunately, the amount of commercially available drug-sIgE assays is limited and they generally show a low sensitivity.[1, 2, 6]

Drug provocation tests

A drug challege is the gold standard for identifying the culprit drug of an allergic reaction. They have the highest sensitivity but need to be conducted under strict surveillance due to its inherent risk of severe, life-threatening reactions. In some cases, it is not possible to perform a drug challenge (e.g., hypersensitivity to neuromuscular blocking agents [NMBAs]). Besides, even DPTs are not 100% predictive and might be contraindicated.[1, 2, 6]

Basophil activation test

Skin tests and sIgE tests are still prone to inaccuracies and the two diagnostic tools often show discrepancies. *Ex vivo* analysis of basophils in the BAT could make diagnosis of an allergy more accurate, especially for drug hypersensitivity reactions. The principle of the BAT is based on the stimulation of basophils with antigen, followed by the flow-cytometric analysis of the upregulation of distinct activation markers such as CD63 and CD203c. There are, however, two major disadvantages connected to the basophil activation test, namely the need for fresh patients' blood and a non-responder status in approximately 5-10 % of the patients that are tested. The non-responder status means that the patients basophils failed to upregulate activation markers upon an IgE-mediated activation, leading to the absence of a positive control.[7] A novel diagnostic tool, the passive mast cell activation test, could possibly circumvent these issues.

Mast cells

All human mast cells originate from a common $CD34^+$ hematopoietic progenitor in the bone marrow. When mast cell progenitors are released into the blood stream, they are undifferentiated and mature further after migrating to specific tissue. Depending on the phenotypical changes a mast cell undergoes when migrating to a certain tissue, mast cells can be divided into two categories. MC_T cells are mast cells that typically reside in mucosal tissue and contain tryptase. MC_{TC} cells contain tryptase as well as chymase, carboxypeptidase and cathepsin and are usually found in connective tissue and smooth muscle.[8, 9]

Mast cell maturation

The differentiation and maturation of mast cells is dependent on several different cytokines. Stem cell factor (SCF) is the major growth cell factor for human mast cells. Ligation of SCF to its receptor CD117 induces enhanced survival, growth, cell migration and effector function.[8] Besides from SCF, cytokines such as interleukin 3 (IL-3) and IL-6 have also been shown to promote the growth, maturation and survival of human mast cell cultures.[10, 11]

Mast cell characteristics

Mast cells can be distinguished from basophils, monocytes and other myeloid cells based on their difference in cell surface phenotype.[12] CD34 is generally regarded as a marker for the selection of hematopoietic progenitor cells.[13] Mature mast cells, however, don't express CD34, but share a couple of characteristics with all leukocytes, such as the leukocyte common antigen (CD45), the hyaluronan receptor (CD44) and leukosialin (CD43). In common with basophils, mast cells express several adhesion antigens, a couple of activation-linked cell surface antigens such as CD9 and CD203c, and the high affinity receptor for IgE, FcεRI. Unique to mast cells are for example KIT (CD117), and the vitronectin receptor (CD51/CD61).[12] Additionally, it has recently been demonstrated that MRGPRX2 is highly expressed on the cell surface of MC_{TC}, while only little expression is reported on MC_T cells. Due to their widespread location, and thus differences in microenvironment, the phenotype of mast cells is heterogenic and can also change during their life time.[9]

Mast cell activation

Since as early as 1878 it has been proven that mast cells play a crucial role in allergic inflammatory reactions.[14] Mast cells can be triggered both by an IgE-mediated and a non-IgE-mediated mechanism, although the latter is not yet well understood.

IgE-mediated mast cell activation

In the IgE driven mechanism, two phases can be distinguished: the sensitisation phase and the effector phase. After a first contact with the allergen, T cells are presented fragments of the allergen by antigen presenting cells. The activated T cells subsequently secrete various cytokines causing a class switch in B cells, turning them into IgE secreting plasma cells. The secreted IgE antibodies bind to high-affinity IgE receptor FcεRI on the surface of mast cells and basophils, creating sensitised cells. Upon a second exposure to the antigen, the effector phase kicks in. During this phase, the antigen crosslinks antigen specific IgE bound to the IgE receptor FcεRI. The aggregation of FcεRI followed by this crosslinking leads to the immediate release of mast cell inflammatory mediators such as histamine.[9, 15]

Non-IgE-mediated mast cell activation

Mast cells can also be triggered by several IgE-independent pathways, such as toll-like receptor (TLR) ligands. TLRs are widely expressed on mast cells and binding by distinct ligands (e.g., TLR2 and TLR4 ligands) can stimulate the release of mediators and cytokine production. Complement factors C3a and C5a can also induce degranulation. In addition, close to sensory nerve endings, mast cells can be stimulated by a variety of neuropeptides, such as substance P (SP), corticotrophin-releasing hormone (CRH) and neurotrophins. Pathogens and their components can directly and indirectly activate mast cells by pattern recognition receptors (PRRs). Moreover, mast cells are responsive to several inflammatory products such as IgG, cytokines and chemokines, or even physical factors like heat and cold.[8, 16] Interestingly, recently it was found that occupation of the Mas-Related G-Protein-coupled Receptor X2 (MRGPRX2) by several drugs, such as neuromuscular blocking agents (NMBAs), fluoroquinolones, icatibant and the opiate morphine, can trigger mast cell activation.[3, 5, 8, 16, 17]

Mast cell mediators

Mast cells secrete a diverse array of inflammatory mediators upon activation. Preformed mediators are stored in their secretory granules and include lysosomal enzymes, proteases, cytokines, and biogenic amines such as serotonin and histamine. The latter is responsible for various allergy-linked effects like vasodilation, increased capillary permeability and smooth muscle contraction. A second class of mast cell mediators are newformed mediators that are derived from membrane phospholipids. As a result of mast cell activation, MCs also synthesize new mediators depending on the type of stimuli. These neosynthesized mediators include cytokines, such as TGF-β, IL-6 an TNF-α, and chemokines, such as CCL5 and CXCL8.[9]

Flowcytometric analysis of mast cell activation

The activation/degranulation of mast cells is measured by flow-cytometric analysis of the upregulation of specific activation markers such as CD63. Flow cytometry is a technique that is based on the light scattering and fluorescent characteristics of cells in suspension. When a (laser) light hits a cell, the light is deflected as forward scatter (FSC) and side scatter (SSC). The FSC is proportional to the size of the cell, while the SSC provides information on the granular content and complexity of the cell that is investigated. Combining the two enables to differentiate cell types in a heterogeneous population. For further identification, the cells can be stained with fluorescent probes, or fluorochromes, such as fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allophycocyanin (APC). These probes are often conjugated to an antibody that can be chosen depending on the properties that are investigated, for example FITC anti-human CD63 Antibody.[18]

Mast cell models

Isolation of tissue mast cells

Idealistically, studies on mast cell biology should be performed on cells isolated directly from human tissue. This technique is typically based on the mechanical and proteolytical digestion of skin tissue, but a gentler method of enzymatic digestion has been shown to generate mast cells of higher purity. However, working with freshly isolated tissue mast cells is limited due to the small number of cells that can be generated from one donor, the donor-to-donor variability, and the availability of donor tissue. Although *in vitro* techniques can provide larger and more homogenous populations of mast cells, the growing conditions cannot completely match their *in vivo* environment. This leads to modifications in the properties and responses of the cell compared to the original cell type. The isolation of tissue mast cells is therefore mainly reserved to study the exact properties of human mast cells.[19, 20]

Rodent mast cell lines

Prior to the establishment of human mast cell lines, research had mostly been done on cells of rodent origin. However, the characteristics of these cells cannot entirely be compared to those of humans and thus data obtained with rodent cells should be critically interpreted when translated to the human situation. While human mast cells, for example, only express three kinds of tryptases and one chymase, the rodent mast cells express several different proteases. Two of the most used rodent lines are shortly summed up below.[21]

The rat basophilic leukemia (RBL) cell line was established in the early seventies but was not yet suitable for degranulation studies until the creation of the histamine releasing subclone RBL-2H3. This cell line demonstrates a similar granular content to that of a mast cell and expresses the high affinity receptor for IgE, FcεRI. It has therefore been used to study the IgE-mediated degranulation of mast cells. Besides, an RBL line transfected with the human MRGPRX2 makes it possible to study the role of the human receptor. Whether the phenotype of the RBL cell line resembles basophils better than MCs is still debated. It depends on the context of what is studied if it can be considered a suitable model for MCs.[21-23]

A more recently developed, but not yet extensively used in research, rodent mast cell line is the NCL-2 line, stemming from bone marrow derived mast cells (BMMC) of Cinnamon coat-colored Nishiki mice. NCL-2 cells have been reported to grow without SCF but show a slow growth due to a non-constitutively active KIT receptor. They express the high-affinity IgE receptor FcεRI and are classified as mucosal MCs.[21]

Human mast cell lines

The first mast cell line that resembled the human mast cell and could be used for research purposes, was established in the late 1980's from the peripheral blood of a patient with MC leukemia. This cell line was named human mast cell-1 (HMC-1) and shows many characteristics of an immature mast cell, such as the (low) expression of histamine and tryptase.[24, 25] HMC-1 cells also express the KIT receptor (alias CD117) that, due to a mutation, is constitutively active. Therefore, there is no need for SCF, leading to a reduced laboratory cost. However, in allergy-related studies, the use of HMC-1 cells is limited due to the lack of expression of FcεRI on their surface.[21]

More than ten years after the establishment of the HMC-1 cell line, Kirshenbaum *et al.* developed two new human MC lineages, namely LAD-1 and LAD-2, originating from a patient with severe mast cell sarcoma/leukemia. This cell line does not have the c-kit activating mutation and therefore needs SCF for promoting growth. Another big difference with the HMC-1 cell line is the fact that LAD-1 and LAD-2 cells express a functional FcεRI on their membrane, thus making IgE-mediated degranulation of the cells possible.[21, 26]

The LUVA cell line, that arose spontaneously during the culture of peripheral blood $CD34^+$ cells from a donor with aspirin exacerbated respiratory disease, was established in 2011. This cell line expresses a functional FcεRI, enabling cross-linkage and subsequent degranulation. LUVA cells will proliferate faster when SCF is added to the growth medium, but they can survive without this growth factor even though they do not show the KIT mutation.[21, 27]

In 90 % of patients suffering from systemic mastocytosis (SM), a mutation in KIT, named D816V, is found, which might explain abnormal MC activation in these patients. To study this disease, two mast cell lines were developed: an SCF-dependent line expressing a functional FcεRI and an SCF-independent line expressing a D816V mutated KIT receptor, named ROSA$^{KIT\ WT}$ and ROSA$^{KIT\ D816V}$, respectively. These cell lines enable comparison of the signalling pathways between wild type and mutant forms of KIT. The ROSA$^{KIT\ WT}$ cell line originates from umbilical cord blood derived hematopoietic progenitor cells. With a doubling time of 24 hours, they show an advantage over LAD-2 cells that have a doubling time of approximately two weeks. In 2014, by transfecting the ROSA$^{KIT\ WT}$ cells with a lentiviral vector encoding KIT D816V, the ROSA$^{KIT\ D816V}$ cell line was obtained, five years after the development of ROSA$^{KIT\ WT}$. Like the parental ROSA$^{KIT\ WT}$, the ROSA$^{KIT\ D816V}$ also expresses a functional FcεRI.[21, 28]

In vitro differentiation of cultured human mast cells

Culturing primary human mast cells from peripheral blood has been practiced since the early eighties.[29] In 2014, Schmetzer *et al.* developed a novel technique to generate and culture human mast cells from peripheral CD34$^+$ stem cells.[30] Our research group has optimized this technique to obtain mast cell cultures from peripheral blood (peripheral blood cultured human mast cells, PBCMCs). Other sources for the isolation of progenitor cells are cord blood and bone marrow, which contain a much higher number of progenitor cells than peripheral blood. However, it must be taken into consideration that these three sources yield mast cells with different characteristics. Besides, cord blood and bone marrow samples are not as easily obtainable as peripheral blood.[31] The expense of culturing progenitor cells is relatively high, but the *in vitro* differentiation of human mast cells has been shown to yield cells that are representative for mature tissue mast cells.[30, 32, 33]

The passive MAT in chlorhexidine allergy

The passive mast cell activation test

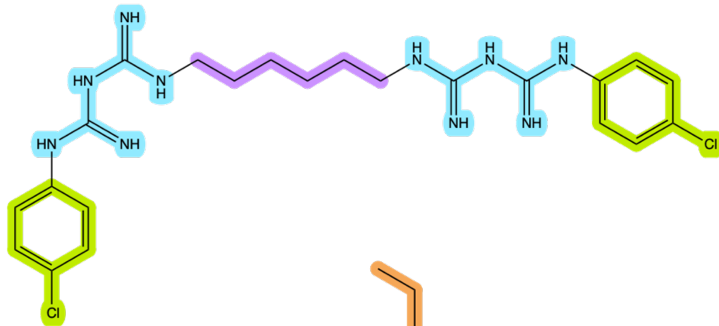
The utility of the pMAT, although nascent, seems promising. Bahri *et al.* developed and validated the mast cell activation test, using human blood-derived mast cells passively sensitised with patients' sera, for the diagnosis of food allergy.[34] Moreover, for peanut, pMAT conferred superior diagnostic accuracy compared with sIgE, SPT and BAT in distinguishing between patients with and without clinical reactivity. A similar approach by Santos *et al.* utilizes passively sensitised LAD2 cells – a mast cell line derived from a patient with mast cell sarcoma/leukemia[26] - in the diagnosis of peanut allergy. Even though the sensitivity of BAT was superior in this study, pMAT showed a high specificity and was able to discriminate between peanut allergic patients and peanut sensitised patients. Additionally, pMAT was able to confirm diagnosis in patients with nonresponding basophils.[35] A proof of concept performed by Elst *et al.* showed that the pMAT could also be extended to small drug molecules, such as chlorhexidine.[36]

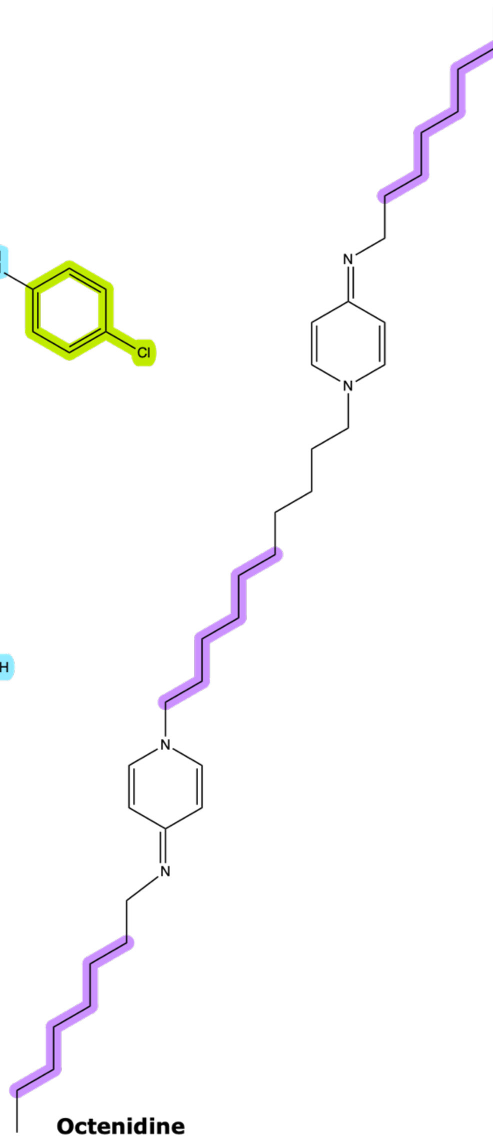
Chlorhexidine allergy

Chlorhexidine - discovered in the 1950s - is a widely used antiseptic, ranging from mouth washes to urethral gels and skin disinfectants.[37] The biguanide consists of two (p-chlorophenyl) guanide units that are linked together by a hexamethylene bridge (see Figure 1).[38] Its bactericidal function is caused by the ability of the agent to cross the cell wall and subsequently attack the cytoplasmic (inner) membrane of the bacteria. As the membrane is damaged, leaking of intracellular constituents occurs because of cell death. With rising concentrations of chlorhexidine, this leakage increases until the intracellular constituents start to coagulate.[39]

Although hypersensitivity to CHX is relatively rare, it has been shown that in up to 10 % of perioperative allergic reactions the culprit drug was found to be chlorhexidine. Nonetheless, due to the exposure to many different drugs and substances during a surgery, an allergy to CHX is often overlooked. This has led to the recommendation for all patients with a suspected perioperative and periprocedural allergic reaction to be tested for CHX-allergy. Currently there is no provocation model available for technical and ethical reasons, so diagnosis is based on skin testing and *in vitro* testing such as the quantification of sIgE and BAT.[37]

CHX-allergy has been proven to be IgE-mediated.[40] Recently, Mueller-Wirth *et al.*[38] suggested the IgE response to CHX to be polyclonal, meaning CHX-allergic patients may not only carry IgE specific to chloroguanide, but also IgE complementary to the biguanide or hexamethylene structure. These findings may be relevant for a potential cross-reactivity with disinfectants carrying similar functional groups.[38] Alexidine is an antiseptic that shows great similarity with chlorhexidine in structure. The difference lies in the two end groups where the biguanide carries ethylhexyl groups instead of chlorophenyl groups, as shown in Figure 1. Although sparsely used, the main use for alexidine is as an antiseptic in mouth washes and as a disinfectant in contact lens solutions.[41] Octenidine dihydrochloride (octenidine) is another antiseptic with some similarities in chemical structure to chlorhexidine. It is not a biguanide like chlorhexidine and alexidine but has a hexamethylene motif in common with both antiseptics (see Figure 1). The areas of applications include the skin, mucous membranes, and wounds.[42]





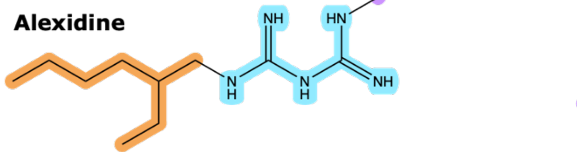


Figure 1: Chemical structures of chlorhexidine, alexidine and octenidine. The biguanide groups are highlighted in blue, the chlorophenyl groups in green, the hexamethylene motifs in purple and the ethylhexyl groups in orange.

Aim of this thesis

There is far to go from the encouraging proof of concept by Elst *et al.*[36] to enter in mainstream clinical use and much of our knowledge about cross-reactivity between drugs and related compounds is based on IgE binding and haptenic inhibition studies. Although interesting, these tests have their limitations as they do not always predict the clinical outcome. An evaluation of effector cell activation by ST and BAT could solve this issue. BATs have been shown to successfully document CHX-allergy and to study cross-reactivity with structurally similar compounds.[43, 44] Nevertheless, with traditional BATs some earlier mentioned weaknesses are encountered. Therefore, the aim of this thesis is to confirm the hypothesis that the pMAT might benefit diagnosis of IgE-mediated chlorhexidine allergy and to study cross-reactivity with ALX and OCT.

Materials and methods

Materials

The following materials have been used in this study.

Material	Manufacturer	Address	Category number
Histopaque-1077	Sigma-Aldrich	St. Louis Missouri, USA	10771
EasySep Human CD34 Selection Kit II	Stemcell Technologies	Vancouver, Canada	17856
MethoCult SF H4236	Stemcell Technologies	Vancouver, Canada	04236
Iscove's Modified Dulbecco's Medium	Thermo Fisher Scientific	Waltham, USA	12440-053
Insulin-Transferrin-Selenium	Thermo Fisher Scientific	Waltham, USA	41400-045
Bovine Serum Albumin Solution	Sigma-Aldrich	St. Louis Missouri, USA	9048-46-8
Pen Strep	Thermo Fisher Scientific	Waltham, USA	15140-122
Human SCF	Miltenyi Biotec	Bergisch Gladbach, Germany	130-096-695
Human IL-3	PreproTech	Cranbury, USA	200-03
Human IL-6	Miltenyi Biotec	Bergisch Gladbach, Germany	130-095-365
Human LDL	Stemcell Technologies	Vancouver, Canada	02698
2-mercaptoethanol	Thermo Fisher Scientific	Waltham, USA	21985-023

Table 1: The materials, with their respective manufacturer, address, and category number, used in the *in vitro* culture of PBCMCs.

Material	Manufacturer	Address	Category number
Tyrode's Salts	Sigma-Aldrich	St. Louis Missouri, USA	T2145
Human IL-33	PreproTech	Cranbury, USA	200-33
Purified Mouse Anti-Human IgE	BD Biosciences	San Jose, USA	555894
Chlorhexidine digluconate solution	Sigma-Aldrich	St. Louis Missouri, USA	18472-51-0
Dulbecco's Phosphate-buff-ered saline (10X)	Thermo Fisher Scien-tific	Waltham, USA	14200-067
CD117 APC (clone: 104D2)	BD Biosciences	San Jose, USA	333233
CD203c PE-Cyanine7 (clone: NP4D6)	Thermo Fisher Scien-tific	Waltham, USA	25-2039-42
CD63 FITC (clone: H5C6)	BD Biosciences	San Jose, USA	557288
Lyse/Fix Buffer (5X)	BD Biosciences	San Jose, USA	558049
Alexidine dihydrochloride	Sigma-Aldrich	St. Louis Missouri, USA	1715-30-6
Octenidine dihydrochloride	Alfa Aesar	Kandel, Germany	70775-75-6

Table 2: The materials, with their respective manufacturer, address, and category number, used in the activation of PBCMCs.

Methods

In vitro culture of peripheral blood cultured human mast cells

PBCMCs were cultured according to Cop *et al.* (2017) with slight modifications.[32] A buffy coat was isolated from 50 mL peripheral blood donated by healthy volunteers. Mononuclear cells were obtained by creating a density gradient using Histopaque-1077. $CD34^+$ cells were isolated from these mononuclear cells using the EasySep Human CD34 Selection Kit II according to the manufacturer's instructions. With this kit, the CD34 surface markers are recognized by an antibody and separated using magnetic particles and an EasySep magnet. The cells were washed and when they showed a minimal purity of 50 %, a colony-forming cell culture was started by adding the $CD34^+$ progenitor cells to a methylcellulose-based medium (MethoCult). This medium was enriched with IMDM containing 1 % ITS, 0.1 % BSA, penicillin (100 units/mL) and streptomycin (100 µg/mL). Additionally, the medium contained SCF (100 ng/mL), IL-3 (100 ng/mL), and IL-6 (50 ng/mL) to enhance the maturation of the progenitor cells into mature mast cells.[11] Finally, LDL (10 µg/mL), with 2-mercaptoethanol (55 µmol/L) in addition to LDL to prevent the oxidization of the lipoprotein, had been added to speed up the process of cytoplasmic granule formation.[30] At a concentration of 10^5 cells/mL, or less, the cells were then brought into a 6-wellplate and incubated in a humidified incubator with 5 % CO_2 at 37°C for 14 days. 300 µL of growth medium containing IMDM, supplemented with IL-3 (20 ng/mL), SCF (20 ng/mL) and IL-6 (50 ng/mL), was added on day 3, 7 and 10. After two weeks, the cells were transferred from the methylcellulose-based medium to a liquid IMDM medium, to which SCF (10 ng/mL) and IL-6 (50 ng/mL) had been added, at a concentration of $0,5\times10^6$ cells/mL. The medium was replaced weekly with fresh medium until the experiments were performed on the fourth or fifth week after the start of the culture. A graphical presentation of the culture protocol is shown in Figure 2.

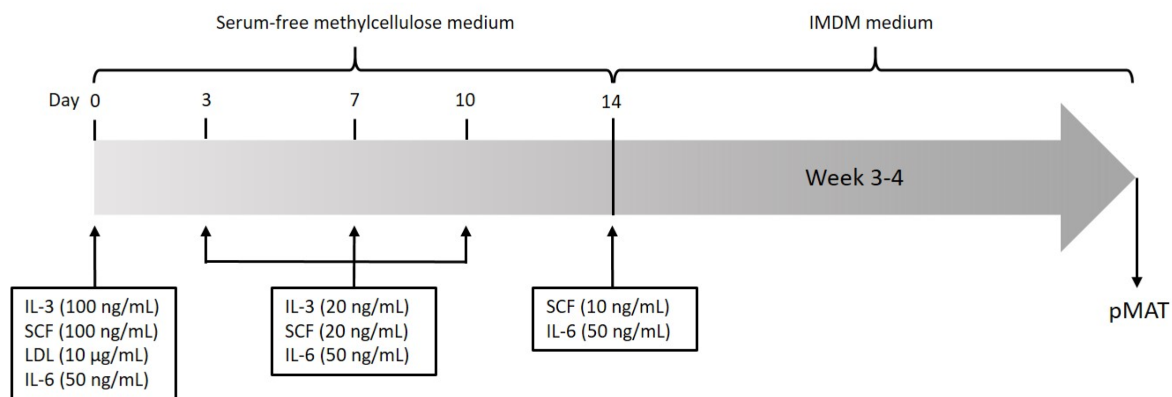


Figure 2: Graphical presentation of the culture protocol. At the start of the culture, the $CD34^+$ cells are brought into a serum-free methylcellulose medium supplemented with IL-3, SCF, LDL and IL-6. On day 3, 7 and 10 the cells are nourished with a mix containing IL-3, SCF and Il-6. After two weeks they are transferred to a liquid IMDM-medium containing SCF and IL-6 (figure based on Schmetzer *et al.*[30]).

Sera from patients with drug hypersensitivity and control individuals

Sera of 59 individuals, of which 39 patients with drug hypersensitivity and 20 control individuals, were selected. 13/39 patients had a confirmed diagnosis of perioperative hypersensitivity to chlorhexidine based upon a positive skin test, sIgE above the threshold of 0.35 kUA/L and a positive basophil activation test ≥5 %, as described.[45] sIge to CHX was quantified using ImmunoCAP assay (Phadia/Thermo Fisher, Uppsala), in which a chloroguanide is coupled to the cellulose as the substrate making it a CHX/GC ImmunoCAP. For 17 patients, the hypersensitivity reaction to CHX was confirmed by positive sIgE and either a positive skin test or positive BAT. 9 sera with CHX-sIgE >0.35 kUA/L were selected from patients that experienced POH but demonstrated negative STs and BATs to CHX. For five of these patients, the neuromuscular blocking agent rocuronium was indicated as being the culprit drug, for the other four the cause of the POH could not be identified. The control individuals contained a group of 15 exposed patients allergic to natural rubber latex (NRL) and a group of five healthy controls, of which all demonstrated a negative ST and sIgE to CHX.

Validation of pMAT in chlorhexidine allergy

In a 1:1 ratio, the PBCMCs were passively sensitised with serum, overnight, in a humidified CO_2-incubator. After centrifugation (500 g; 5 min; 20°C) of the cells, the cell pellet was suspended in pre-warmed Tyrode's buffer at a concentration of 5×10^5 cells/mL. The cells were pre-incubated with IL-33 (100 ng/mL) for 20 min at 37°C and subsequently stimulated with pre-warmed Tyrode's buffer (negative control), aIgE (1 µg/mL) (positive control), 2.8×10^{-6} mol/L CHX or 2.8×10^{-8} mol/L CHX and incubated for 20 min at 37°C. The concentrations of CHX were determined in dose finding experiments in a proof of concept done by our research group.[36] To stop the reaction, the cells were placed on ice. After centrifugation (500 g; 4°C), the supernatants were removed and the cell pellet was suspended in PBS with 0.1 % BSA and stained with monoclonal anti-human CD117-APC, anti-human CD203c-PE-Cy7 and anti-human CD63-FITC for 20 min at 4°C. Lyse/Fix buffer (1X) was added after staining and left to incubate for 20 min at room temperature. The cells were then centrifuged (500 g; 10 min; 20°C) and supernatant was subsequently aspirated. Finally, a washing step with PBS + 0.1 % sodium azide was performed and the mast cell activation was measured as surface upregulation of the lysosomal degranulation marker CD63.

Chlorhexidine cross-reactivity

Sera of ten patients with a confirmed allergy to CHX were chosen, of which all tested positive in the pMAT in our validation experiments. Additionally, 5 sera were selected from patients with a positive sIgE to CHX, but a negative ST and BAT. These patients tested negative in the pMAT. Finally, 5 healthy control individuals were included, that all demonstrated a negative ST, sIgE and pMAT to CHX.

The PBCMCs were passively sensitised and activated as described above, including four concentrations of alexidine and octenidine at 2.8×10^{-6}, 2.8×10^{-8}, 2.8×10^{-10} and 2.8×10^{-12} mol/L.

Flow cytometric analysis

For this project, 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 was analysed using Kaluza Analysis 2.1 software (Beckman Coulter, Brea, CA, USA).

To measure the surface upregulation of CD63 in our pMAT, a first selection was made based on FSC-height and FSC-area to select the single cells. A FSC-SSC plot was then used to distinguish the cells from debris. Since the cells were stained with anti-human CD117-APC and anti-human CD203c-PE-Cy7, the mast cells could be selected based on double positivity for CD117 and CD203c. This selection/gating strategy is shown in Figure 3. The fluorescence minus one (sample not stained with anti-human CD203c-PE-Cy7), was used to distinguish between $CD203^-$ and $CD203^+$ cells by setting the marker on the 95th percentile. Depending on the negative control, the upregulation of CD63 in samples activated with CHX was determined. The diagnostic threshold was set on ≥3 %, based on the expression of CD63 of the blanks of all sera used in the validation + 3.3 SD.[46, 47]

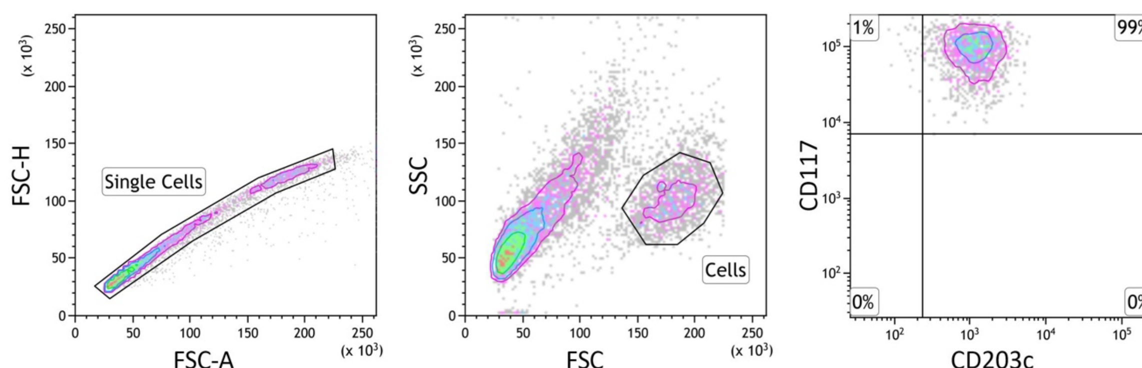


Figure 3: Gating strategy for mast cells. Single cells were gated based on the forward scatter (FSC)-H and FSC-A plot. Cells were gated based on FSC-side scatter (SSC). Mast cells were $CD117^+CD203c^+$.

Results

Validation of pMAT in chlorhexidine allergy

A total of 30 patients had their diagnosis of CHX-allergy confirmed by positive sIgE, along with either a positive skin test or a positive CHX-BAT. Table 3 shows that the specific IgE to CHX varied between 0.45 and 250 kUA/L and the total IgE between 12 and 2574 kU/L in these patients. As described above, the diagnostic threshold for both stimulation concentrations was set on ≥3 %. Considering this threshold, the pMAT showed to be positive for 12/13 (92.3 %) patients with positive STs and BATs (see Table 3 and Figure 4; ST^+, BAT^+, n=13). For a stimulation concentration of 2.8×10^{-6} and 2.8×10^{-8} mol/L, the mean (range) of CD63 upregulation for this group was 44 % (0-85) and 45 % (0-88), respectively (Figure 5). In the group with positive STs but no BATs, the pMAT was able to confirm CHX-allergy in 7/11 (63.6 %) patients. 3/6 patients with a negative ST but positive BAT also tested positive in the pMAT. As shown in Table 4 and Figure 4, the pMAT using sera from patients with a positive sIgE to CHX but negative ST and BAT did not demonstrate any upregulation of CD63. This shows that the pMAT can discriminate between clinically relevant and irrelevant sIgE results. For the 20 CHX-sIgE negative control sera, the percentage of CD63 upregulation by passively sensitised PBCMCs remained comparable to spontaneous expression (Figure 4; Tolerant Patients + Healthy Controls, Figure 5; Tolerant Patients + Healthy Controls, Table 5). PBCMCs that were not passively sensitised with patient's sera remained unresponsive to CHX (data not shown).

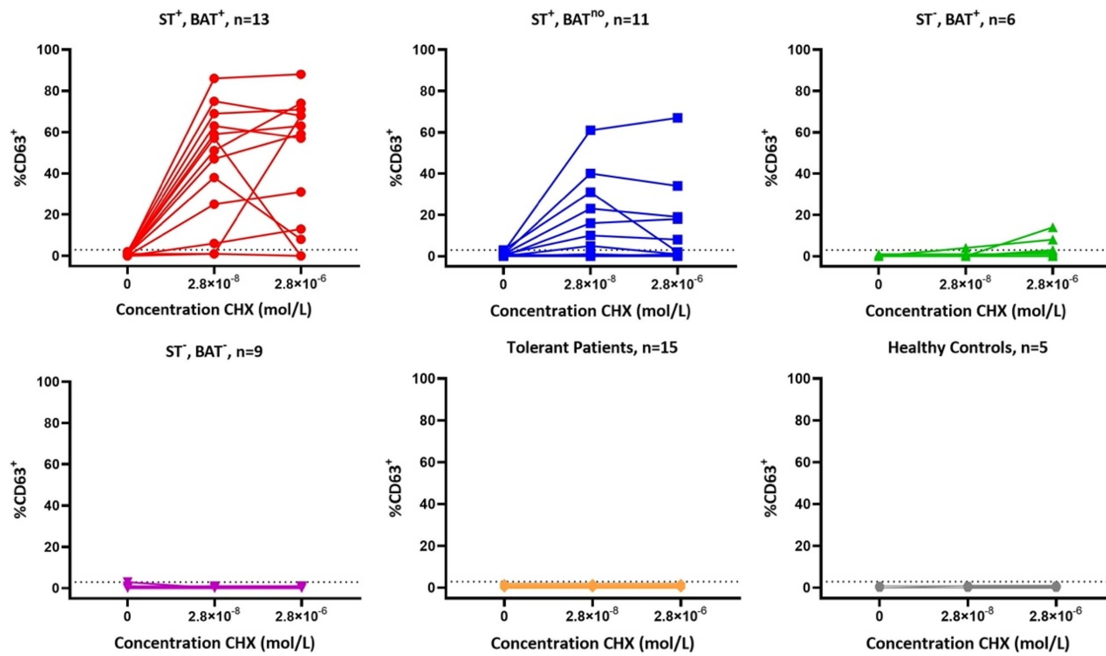


Figure 4: Individual plots of mast cell activation with chlorhexidine. Cultured human-derived mast cells were activated with chlorhexidine after passive sensitisation of the cells with sera of patients with positive skin test and basophil activation test (ST^{+}, BAT^{+}), sera of patients with positive skin test but without BAT data (ST^{+}, BAT^{no}), sera of patients with negative skin test but diagnostic positive BAT (ST^{-},BAT^{+}), sera of patients with positive IgE but negative skin test and basophil activation test (ST^{-}, BAT^{-}) or sera of healthy controls and exposed (tolerant) patients allergic to natural rubber latex. The dotted line represents the threshold of 3 %.

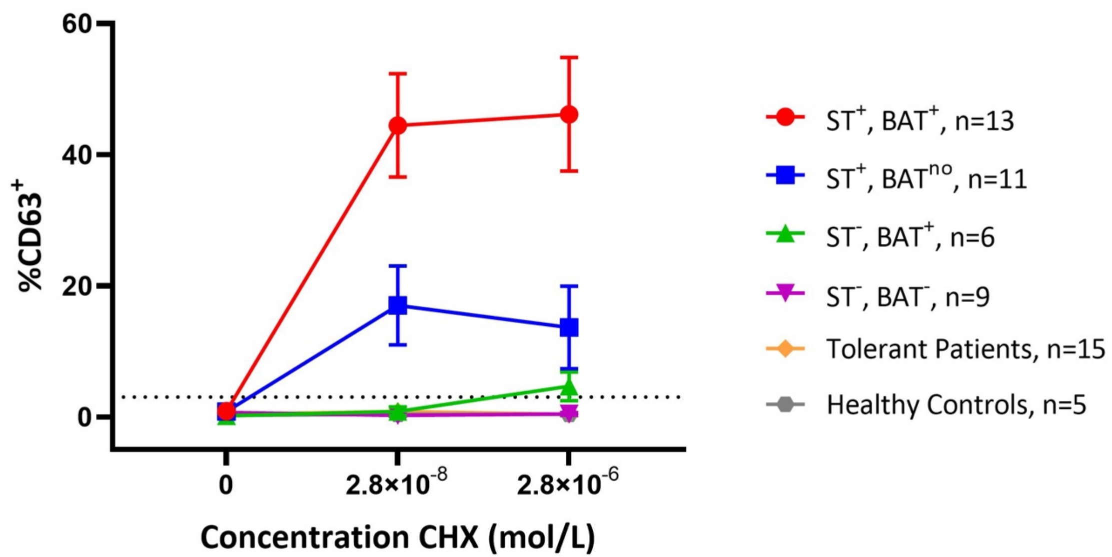


Figure 5: Combined plot of mast cell activation with chlorhexidine. Cultured human-derived mast cells were activated with chlorhexidine after passive sensitisation of the cells with sera of patients and control individuals. The results are expressed as mean + standard error of mean (SEM). The dotted line represents the threshold of 3 %.

P	Sex/ Age (yr.)	Total IgE (kU/L)	sIgE CHX[a] (kUA/L)	Delay (d.)	ST CHX	Severity grade	Acute Tryptase (ng/mL)[b]	Basal Tryptase (ng/mL)	BAT CHX[c] (%) (2.8×10^{-5} mol/L)	BAT CHX[c] (%) (2.8×10^{-6} mol/L)	pMAT[d] (%) (2.8×10^{-6} mol/L)	pMAT[d] (%) (2.8×10^{-8} mol/L)
1	M/63	195	3.28	5	+	3	ND	7.7	55	51	31	25
2	M/68	65	1.2	37	+	4	**41**	6	47	24	13	6
3	M/57	60	8.77	69	+	4	**34**	9.2	44	0	0	57
4	M/72	149	0.66	66	+	4	**59.6**	4.8	78	64	63	59
5	M/41	68	10.3	161	+	2	ND	6	55	65	88	86
6	M/62	582	18.5	18	+	3	21.9	3.4	64	57	68	75
7	M/62	252	8.38	14	+	3	6.5	4.2	43	34	71	69
8	M/19	103	7.85	147	+	2	ND	ND	57	50	74	51
9	F/56	106	6.7	60	+	3	**6.4**	3.35	81	84	59	47
10	M/52	66	1.14	35	+	4	**31.5**	3.6	54	66	8	38
11	M/21	900	1.68	56	+	3	**27.2**	10.7	71	80	57	63
12	F/68	232	0.46	455	+	1	ND	30.2	33	5	68	1
13*	M/59	131	0.45	13	+	3	**56.4**	3.5	30	27	0	1
14	M/67	190	3.5	177	+	3	ND	ND	ND	ND	8	10
15	M/66	175	28.8	192	+	3	ND	8.4	ND	ND	67	61
16	M/57	110	15.1	27	+	4	ND	6	ND	ND	19	23
17	F/42	167	4.94	115	+	3	**37**	5.6	ND	ND	34	40
18	F/9	59	5.6	21	+	No POH	ND	ND	ND	ND	18	16
19	M/45	41	1.43	70	+	3	ND	ND	ND	ND	2	31
20	M/70	248	2.11	140	+	3	ND	ND	ND	ND	1	5
21	M/69	12	0.66	36	+	3	**50.2**	5.5	ND	ND	1	0
22*	M/27	202	0.55	44	+	4	ND	5.6	ND	ND	0	1
23	F/12	430	2.42	Unk	+	No POH	ND	ND	ND	ND	0	0
24	F/24	119	5.43	289	+	No POH	ND	ND	ND	ND	0	0
25	F/42	568	0.48	98	-	1	4.9	2.8	14	0	0	0
26	M/35	704	250	136	-	1	ND	ND	25	0	14	0
27**	F/52	2574	9.32	10	-	3	3	13.1	18	0	3	0
28	M/73	117	1.83	569	-	4	ND	7.6	18	12	8	4
29***	F/35	122	6.39	107	-	3	**26.7**	3.5	21	1	1	1
30*	F/51	981	1.22	179	-	2	ND	5.4	8	0	2	0

Table 3: Patient characteristics, results of confirmatory testing and pMAT results. CHX is the culprit of the reaction for patients 1-30. *rocuronium as a second culprit, **cefazolin as a second culprit, ***natural rubber latex (NRL) as a second culprit. P, patient; yr., years; IgE, immunoglobulin E; CHX, chlorhexidine; sIgE, specific IgE; d., days between the index reaction and the confirmatory tests; ST, skin test; BAT, basophil activation test; pMAT, passive mast cell activation test; M, male; F, female; ND, not determined; POH, perioperative hypersensitivity; unk, unknown. [a]the threshold for sIgE positivity is set on 0.35 kUA/L; [b]acute tryptase levels in bold indicate mast cell activation (MCA) according to the consensus formula (tryptase level > 1.2×baseline level + 2 ng/mL); [c]the threshold for BAT positivity is set on ≥5 %; [d]the threshold for pMAT positivity is set on ≥ 3%. Severity grade according to Garvey L. H. *et al*. [48] Of note, a low titer of sIgE CHX still resulted in a significant effector cell activation in both BAT and MAT (patients 4 and 12).

P	Sex/ Age (yr.)	Total IgE (kU/L)	sIgE CHX[a] (kUA/L)	Delay (d.)	ST CHX	Severity grade	Acute Tryptase (ng/mL)[b]	Basal Tryptase (ng/mL)	BAT CHX[c] (%) (2.8×10^{-5} mol/L)	BAT CHX[c] (%) (2.8×10^{-6} mol/L)	pMAT[d] (%) (2.8×10^{-6} mol/L)	pMAT[d] (%) (2.8×10^{-8} mol/L)
31*	M/77	4848	1.71	44	-	2	8.7	14.3	1	0	0	0
32*	F/54	815	6.8	114	-	4	ND	2.4	0	0	0	0
33**	M/63	6079	24.8	121	-	4	**20**	4.9	0	0	0	0
34**	F/44	2483	2.17	72	-	4	**7.5**	2.2	1	1	1	0
35*	F/51	832	32	153	-	3	4.1	2.6	1	1	1	0
36*	M/63	3014	30	31	-	4	ND	4	2	3	0	0
37**	M/65	3217	11.1	41	-	4	**90**	6.9	0	0	1	1
38**	F/60	657	4.01	10	-	3	3	3.2	0	1	1	1
39**	F/51	188	7.07	36	-	3	**132**	4.6	0	0	0	0

Table 4: Patient characteristics, results of confirmatory testing and pMAT results. *the culprit of the suspected POH reaction is unknown **the culprit of the reaction is rocuronium. P, patient; yr., years; IgE, immunoglobulin E; CHX, chlorhexidine; sIgE, specific IgE; d., days between the index reaction and the confirmatory tests; ST, skin test; BAT, basophil activation test; pMAT, passive mast cell activation test; M, male; F, female; ND, not determined. [a]the threshold for sIgE positivity is set on 0.35 kUA/L; [b]acute tryptase levels in bold indicate mast cell activation (MCA) according to the consensus formula (tryptase level > 1.2×baseline level + 2 ng/mL); [c]the threshold for BAT positivity is set on ≥5 %; [d]the threshold for pMAT positivity is set on ≥3 %. Severity grade according to Garvey L. H. *et al.* [48]

C	Sex/ Age (yr.)	Total IgE (kU/L)	Severity grade	Culprit	Acute Tryptase (ng/mL)[a]	Basal Tryptase (ng/mL)	pMAT[b] (%) (2.8×10^{-6} mol/L)	pMAT[b] (%) (2.8×10^{-8} mol/L)
40	M/74	630	1	NRL	**31**	9.6	0	1
41	F/40	484	3	NRL	**49.9**	3.6	0	1
42	F/23	740	4	NRL	ND	ND	0	0
43	F/67	540	3	NRL	ND	12.2	1	1
44	F/56	310	2	NRL	24	19	0	1
45	F/45	67	3	NRL	ND	ND	0	1
46	F/54	142	3	NRL	**14.4**	6.2	1	1
47	F/30	114	1	NRL	ND	3.8	0	1
48	M/16	1551	3	NRL	ND	2.8	1	1
49	F/52	206	2	NRL	ND	ND	0	0
50	F/44	1096	3	NRL	ND	ND	2	2
51	F/27	101	1	NRL	ND	3.2	0	1
52	F/45	52	3	NRL	ND	5.8	0	0
53	F/44	773	1	NRL	ND	3.5	1	1
54	F/59	224	4	NRL	ND	16.6	0	0
55	F/49	3.88	NA	NA	NA	NA	0	0
56	M/38	511	NA	NA	NA	NA	1	1
57	F/50	85	NA	NA	NA	NA	0	0
58	M/56	19	NA	NA	NA	NA	0	0
59	F/23	5	NA	NA	NA	NA	1	1

Table 5: Control characteristics, results of confirmatory testing and pMAT results. C, control; yr., years; IgE, immunoglobulin E; CHX, chlorhexidine; pMAT, passive mast cell activation test; M, male; F, female; ND, not determined; NA, not applicable. [a]acute tryptase levels in bold indicate mast cell activation (MCA) according to the consensus formula (tryptase level > $1.2\times$baseline level + 2 ng/mL), [b]the threshold for pMAT positivity is set on ≥3 %. Severity grade according to Garvey L. H. *et al.* [48]

Chlorhexidine cross-reactivity

As shown in Table 6 and Figure 6, 4 out of 10 patients with a confirmed CHX-allergy showed responsiveness to ALX by upregulating the lysosomal degranulation marker CD63 upon activation with the drug. The percentages of degranulating MCs varied between 12 and 34 % for the corresponding concentration of 2.8×10^{-6} mol/L. At a lower concentration of 2.8×10^{-8} mol/L, only one serum showed to be positive in the ALX pMAT. For OCT, 3 out of 10 sera demonstrated an increased expression of CD63, with a variation of degranulating MCs between 4 and 22 % for a concentration of 2.8×10^{-6} mol/L. In contrast, all patients with a positive sIgE for CHX, but negative skin test, BAT and pMAT, showed no activation in the pMAT after stimulation with ALX or OCT. Sera from 5 healthy controls, with no CHX-sIgE and negative skin test and pMAT, demonstrated no upregulation of CD63. PBCMCs that were not passively sensitised with patient's sera remained unresponsive to all three antiseptics (data not shown). Representative individual plots are shown in Figure 7.

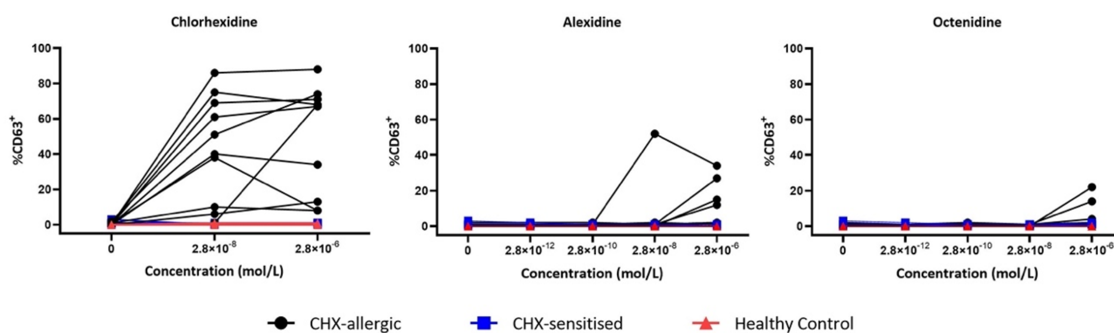


Figure 6: Mast cell activation with chlorhexidine, alexidine and octenidine. Cultured human-derived mast cells were activated with chlorhexidine, alexidine or octenidine after passive sensitisation of the cells with sera from 10 CHX-allergic patients with positive CHX sIgE, skin test and basophil activation test/pMAT CHX, sera from 5 patients sensitised to CHX (i.e., positive sIgE to CHX, but negative skin test and basophil activation test) or with sera from 5 healthy controls.

P/C	Number[a]	sIgE CHX[b]	ST CHX	pMAT CHX[c] (%) (2.8×10^{-6} mol/L)	pMAT ALX[c] (%) (2.8×10^{-6} mol/L)	pMAT OCT[c] (%) (2.8×10^{-6} mol/L)
P	2	+	+	13	0	0
P	5	+	+	88	27	14
P	6	+	+	68	2	2
P	7	+	+	71	0	0
P	8	+	+	74	15	22
P	10	+	+	8	2	1
P	12	+	+	68	34	4
P	14	+	+	8	1	1
P	15	+	+	67	12	2
P	17	+	+	34	0	1
P	33	+	-	0	0	1
P	34	+	-	1	0	1
P	35	+	-	1	1	2
P	38	+	-	1	1	1
P	39	+	-	0	1	1
C	55	-	-	0	0	0
C	56	-	-	1	0	0
C	57	-	-	0	0	0
C	58	-	-	0	0	0
C	59	-	-	1	0	0

Table 6: Patient/control characteristics, results of confirmatory testing and pMAT results. P, patient; C, control; sIgE, specific immunoglobulin E; ST, skin test; CHX, chlorhexidine; pMAT, passive mast cell activation test; ALX, alexidine; OCT, octenidine. [a]the number corresponds to the patient/control number in Table 3, Table 4 or Table 5, [b]the threshold for sIgE positivity is set on 0.35 kUA/L; [c]the threshold for pMAT positivity is set on $\geq$3 %.

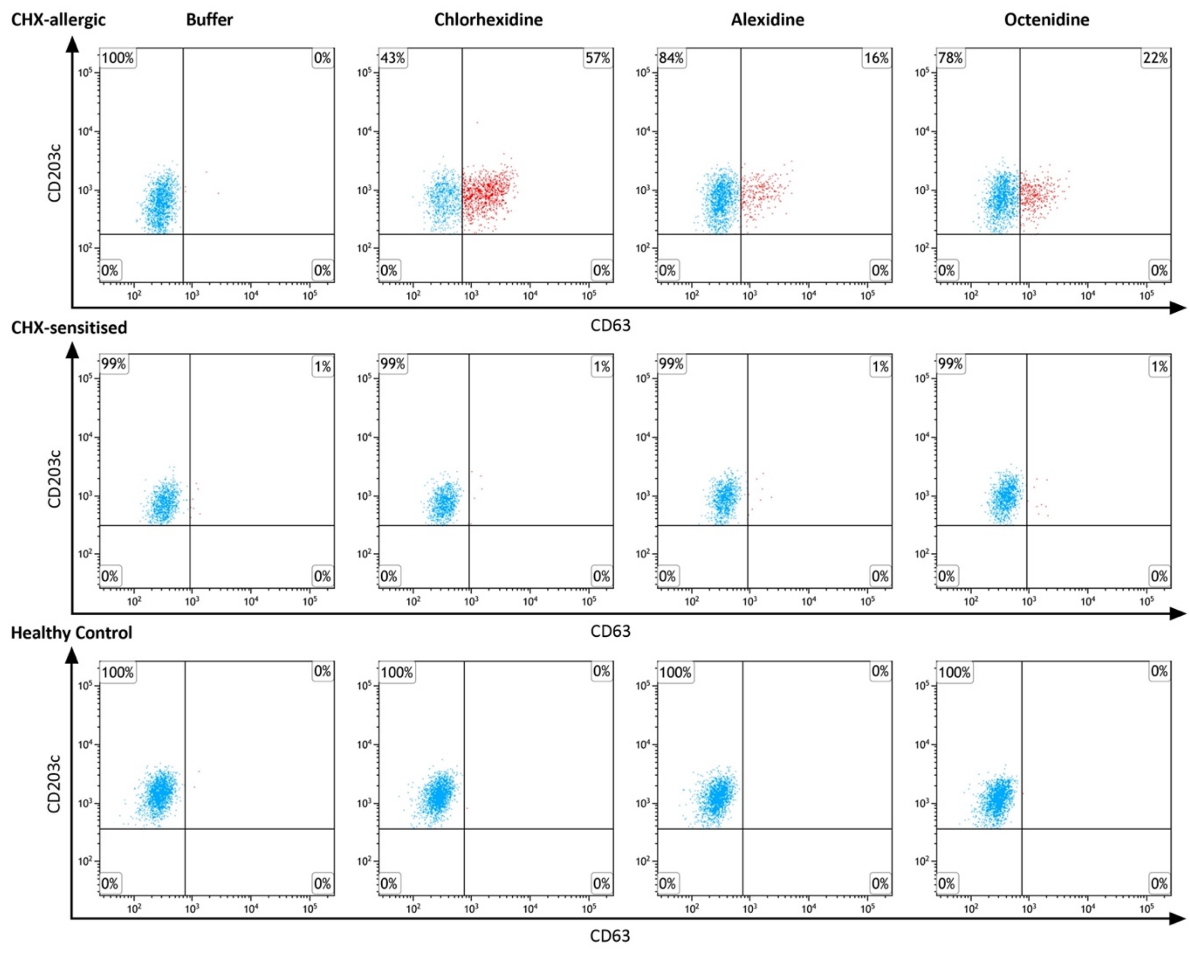


Figure 7: Representative plot of the mast cell activation test with chlorhexidine, alexidine or octenidine. Cultured human mast cells were activated with chlorhexidine, alexidine or octenidine 2.8×10^{-6} mol/L after passive sensitisation of the cells with serum of a patient with established CHX allergy, a CHX-sensitised patient, and a healthy control with a negative sIgE and negative skin test CHX.

Discussion

Diagnosis of an allergy to CHX is currently based on the results of skin tests and quantification of CHX-sIgE antibodies since there is no provocation model available.[37, 49, 50] However, these tests often show discrepancies, and both lack sensitivity and specificity. Because CHX has recently been proven to be an important cause of perioperative hypersensitivity and thus re-exposure for CHX-allergic patients must be avoided, an additional tool for diagnosis is required. Quantification of histamine release and flow-assisted activation of *ex vivo*-activated basophils has long been the focus since effector cell assays simulate the *in vivo* situation better than sIgE-binding assays do.[7] However, Garvey *et al.*[40] showed that the measurement of histamine release induced by CHX after passive sensitisation of IgE stripped basophils with patients' sera shows less sensitivity than the quantification of CHX-sIgE. Additionally, the use of BAT has its limitations, with the major ones being the need for fresh patients' blood and the non-responder status. For this reason, the use of BAT as a diagnostic test has not been implemented in mainstream use.

The passive mast cell activation test, or pMAT, has previously been proven by our research group to show great potential in diagnosing CHX-allergy.[36] Here it is shown that the pMAT constitutes a reliable substitute for BATs to diagnose CHX-allergy. Actually, comparison between the pMAT results of CHX-allergic patients with positive STs and BAT and the results of control patients, considering both stimulation concentrations, led to a sensitivity of 92.3 %, a specificity of 100 %, a positive predictive value of 100 % and a negative predictive value of 95.2 %. For most patients who did not receive a BAT and 3/6 patients who had negative STs, the pMAT also confirmed diagnosis. These findings show that the pMAT could be a reliable substitution for BAT, which aligns with the observations of Barhi *et al.*[34], who demonstrated the pMAT to show superior diagnostic results compared with traditional BAT in the assessment of peanut allergy. Due to a lack of sera from CHX-allergic patients (ST^+, CHX-$sIgE^+$) who had received a negative BAT or were non-responding in BAT, it was not possible to determine whether pMAT could show superiority when BAT failed to confirm diagnosis. Further analyses are therefore required to verify these findings, especially in these cases with equivocal or negative tests. Moreover, our MAT method demonstrates a high analytical sensitivity, as successful passive sensitisation was attained for titres of CHX-reactive sIgE as low as 0.45 and 0.46 kUA/L in the traditional ImmunoCAP assay. On the other hand, MCs sensitised with CHX-reactive sIgE antibodies obtained from patients with negative skin test, remained unresponsive to CHX. In other words, the pMAT shows to potential to discriminate between clinically relevant CHX-sIgE and irrelevant sIgE. Challenge testing would also add more rigor to the validity of the findings, however, currently there is no such validated safe CHX

challenge procedure. Nevertheless, it is believed that a witnessed, convincing reaction, in combination with the ST, sIgE and BAT outcomes, is a valid approach to explore the pMAT in CHX-allergy.[50]

In the first part of this thesis, it was shown that the pMAT could be a valid diagnostic tool in immediate drug hypersensitivity. The second part, covering potential chlorhexidine cross-reactivity with other structurally similar disinfectants, reveals that the use of cultured donor MCs reaches further than that. With a limited number of experiments, it was demonstrated that ALX and OCT can activate human MCs that were incubated with sera from patients with an established CHX-allergy. It is interesting to note that not all patients reacted to all three tested compounds. For some patients, the upregulation of the activation marker CD63 was restricted to CHX and ALX, or to CHX only. These findings show that the CHX cross-reactivity pattern is unpredictable. However, they do align with the observations by Mueller-Wirth *et al.* that the IgE response is likely to be polyclonal and extends beyond the chloroguanide parts and includes the biguanide and hexamethylene structures present in CHX, ALX and/or OCT.[38] The route of sensitisation is uncertain in the majority of CHX-allergic patients.

This application of pMAT is an interesting proof of concept due to the limitations of the techniques that are currently available to study cross reactivity. In ImmunoCAP and inhibition assays, the substrate is bound to a solid phase and the presence of sIgE in patients' sera is measured by its ability to bind to the substrate or the inhibition of its binding by the drug in question resp. Its weakness lies in the difficulties coupling the drug to the solid phase and in the fact that results of sIgE inhibition studies are not always predictive for the clinical outcome.[38] The latter was shown in a study where cisatracurium significantly inhibited rocuronium-sIgE in sera from rocuronium-allergic patients with negative cisatracurium STs and who were uneventfully exposed to the drug during anaesthesia.[51] STs, on the other hand, mirror the *in vivo* situation better. However, histamine release by skin MCs might not only result from IgE mediated reactions but could also be the result of the occupation of the MRGPRX2 receptor.[52, 53] To overcome these issues, the application of traditional, or direct, BAT was extended from a diagnostic tool in CHX-allergy, to the study of cross-reactivity with structurally related compounds including ALX and OCT.[38, 44] As mentioned earlier though, BATs come with some major limitations including the need of fresh viable cells and the non-responder status. The passive BAT, using stripped donor cells passively sensitised with patients' sera, are conducted by some groups to resolve some weaknesses encountered with traditional BAT. However, they are highly dependent on the donor and are usually less sensitive than direct BATs.[38]

In general, the PBCMCs used in our applications of pMAT are the result of a time intensive protocol and are not able to be maintained over longer time. The source of mast cells is therefore an important factor to be taken into consideration. In this regard, the use of a mast cell line expressing a functionally active FcεRI could be an alternative for better standardization, although in the context of passively sensitizing mast cells, the sole attempt with LAD-2 cells seemed unsuccessful.[54] Additionally, the pMAT is technically more difficult than BAT considering an overnight incubation of patients' sera. Neverthe-less, as our technique does not require fresh blood samples, it should ease collaborative multicentric studies with shipping of (historical) patient sera to the laboratory responsible for further analyses.

Conclusion

The pMAT, that uses donor MCs passively sensitised with patients' sera, is a reliable diagnostic of which the application extends beyond traditional proteinaceous allergens but might also involve small molecules such as drugs. Unlike *ex vivo* basophil activation assays, the pMAT enables deferred and more standardized batch analyses and ultimately translates easier into a clinically valuable diagnostic. Additionally, this study provides encouraging evidence that the pMAT can benefit exploration of cross-reactivity patterns. Although it is far to go from this proof of concept to more systematic use, collaborative studies involving clinical centers and centralized experienced laboratories can ease promotion and breakthrough of this attractive technique.

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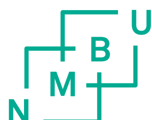

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