University of Southampton

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Measurement of mast cell and basophil activation *in vitro* as means for investigation of drug hypersensitivity

by

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Abstract

Allergic drug reactions to drugs are becoming more frequent throughout the world and are among the most common and serious form of immuno-pathological process in modern clinical medicine. These reactions can lead to life-threatening anaphylaxis which can occur within minutes of receiving the drug. Symptoms provoked by the explosive release of mediators from mast cells and basophils release may include itchiness, angioedema, difficulty breathing and circulatory collapse. There are few reliable laboratory tests available to identify those with drug hypersensitivity who may be at risk of anaphylactic shock, or even to establish if such a reaction has occurred. Confirmation of drug hypersensitivity by skin testing or oral challenge can put the patient at risk of serious reaction, and as such are expensive and performed at few centres.

The aim of the study was to seek to develop new tests for drug allergy based on assessment of mast cell or basophil activation either *in vitro* or *in vivo*. Patients were recruited who were suspected of having suffered an allergic reaction to medicine and who were undergoing skin testing or oral drug challenge. Blood was collected before and following the challenge, the nature of symptoms provoked were recorded, and clinical history and demographic data obtained. The activation of basophils *in vitro* in response to the drug was assessed by measurement of histamine release by immunoassay or up-regulation of CD63 by flow cytometry. In parallel studies, cells of a rat basophil leukaemia cell line (RBL-703-21) that had been transfected with the α chain of the human IgE receptor, were passively sensitised with serum and challenged with the drug implicated. The release of β-hexosaminidase in cell supernatants was measured to determine the degree of drug-induced cell activation. Mast cell tryptase was successfully purified from human lung tissue, and preliminary attempts made to optimise the detection of the basophil product basogranulin by dot blotting with specific antibodies BB1 and BB5, but time did not allow measurement of either of these markers of allergic reaction in the patients studied.

Skin weals were provoked in some 40% of patients following skin testing, though other symptoms were frequently recorded in the presence or even absence of a weal. These included hives, post-nasal drip, abdominal pain, vomiting, cough, shortness of breath, wheeze and stridor, and there was considerable heterogeneity in clinical responses. In patients with a positive test outcome on skin or oral challenge, raised levels of serum IgE specific to that drug were commonly observed (where the test was available). Increased expression of CD63 was provoked by *in vitro* challenge of basophils in a minority of patients who responded on *in vivo* challenge, but no histamine release into cell supernatants was detected where this was examined. Drug challenge of RBL-703-21 cells passively sensitised with patient serum resulted in the release of only a small quantities of β-hexosaminidase, and no evidence was found for direct activation of cells by the drugs themselves. There was, however, significantly higher net release of β-hexosaminidase from patients with positive skin reactions to the neuromuscular blocking agent rocuronium than in those who did not respond on skin challenge.

The reliable diagnosis of drug hypersensitivity remains a formidable challenge, and there is no ready replacement of skin or oral challenge of patients with drugs suspected of causing a reaction. The present study indicates some of the difficulties as well as ways forward in developing new laboratory tests. *In vitro* challenge of blood basophils or passively sensitised cells of mast cell or basophil-like cell lines hold some promise, but it will be crucial for the sensitivity of such procedures to be increased and optimised with a range of drugs. Assays for mast cell or basophil products in the circulation of patients experiencing reactions should also be valuable as diagnostic tools.

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Declaration of Authorship

I, Desiree Ludwig declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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I confirm that:

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3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

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List of abbreviations

BSA	Bovine serum albumin
CCR3	Chemokine receptor 3
CD63	Cluster of differentiation 63 (gp53)
CD203c	Cluster of differentiation 203c
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide
DPPI	Dipeptidyl peptidase I
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FcεRI	Fc-epsilon receptor I
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
HRP	Horse radish peroxidise
IDT	Intradermal test
IgE	Immunoglobulin E
IgM	Immunoglobulin M
IgG	Immunoglobulin G
MC_{TC}	Connective tissue mast cells (containing tryptase and chmyase)
MC_T	Mucosal mast cells (containing only tryptase)
NMBA	Neuromuscular blocking agent
NSAID	Nonsteroidal anti-inflammatory drug
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
PBS-T	Tween in PBS
PE	Phycoerythrin
RBL	Rat basophil leukaemia cells
SI	Stimulation index
SPT	Skin prick test
TEMED	Tetramethylethylenediamine
TMB	Tetramethlbenzidine
α-CCR3 PE	Monoclonal anti-human CCR3 antibody, PE conjugated
α-CD63 FITC	Monoclonal anti-human CD63 antibody, FITC conjugated

1. Introduction

1.1 Allergic drug reactions and anaphylaxis

Allergic drug reactions are occurring with increasing frequency and can have serious consequences. Around 10% of hospitalised patients have been reported to have had adverse drug reactions and about 7% may require ambulatory care [1, 2]. Allergic drug reactions can cause a range of symptoms such as angioedema, urticaria, and rhinitis, and the most common symptoms are itchiness, swelling, bronchospasm and headache. These reactions can rapidly become life-threatening, especially if the event occurs during surgery.

The aim of this study is to develop and validate new detection methods for drug hypersensitivity and anaphylaxis, focusing on investigation of allergic sensitivity. The primary approach will involve investigation of mast cell or basophil activation *in vitro*, and analysis of levels of specific biomarkers such as β–hexosaminidase, and histamine, that may be released from these cells in patients suffering allergic drug reactions.

1.1.1 Allergy

Allergy is a common disease involving hypersensitivity to antigens that would normally be tolerated in healthy individuals, such as those present in foods, drugs or insect venoms. The Nomenclature Review Committee of the World Allergy Organisation defined allergy as ‘a hypersensitivity reaction initiated by specific immunologic mechanisms’ [3]. Hypersensitivity was described as ‘objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons’. Coombs and Gell categorised hypersensitivity reactions into three types which were antibody-mediated and one type which was cell-mediated (Table 1.1) [4].

Table 1.1: Classification of hypersensitivity by Coombs and Gell

Antibody-mediated hypersensitivity			Cell-mediated hypersensitivity
Type I	Type II	Type III	Type IV
Immediate hypersensitisation IgE-mediated	Humoral cytotoxic immune response	Immune complex-mediated immune response	Delayed hypersensitisation T-cell mediated

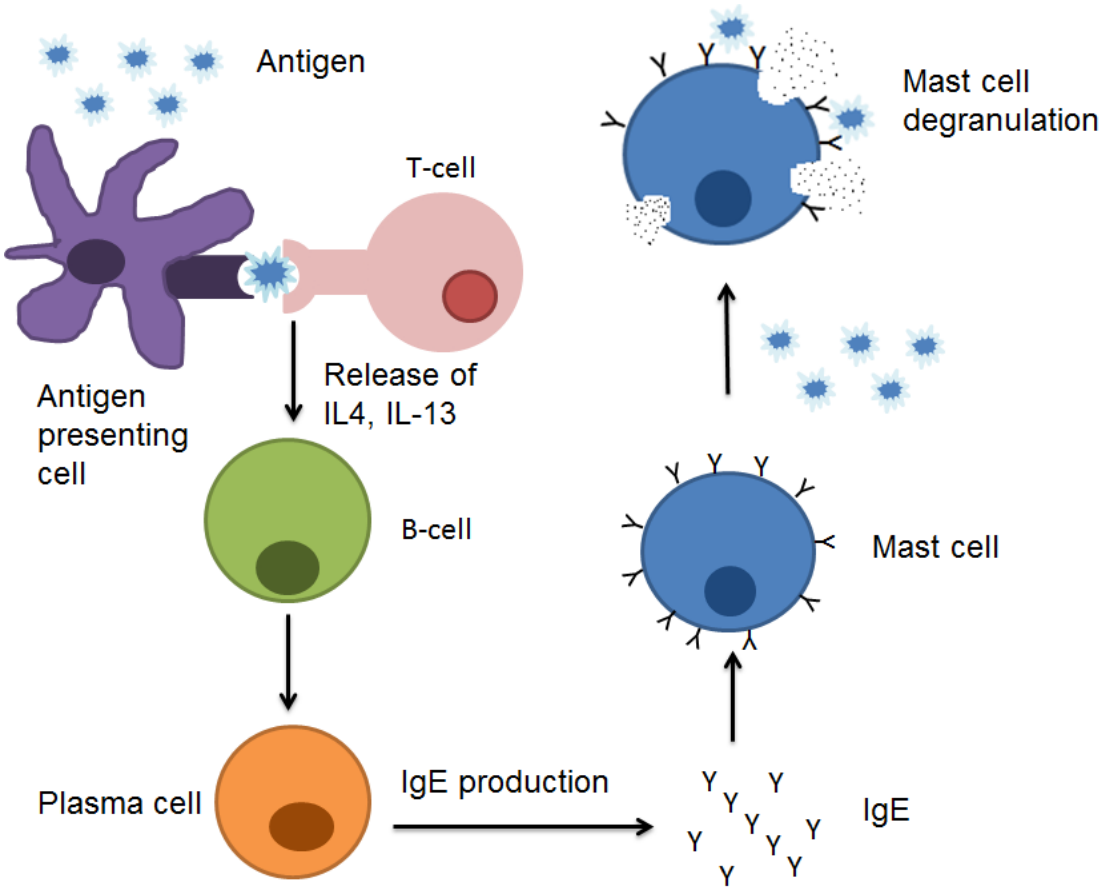

Figure 1.1: IgE mediated mechanism. The antigen connects an antigen presenting cell with a T-cell which triggers release of cytokines IL-4 and IL-13. Both cytokines activate B-cell which leads to IgE production in plasma cells. The specific IgE can bind to the mast cell through the FcεRI receptor and after second exposure the antigen binds to the specific IgE. The cross-linking of two specific IgE with the antigen triggers mast cell degranulation.

Immediate hypersensitivity involves a reaction mediated by immunoglobulin E (IgE). In 1967, Johannson et al. identified a new immunoglobulin class which was not reacting with existing immunoglobulin A, G, M or D [5]. The group found that the new immunoglobulin (Ig) had an molecular weight of 196,000 and in the blood of one patient who suffered from extrinsic asthma the level of this new immunoglobulin as fifteen times higher than average [6]. One year later, the newly discovered immunoglobulin was named IgE [7] and has a structure similar to the other immunoglobulins including both heavy and light chains with variable and constant regions (Figure 1.2 a) [8]. The group of Ishizaka discovered that IgE is involved in hypersensitivity. It was deduced that IgE was the only immunoglobulin that induced skin reactions after applying anti-IgE to the skin and observing the development of weals.

The type I mechanism can be divided into two stages: sensitisation and secondary exposure. The sensitisation step includes the first exposure to the allergen (Figure 1.1). The allergen is detected by antigen presenting cells and these cells activate Th2 lymphocytes. Th2 lymphocytes secrete IL-4 which induces antibody class-switching in B-cells to create antigen-specific IgE. B-cells require two steps for class-switching to occur; cytokines IL-4 and 13 send a signal, followed by ligation of B cell CD 40 by T cell CD40L [9]. Once IgE antibody has been generated the Cε3 domain binds to the α domain of the high affinity IgE receptor FcεRI (Figure 1.2 b) which is expressed on the surface of mast cells and basophils [10]. If the individual is then exposed to the allergen again, the allergen cross-links with the Fab region of IgE on the cell surface and induces degranulation. Mast cells and basophils release a range of mediators, including histamine, tryptase, β-hexosaminidase and basogranulin.

In some immune reactions, the cell is destroyed after the antibody is bound to it. This type of reaction is called antibody-dependent cell mediated cytotoxicity (ADCC) and belongs to the category of humoral cytotoxic immune response (type II) [11]. The drug can bind to proteins and leading to formation of an antigenic complex which causes IgG or IgM synthesis [12]. The cross-reaction of IgG or IgM with the antigenic complex leads to cell lysis and is usually mediated by FcγR or FcμR expressing on macrophages [13, 14]

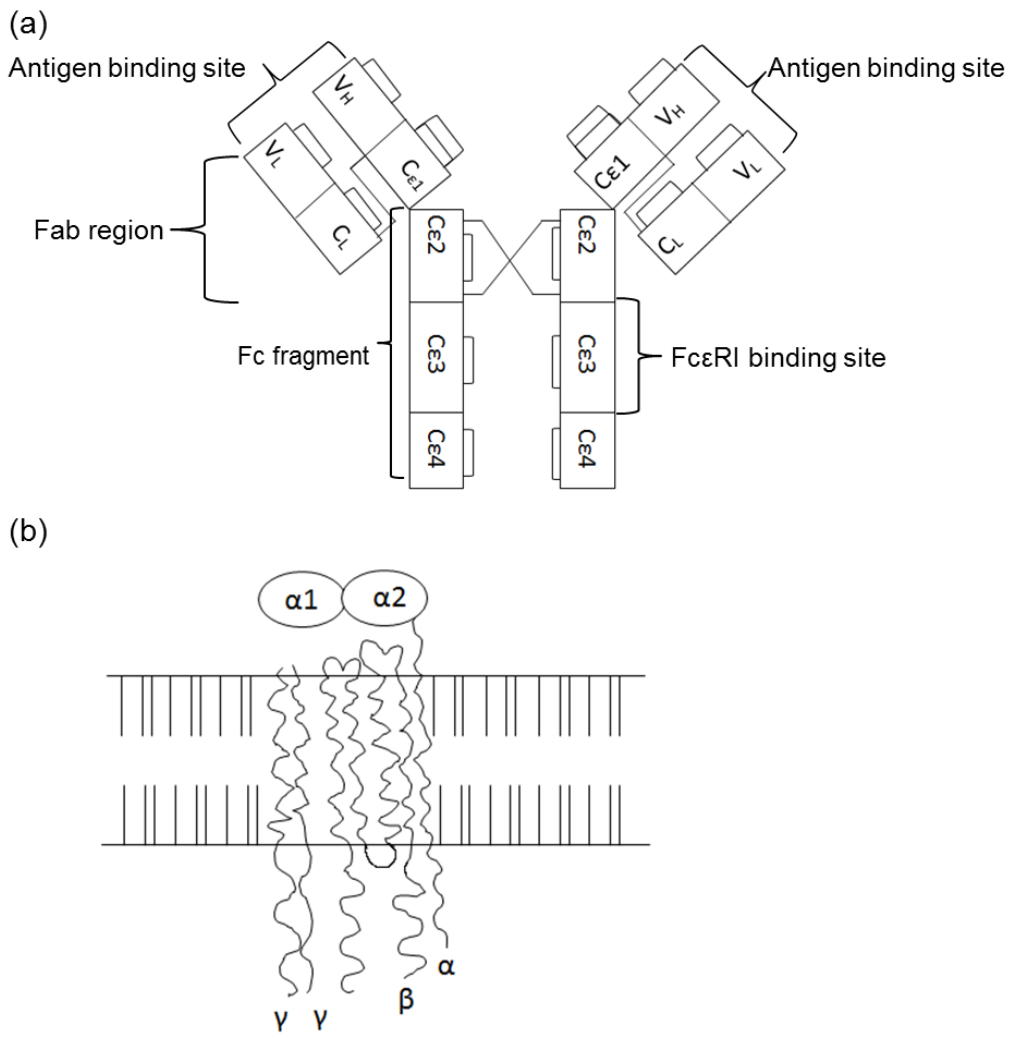

Figure 1.2: IgE and the FcεRI receptor. (a) The structure of IgE with Fab region, Fc fragment, antigen binding site and FcεRI binding site. (b) Schematic structure of high affinity IgE FcεRI receptor

Immune complex reactions (type III) are caused by antigen and antibody interactions that form aggregates in vessels and tissues. These immune complexes are recognised by Fcγ receptors on mast cells and other leukocytes and induce activation of these cells [11].

Type IV reactions also known as delayed hypersensitivity are antibody-independent [12]. A complex of macrophage cell membrane, antigens and helper T cells induce lymphocyte mitosis which release lymphokines such as IL-1β and TNFα. These lead to local inflammatory reactions that appear within 24 to 48 hours after exposure to the antigen [11].

The focus of the present study is drug hypersensitivity, particularly immediate hypersensitivity IgE-mediated (Type I) responses.

1.1.2 Adverse drug reactions and drug hypersensitivity

Adverse drug reactions (ADR) comprise many types of reactions that are caused by taking medication. The World Health Organization (WHO) defines ADR as 'any response to drug which is noxious and unintended, and which occurs at doses normally used in man for prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function' [15]. In 1977 Rawlins separated ADR into two groups: augmented reactions (type A) and bizarre reactions (type B) [16]. Type A drug reactions can occur in anyone, and can include side effects and drug interactions [12, 17, 18]. Drug reactions which appear in patients, with a certain predisposition e.g. a specific intolerance or hypersensitivity are included in type B. An Australian study by Miller et al. investigated the frequency of ADR over 6 months and found that 852 (10.4%) patients out of 8215 had a confirmed ADR as diagnosed by their general practitioners [19]. Drug hypersensitivity was in 11% of these cases the reason for the ADR. There are various drug groups which can cause drug hypersensitivity. The most common drug groups that can lead to allergic reactions are nonsteroidal anti-inflammatories and antibiotics (Table 1.2) [20-22]. However, some of these studies based their results on clinical history and did not confirm the allergen that caused the allergic reaction with either skin testing or *in vitro* testing. A study performed by Messaad et al. analysed 898 patients who had drug hypersensitivity diagnosed by clinical history and performed oral or injected drug provocation tests [23]. It was discovered that out of 1372 challenges performed only 241 were positive, suggesting that diagnosis of drug hypersensitivity based on the clinical history could lead to misdiagnosis and unnecessary avoidance of the suspected drug.

The prevalence of drug hypersensitivity and factors that could increase the likelihood of developing drug hypersensitivity are not well investigated. Kurt et al. investigated the prevalence and risk factors for drug hypersensitivity by analysing 1052 questionnaires

obtained from adults, and found that itchiness was the most commonly experienced allergic symptom (55 patients), followed by hives or urticarial complaints (53), angioedema (37), shortness of breath (32) and hypotension or loss of consciousness (21) [21]. Furthermore, the group analysed factors that may be associated with drug hypersensitivity reactions and noted a link between allergic rhinitis and eczema with any type of allergic reaction. Additionally, it was also found that angioedema was associated with eczema and systemic hypertension and shortness of breath was linked with asthma.

Table 1.2: Categories of drugs most commonly implicated as triggers of drug hypersensitivity

Most common drug hypersensitivity	Confirmation by clinical history or skin prick test or other *in vitro* tests	Country	Reference
Nonsteroidal anti-inflammatory drug (NSAID) (37%) β-lactam antibiotics (29.8%) Other drugs (18%)	Clinical history (44%), drug provocation testing (30.8%), skin testing (14.6%), and *in vitro* tests (10.4%)	Spain	Doña et al.[20]
NSAID (46%) Antibiotics (30%) Other drugs (25%)	Clinical history	Turkey	Kurt et al.[21]
β-lactam antibiotics (63%) NSAID (29%) Pyrazolones (five-membered-ring lactam) (10%)	Diagnosis was confirmed in 26% (No information about diagnosis method)	Spain	Gamboa et al.[22]

1.1.3 Anaphylaxis

Anaphylaxis is described as 'a severe, life-threatening generalized or systemic hypersensitivity reaction' [3]. The European Academy of Allergology and Clinical Immunology (EAACI) categorised anaphylaxis as nonallergic and allergic anaphylaxis [3]. Nonallergic anaphylaxis was described as a life-threatening reaction which is not caused by an immunological mechanism. The definition of allergic anaphylaxis encompasses all anaphylactic events that are triggered by an immunological mechanism such as with IgG immune complexes, immune cell-mediated mechanisms, or IgE-mediated [24, 25]. The EAACI executive committee subdivided the term allergic anaphylaxis into further two subclasses: IgE-mediated anaphylaxis or non-IgE-mediated allergic anaphylaxis [24] and these terms were adopted by Johansson et al. in their report of the nomenclature review in October 2003 [3]. Research by Tsujimura et al. have suggested that IgG1-mediated systemic anaphylaxis may be dependent on basophil activation and the generation of PAF, whereas IgE-mediated anaphylaxis may be dependent on mast cell activation in mice [26]. The extent to which there may be basophil activation in human anaphylaxis in the absence of extensive mast cell activation remains to be determined.

Under-diagnosis, under-reporting and variability in case definitions complicate the recording of anaphylactic events. The group of Peng et al. investigated the frequency, type and severity of anaphylactic reactions over ten years and analysed patients with a reported anaphylactic event at the UK General Practice Research Database [27]. The group found 897 patients had a computer-recorded diagnosis of anaphylaxis. Based on this result they estimated that 8.4 per 100 000 people per year in the UK are affected by anaphylaxis. In a 10-year study in the United States of America, there was a higher incidence estimated with 49.8 cases per 100,000 people per year [28]. Furthermore, the American group also found that the annual incidence rate was increasing over the ten years at an average of 1.2 per 100,000 people per year. These results deviate from the findings of the UK study and differences in study designs, criteria and codes for an anaphylactic event in the database could be the reason for the difference.

Triggers and symptoms of anaphylactic events

In a study of 2,012 anaphylactic events in Central Europe, insects (50.4%) were the most common eliciting agent of anaphylactic shock, followed by food (24.3%) and drugs (16.7%) [29]. Painkillers and antibiotics were the most frequent agents implicated in anaphylactic

reactions (Table 1.3). Similar observations were made by Brown et al. [30]. A study by Harboe et al. investigated 83 patients who suffered from anaphylactic shock induced by drugs [31]. The most frequent clinical symptom was bronchospasm with 78.3% patients affected. Systolic blood pressure (< 60 mmHg) was seen in 53 cases (63.9%) and hypoxemia (saturation < 90%) as a clinical symptom was reported in 41 cases. Skin reactions (rash or angioedema) were described in 52 cases. A French study by Laxenaire et al. investigated anaphylactic events during anaesthesia, and found that in 477 patients cardiovascular symptoms (73.6%) were the most common anaphylactic symptoms followed by cutaneous symptoms (69.6%), bronchospasm (44.2%), angioedema (11.7%) and cardiac arrest (4%) [32]. A bigger study performed by a consortium of 79 specialised allergy clinics in Germany, Austria and Switzerland analysed 2012 severe and systemic anaphylactic reactions which occurred in less than 12 months showed that skin was the most affected organ with 84% affected [29]. Second was the cardiovascular system with 72% and third with 68% the respiratory system. It was also found that patients for whom food and drugs elicited anaphylaxis were more likely to have respiratory symptoms than circulatory symptoms.

Table 1.3: Categories of medication most commonly implicated as triggers of anaphylactic reactions

Common drug agents of an anaphylactic reactions	Study region	Reference
Analgesics (44.5%) Antibiotics (20.2%) Local anaesthetics (12.8%) Others (5.7%)	Central Europe	Worm et al. [29]
Antibiotics (58%) NSAID (12.8%) Narcotic (4.4%)	Australia	Brown et al.[30]

1.2 Mast cells and Basophils

Basophils and mast cells share similarities such as expression of the αβγ2 form of FcεRI [33] which binds IgE with high affinity [34]. This feature enables both cell types to play a central role in inflammatory and immediate allergic reactions. The two cell types will be considered separately in the following sections.

1.2.1 Mast cells

In 1878, Paul Ehrlich gave the first description of mast cells and basophils in his doctoral thesis and since then understanding has increased greatly as to how mast cells develop and differentiate [35]. Kempuraj et al. identified that cells with the mast cell phenotype arise from CD34+ cell progenitors in the presence of stem cell factor and IL-6 *in vitro* [36]. Irani et al found also that stem cell factor is an important cytokine for mast cell differentiation [37]. The group investigated foetal liver cells treated in culture with recombinant human stem cell factor (rhuSCF) and found that the cells that developed were tryptase-positive suggesting that they are mast cells. It was shown that in the presence of rhuSCF the number of c-kit positive cells increased over time [37]. Kirshamer et al. found that a cell population positive for CD34, c-kit and CD13 (CD34+/c-kit+/CD 13+) on the cell surface differentiated into a culture comprising 90% mast cells [38]. In addition, the group found that CD34+/c-kit+/CD 13+ subpopulations had increased FcεRI expression compared to the cell population positive for CD34 und c-kit but negative for CD13 (CD34+/c-kit +/CD13-) cells population *in vitro*. Mast cells in culture differentiated from CD34+/c-kit+ progenitor cells and had FcεRI expression on the cell surface.

Mast cell heterogeneity

Human mast cells have been categorised into two major subtypes: those which contain the neutral proteases tryptase and chymase (named MC_{TC}) and those which contain tryptase alone (named MC_T) when examined by immunohistochemistry [39]. The group of Irani examined mast cells in skin, lung and small intestine, and reported that approximately one third of tryptic protease activity and two thirds of chymotryptic protease activity was located in skin mast cells and in the submucosa tissue of the small intestine. In contrast, only tryptic activity was found in mast cells which are located in mucosa tissue. The group also showed that MC_T mast cells were present in the lung epithelium, lumen of bronchioles and bronchi, but no MC_{TC} mast cells were present. Similar findings were replicated three years later by the same group and it was reported that it is predominantly MC_T mast cells that are located in the lung, whereas skin mast cells were of the MC_{TC} phenotype [40]. Therefore, mast cells present in the mucosa of the respiratory and gastrointestinal tracts (which are likely to have a role in induction of mucosal inflammation) contain the protease tryptase but not chymase

[30]. It was also found that 90% of the mast cells in heart tissue contain tryptase and chymase [41].

Cathepsin G can also be stored in mast cells and has been found to be present in MC_{TC} cells in the skin and small intestine, but not in MC_T cells in the human lung [42]. Irani et al. investigated the location of the mast cell protease carboxypeptidase in skin, lung and gastrointestinal tissue by immunohistochemistry, and found that was stored predominantly in MC_{TC} cells and only 2% of carboxypeptidase was located in MC_T cells [43].

Activation of mast cells

Mast cells can be activated by various substances directly without cross-linking the FcεRI receptor, and release a range of mediators (Figure 1.3). However, in this section only a selection of these substances will be discussed.

IgE-dependent mast cell activation

Mast cell activation can be induced by cross-linking the high affinity IgE receptor on the mast cell surface. Human lung mast cells show a bell-shaped histamine release response with IgE dependent stimuli such as antibody specific for IgE [44]. Addition of anti-IgE can lead to activation of all tissue mast cells [45]. Rees et al. found that anti-IgE triggers mucosal mast cell activation faster than intestinal muscle mast cells. However, both mast cells types released histamine after stimulation with anti-IgE [46]. The β-adrenergic receptor agonist salbutamol inhibited histamine release after pre-incubation with the agonist and following stimulation with anti-IgE in human lung mast cells. The group of Church et al. found 39% inhibition of histamine release at a concentration of 10 µg of salbutamol [47].

Yamaguchi et al. investigated the relationship between IgE and FcεRI expression on mouse mast cells and found that the expression of FcεRI was increased when isolated mouse mast cells where incubated with mouse monoclonal IgE antibody [48]. In contrast, incubation with IgG2a antibody was not associated with an increase in FcεRI expression on mouse bone marrow derived mast cells. A study by Krohn et al. showed that human mast cells from peripheral blood (from allergic asthmatics) incubated with IL-4 and various recombinant human immunoglobulin E (rhuIgE) concentrations have after 2 weeks an increase of IgE-FcεRI density on mast cells [49]. Similar results were found by the group of Frandsen et al. who investigated the influence of IgE on cultured human mast cells [50]. It was found that human mast cells isolated from peripheral blood and incubated with different IgE concentrations on the last 10 days of the maturation step had an increase in FcεRI expression that was dependent on IgE concentration. Moreover, it was found that human

mast cells cultured with IgE and activated with anti-IgE released histamine in a dose-dependent manner. The group also showed that there was a decrease in histamine release at IgE concentrations above 250 ng/ml. In contrast, release of PGD_2, tryptase and chymase seem to not be affected by IgE concentration. These results seem to suggest that IgE is not only involved in mast cell activation it might be also involved in stability of FcεRI expression on the cell surface. Therefore, IgE levels may be related to the severity of an allergic reaction.

IgE-independent mast cell activation

Benyon et al. have reported histamine release by human skin mast cells after challenging with calcium ionophore A23187, compound 48/80, poly-L-lysine, morphine and substance P [51]. All of these were stimuli for histamine release from mast cells from human breast and skin tissue as well as from human infants foreskin tissue [45, 51]. However, it was found that the 1-4 peptide of substance P induced less histamine release from skin mast cells than the 4-11 peptide of substance P [45, 51]. Additionally, substance P was found to induce concentration-related histamine release until a plateau was reached at 30 to 100 µM [51]. 48/80 showed similar patterns to substance P and skin mast cells challenged with poly-L-lysine at a concentration of 10 µM also reached a maximum histamine release after 10 s. No cytotoxic effects were noted at the highest concentrations employed of substance P (30 µM), poly-L-lysine (10 µM), and compound 48/80 (30 µg/ml) as indicated by pre-incubating cells with the metabolic inhibitors 2-deoxy-D-glucose and antimycin A. The narcotic analgesic morphine at a concentration range of 10 to 1000 µmol/l can also induce histamine release from skin mast cells [45, 51]. Maximal quantities of histamine were released in less than 30 s., and this could be inhibited by treating the skin mast cells with the morphine antagonist naloxone. However, substance P, compound 48/80, poly-L-lysine and morphine did not induce any histamine release from lung, adenoid, tonsil or colon mast cells [45]. In contrast, calcium ionophore has been found to trigger histamine release from all mast cells examined [45, 51]. Lowman et al. investigated the activation of mast cells by formyl-methionyl-leucyl-phenylalanine (FMLP) at a concentration range of 0.1-100 µmol/L and found no histamine release from human skin, lung, adenoid, tonsil or colon mast cells [45].

IgG induced mast cell activation (through the FcγRIII receptor) seems to be involved in anaphylaxis in mice but the involvement of IgG and the Fcγ receptors for mast cell activation in humans is not completely understood [25]. Miyajima et al. have investigated the ovalbumin-induced IgG1-dependent anaphylaxis in mice which had no expression of FcεRI, or no expression of either FcεRI and FcγRI/III, or which lacked mast cells altogether. Their findings suggested that IgG1 antibodies can induce passive systemic anaphylaxis. Human

mast cells can also express FcγRI and FcγRII [52], and Okayama et al. have shown that human mast cells cultured from CD 34-positive progenitors have increased expression of FcγRI after IFN-γ treatment but not an increase in FcεRI, FcγRII or FcγRIII receptor expression [52]. IFN-γ treated mast cells were found to release histamine following anti-FcγRI challenge and there was up-regulation of mRNA for certain cytokines.

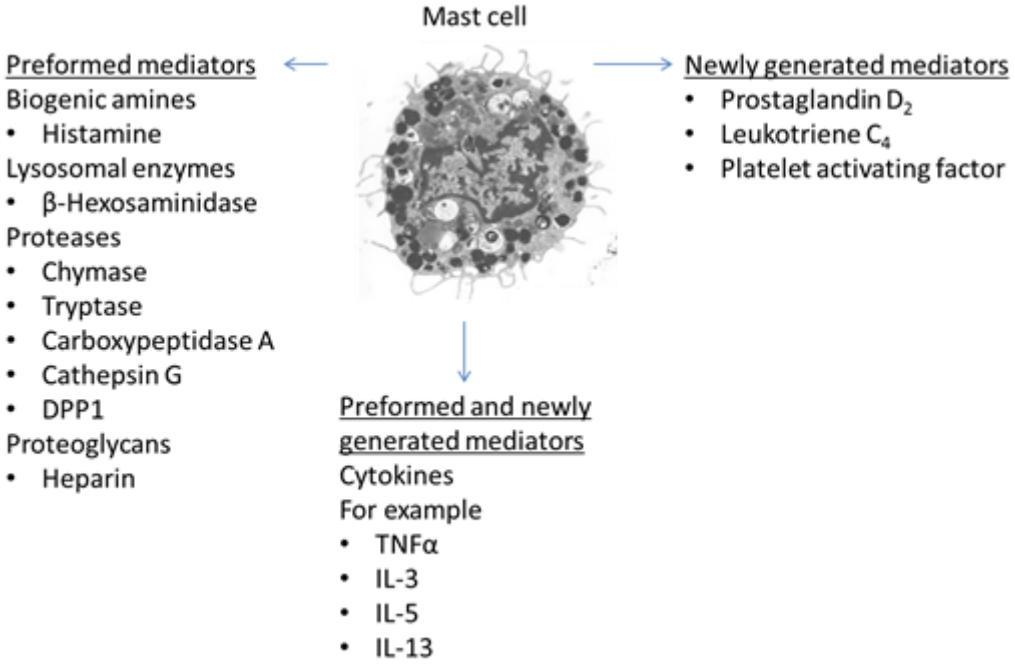

Figure 1.3: Mediators released after activation of mast cells. Mast cells can release preformed and newly generated mediators, and some mediators which are both preformed and newly generated.

Table 1.4: Tryptase subtypes. Adapted from Caughey et al. [53].

Variant	Gene	Nature
α–tryptase	TPSAB I	Soluble
βI–tryptase	TPSAB I	Soluble
βII–tryptase	TPSB 2	Soluble
βIII–tryptase	TPSB 2	Soluble
γ–tryptase	TPSG I	Membrane anchored
δ–tryptase	TPSD I	Truncated

1.2.2 Basophils

Like mast cells, basophils also develop from haematopoietic CD34-positive progenitors which are present in bone marrow. Interleukin 3 (IL-3) has been found to be an essential factor for basophil differentiation from CD34-positive progenitors [54-57]. Valent et al. showed that cells from human bone marrow incubated with IL-3 for 3 hours can give rise to basophils. It was also found that cultures which were exposed for longer periods to IL-3 contained mainly eosinophils [56]. Mononuclear cells which were isolated from umbilical cord blood also differentiated after incubation with IL-3 to basophils [54]. Kepley et al. identified these cells by using the basophil-specific antibody 2D7. The group also found that cell culture which was only incubated for 3-4 hours with IL-3, washed and re-suspended in IL-3 medium had the highest amount of histamine and FcεRI expression. The c-kit receptor plays an important role in mast cell differentiation so it might also influence basophil differentiation. Fuereder et al. reports that blood basophils isolated from non-allergic donors do not express the receptor [58]. However, Kirschenbaum et al. investigated the effect of IL-3 and SCF on CD34-positive pluripotent progenitor cells which were isolated from bone marrow by an immunomagnetic method [55]. Cell cultures incubated with IL-3 and SCF showed an increase in basophil number after 1 week and this peaked at 3 weeks. Treatment of CD34-positive progenitor cells with SCF alone resulted in just small numbers of basophils. These findings and the observation that c-kit receptor is absent from blood basophils suggests that the pattern of receptor expression may change over the differentiation process.

Basophil activation

Like mast cells, basophils can also be activated through the FcεRI receptor in an IgE-dependent mechanism or by various agents in IgE-independent mechanisms. Basophil degranulation can result in release of preformed mediators, newly generated mediators or both (Figure 1.4). However, in this section only a selection of these agents that can lead to direct basophil degranulation will be described.

IgE-dependent basophil activation

Cross-linking of FcεRI-bound IgE by antigen or anti-IgE can lead to basophil degranulation. A study by Mochizuki et al. showed that isolated blood basophils release histamine with a bell-shaped curve after incubation with anti-IgE [59]. The group showed that the basophil product basogranulin was also released in a bell- shaped curve in response to anti-IgE. Warner et al. found that anti-IgE can induce release of leukotriene C_4 (LTC_4) as well as histamine from isolated blood basophils [60]. However, IgE seems to have key roles not only in activation of basophils, but can also regulate expression of FcεRI receptors. The work of Malveaux et al. identified that the total number of FcεRI receptors correlated with serum IgE

levels in 26 blood donors [61]. In atopic subjects, it was found that the FcεRI expression on basophils was higher than in nonatopic controls and correlated with serum IgE concentration [62]. However, the group of Sihra et al. found that also the FcεRI expression on monocytes is associated with the IgE concentration in the patient serum. [63]. The group of Saini et al. investigated serum IgE levels and FcεRI receptor expression in various diseases including atopic asthma, dermatitis, hypereosinophilia, hyper–IgE syndrome parasitized hosts and IgE myelecoma. The group found that IgE and FcεRI receptor expression on basophils correlated with the serum IgE levels in all of these diseases. This study suggests that IgE not only regulates FcεRI expression on basophils in atopic allergic diseases but also in other diseases. However, all this supports the hypothesise that there is an association between serum IgE levels and the density of FcεRI receptor on basophils, and this association could help to identify patients with a higher risk of anaphylaxis.

IgE-independent basophil activation

Calcium ionophore triggers degranulation of basophils as well as of mast cells. Mochizuki et al. showed a dose dependent histamine release after incubation of blood basophils with 0.01 to 1 µM calcium ionophore [59]. The greatest amount of histamine release was seen with 1 µM. Additionally, the group showed that anaphylatoxin C5a induced concentration-dependent basophil activation. Schulman et al. investigated the activation of C5a in basophils and mast cells and found that C5a induced degranulation in basophils but not in mast cells [64]. The group examined the effects on both skin and lung mast cells, and no release of histamine, LTC_4 or PGD_2 was detected. Mochizuki et al. showed that fMLP induced basophil activation and led to dose-dependent histamine release with the highest release at 1 µM [59].

Chirumbolo et al. has demonstrated basophil activation in response to different stimuli is associated with up-regulation of CD63 and CD203c membrane expression as detected by flow cytometry [65]. Calcium ionophore induced up-regulation of CD63 and CD203c expression in a similar manner. In contrast, fLMP induced full CD63 up-regulation in the first minute, but expression of CD203 increased more slowly and reached a plateau after 3 minutes.

Basophils express not only FcεRI, the high affinity IgE receptor, but also the low affinity FcγRII receptor. A study by Anselmino et al. showed that human blood basophils express FcγRII on their cell surface [66]. These authors reported that only complexes or aggregated IgG can bind to the FcγRII receptor on human blood basophils. Research by Tsujimura et al. has identified that at least in a mouse model there may be a form of anaphylaxis that is mediated primarily by basophil activation that is distinct from that mediated primarily by mast

cells [26]. IgG1-ediated systemic anaphylaxis was proposed through FcγRII and FcγRIII which was dependent on basophil activation and the generation of PAF, whereas IgE-mediated anaphylaxis was dependent on mast cell activation. The extent to which there may be basophil activation in human anaphylaxis in the absence of extensive mast cell activation remains to be determined and requires better means for assessing basophil activation in humans.

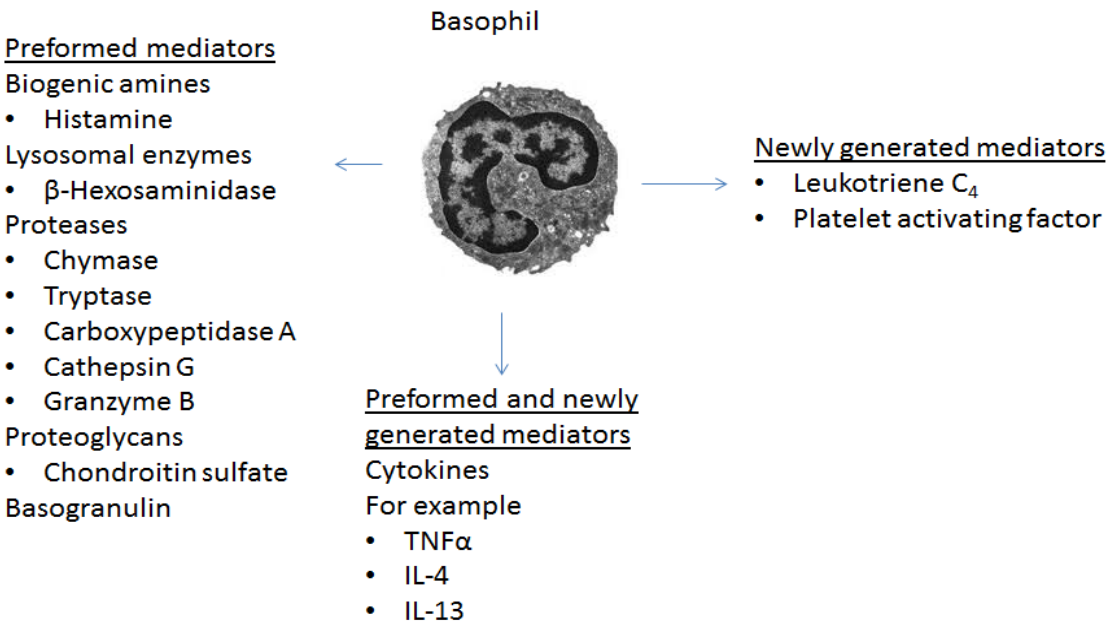

Figure 1.4: Mediators released after basophil activation. Basophils can release preformed and newly generated mediators, and some mediators which are both preformed and newly generated.

1.3 Mediators released after basophil and mast cell activation

1.3.1 Preformed mediators

Histamine

Histamine is an amine and plays an important role in allergic reactions. A quantity of 3 to 5 pg is present in each human mast cell [67] and it is synthesized from L-histidine by histidine decarboxylase [68]. This is released by mast cells and basophils after activation. Histamine can be degraded by histamine N-methyltransferase to tele-methyl histamine and also by diamine oxidase to imidazole acetaldehyde [69] .

The actions of histamine are mediated through binding to specific membrane receptors on a wide variety of target cell types. Four histamine receptors have been described: HR1, HR2, HR3, and HR4. Antagonism of HR1 and HR2 receptors in cells of the human leukemic mast cell line HMC-1 has been reported to lead to a decrease in production of the cytokines TNF-α, IL-8 and IL-6 [70]. Human H4 receptor cDNA expression has been found in basophils purified from human peripheral blood mononuclear cells, in cord blood-derived mast cells and in HMC-1 cells [71].

Symptoms of urticaria, erythema and high heart rate have been found to be correlated with the levels of histamine measured in plasma [72]. The group of Lin et al. investigated histamine levels in 89 patients and showed that those who suffered from angioedema and wheezing during an allergic reaction have a high concentration of histamine in plasma.

Value as marker

Tests for histamine are commercially available, but the rapid metabolism of histamine by N-methyltransferase and diamine oxidase makes it difficult to measure levels in biological fluids after an allergic reaction. Thus, any increase in histamine levels following an allergic reaction transitory and there is a return to the baseline levels within minutes [73].

Tryptase

Tryptase is a serine peptidase and the gene for tryptase is located on chromosome 16 [74]. Four genes for human tryptase have been described: TPSG I, TBSB 2, TPSAB I, and TPSD I. These encode α, β, γ and δ tryptase respectively and subvariants have been described for β-tryptase (Table 2.1). The β-tryptase is the most extensively studied form of the human protease. It is largely unique to mast cells though may be present in small quantities in basophils [53]. A range of pro-inflammatory actions has been demonstrated, and β-tryptase has been proposed to have important mediator roles in asthma and other allergic conditions [53, 74]. It appears that α-tryptase is an enzymatically inactive form that may be encoded at

the same locus as β-tryptase. Y-tryptase is membrane-anchored and has been proposed to have inflammatory potential on the basis of studies involving transfer into animal models [75].

Some human subjects lack the gene for α-tryptase and the human mast cell line termed HMC-1 (derived from a patient with monoblastic leukaemia) has been noted to be deficient in the α-tryptase gene [76]. Tryptase purified from human tissues (and likely to be predominantly β-tryptase) has been found to have a range of biological actions, including direct actions on various cell types, e.g. the fibroblast which can be induced to stimulate the synthesis of collagen [77].

Value as a marker

The measurement of circulating levels of tryptase has been found to be useful in the diagnosis of anaphylaxis if an increase in levels is detected in the hours following the event [78]. Sahiner et al. investigated serum tryptase levels in children with food allergy and found that serum basal tryptase levels were correlated with the severity of reactions which the patients suffered in the past [79]. The group investigated the basal tryptase level of children and compared the levels between control group, patients who suffered of food allergy and an anaphylactic shock previously. The tryptase level was significantly higher in the group who had food allergy and an anaphylactic shock compared to the control group. This suggests that tryptase might be a good marker to identify patients who might have a higher risk of a severe allergic reaction. However, samples for the measurement of tryptase should be collected in the hours following a reaction since the half-life of tryptase after release into the blood stream is about 2 hours [80].

Chymase

Chymase, like tryptase, belongs to the serine peptidase family [53, 81]. Just one gene has been identified for this protease in humans, whereas multiple genes are known for distinct variants of chymase in rodents. Chymases cleave their targets mostly after aromatic residues [82]. Endogenous substrates include angiotensin I, and the cytokine IL-6 and exogenous targets include allergens (e.g. birch pollen profilin) [82]. Mouse mast cell protease 4 (MCP-4) has the greatest similarity to human chymase and in MCP-4 knock-out mice there are low levels of IgE and IgG levels suggesting a role for chymase in generation of these immunoglobulins [83]. MCP-4 has also been proposed to regulate MMP-9 activity through activating the proform of this metalloproteinase [84].

Value as a marker

Being stored in the mast cell secretory granules, chymase could be a useful serum marker in anaphylaxis. Chymase concentrations in serum have been found to be increased in the hours following onset of anaphylaxis, though levels were not associated with those of tryptase [85].

Carboxypeptidase A3

The protease carboxypeptidase A3 (CPA3) is a member of the family of zinc-containing carboxypeptidases [86]. CPA3 is located in MC_{TC} mast cells and expressed in granules that have both tryptase and chymase [43, 82]. Irani et al. investigated the location of CPA3 in MC_T or MC_{TC} and found an association between numbers of CPA3-containing cells and chymase-containing cells in certain tissues, therefore it was concluded that all chymase - containing MC_{TC} cells may contain CPA3 [43].

CPA3 may be involved in the processing of angiotensin I because it cleaves at the carboxyl - terminal of His-Leu bond of angiotensin I [43] and it is possible that CPA3 processes the proteins or peptides after processing by chymase [87]. There have been few studies of human CPA3, but it is of interest that the structurally related bovine pancreatic carboxypeptidase A can stimulate human dermal fibroblast proliferation as assessed by an increased degree of [^{3}H]-thymidine incorporation (but there was no effect the production of type I collagen) [88].

In N-deacetylase/N-sulphotransferase 2 deficient mice, which have no heparin, it was found that mature CPA3 is absent [89]. As pro-CPA3 is present in wild type and in knock-out mice, it has been suggested that heparin may be involved in processing of pro-CPA3, but it could be also that pro-CPA3 is correctly processed but is dependent on heparin at another step.

Value as a marker

In recent studies, increased serum levels of CPA3 have been found, and unlike those for tryptase which decline to baseline levels within a few hours, CPA3 levels remain elevated at least until 24 hours after the triggering event [90].

Dipeptidyl peptidase 1

Dipeptidyl peptidase 1 (DPP1; also known as cathepsin C) belongs to the papain family of proteases and is a lysosomal cysteine dipeptidyl amino peptidase. DPP1 has four identical subunits. It cuts off dipeptide moieties from the N-terminal of target proteins. It is expressed

by various cell types including mast cells, basophils and neutrophils and has been implicated in the activation of various proteases.

Studies with DPP1 knockout mice have suggested that DPP1 is involved in activation of chymase but not tryptase [91], though DPPI can activate pro forms of human β-tryptase *in vitro* and may fulfil this function in man. In addition, roles for DPPI in activation of neutrophil-derived serine proteases have been shown, including cathepsin G and elastase which are associated with induction of tissue damage and processes of chronic inflammation [92]. In cytotoxic lymphocytes processed from DPPI-deficient mice, the propeptide domain of granzymes A and B are inactive, suggesting that DPPI is essential for the processing and activation of these two granzymes [93]. A defect in the gene for DPP1 has been found in patients with Papillon-Lefèvre syndrome, a condition associated with palmoplantar keratoderma and periodontitis [92].

Value as a marker

In preliminary studies high DPP1 concentrations in serum have been found in patients following anaphylactic shock, and levels did not correlate with those of tryptase [85]. Further studies are needed to evaluate the potential of DPP1 as a useful serum marker in anaphylaxis.

Cathepsin G

Though best known as a product of neutrophils, cathepsin G can also be produced and released by mast cells and basophils. Cathepsin G can cleave substrates of chymotryptic enzymes [82]. *In vitro* cathepsin G purified from human leukocytes has been found to decrease production of type I collagen in human dermal fibroblasts [88].

Value as a marker

Though not strictly a marker of mast cell activation, the measurement of cathepsin G in biological fluids in allergic reactions deserves consideration.

Heparin

Heparin is a proteoglycan that is secreted on activation of mast cells but not from basophils. Heparin has various actions including stimulation of lung fibroblast proliferation at low concentrations *in vitro*, and suppression of proliferation at high concentrations [26].

β-hexosaminidase

β-Hexosaminidase is a lysosomal enzyme and three isoforms are known: β-hexosaminidase A (HexA), β-hexosaminidase B (HexB), and β-hexosaminidase S (HexS) [94]. β-hexosaminidase catalyses the terminal β-glycosidically linked N-acetylglucosamine and N-acetylglucosamine residues from a number of glycoconjugates and is released as a preformed meditator from mast cells and basophils. HexA and HexB are found in equal amounts in normal human tissues. Small levels of HexS have been detected in tissue from patients with Sandhoff disease [94].

β-Hexosaminidase digests 4-nitrophenyl-N-acetyl-β-D-glucosaminide to produce 4-nitrophenol which can be readily detected spectrophotometrically at 405 nm [94]. This method is widely used to measure β-hexosaminidase release after mast cell and basophil degranulation *in vitro*.

Value as a marker

β-Hexosaminidase is useful as a marker for mast cell or basophil activation *in vitro*, though its value as an *in vivo* marker has yet to be established.

Basogranulin

The production of basophil-specific monoclonal antibody BB1 allowed detection of a cytoplasmic antigen whose selective presence in basophil secretory granules led to the term basogranulin being applied to it [95]. Peripheral blood basophils were found to express basogranulin in the granules by light and immuno-electron microscopy, and it was released along with histamine with IgE-dependent and IgE-independent stimulation of basophils [59].

Value as marker

Basogranulin has been shown to be valuable as a cell-specific marker for basophils [95, 96] and its measurement in biological fluids could allow for the first time an assessment of the role of basophil activation in allergic reactions.

1.3.2 Mediators that are both preformed and newly generated

Cytokines

Cytokines are produced by many cell types and are responsible for the induction and maintenance of immune responses [97]. Among those produced by mast cells is TNF-α. This is stored in mast cells and both secreted and newly generated in response to various stimuli, and can mediate up-regulation of endothelial and epithelial adhesion molecule expression. Basophils release IL-4, IL-13 and CD40L which could be involved in stimulating IgE synthesis [69].

Value as a maker

Increased levels of IL-4 and IL-13 have been reported in patients with positive skin tests to penicillin, with the degree of increase related to the size of the skin reaction [82]. High serum levels of IL-4 and IL-13 have also been noted in cases of allergic rhinitis, IL- 13 in patients with urticaria and dermatitis, and IL-10 and TNF-α in patients with asthma, allergic rhinitis, urticaria and allergic rhinitis and dermatitis [98]. Stone et al. have reported increased serum levels of IL-2, -4, -5, -6, -10, -13, TNF-α in anaphylaxis, even up to 10 hours after its initiation [81]. Application of sensitive assays for selected cytokines in allergic reactions could provide useful information on mechanisms, though in view of the range of cellular sources and the diverse stimuli leading to their generation such an approach may be less productive in diagnosis unless patterns of several cytokines are investigated.

1.3.3 Newly generated mediators

Prostaglandin D_2 (PGD_2)

Following release from mast cells, PGD_2 can be converted by 11-ketoreductase to 9α, 11β-prostaglandin F_2 (9α,11β-PGF_2) which can be measured in urine [99]. Ono et al have detected a higher 9α,11β-PGF_2 concentration in urine samples from patients following anaphylaxis than in those from their healthy counterparts [99]. PGD_2 can stimulate increases in cytosolic calcium in eosinophil's and has been found to be a major contributor to this effect in superntants from anti-IgE–activated human lung mast cells [100]. Interleukin 33 (IL-33) has also been found to stimulate PGD_2 release from mast cells, at least in primary mouse bone marrow-derived mast cells (BMMC) and after 24 hour periods [101]

Value as a marker

PGD_2 has potential as a serum maker for measurement of mast cell activation, though there have been suggestions that PGD_2 and 9α,11β-PGF_2 can be a product also of activated T cells or macrophages/monocytes [99].

Cysteinyl leukotriene C_4 (LTC_4)

LTC_4 is a product of the catalysis of leukotriene A_4 by leukotriene C_4 synthase; and γ-glutamyl transpeptidase transforms LTC_4 into LTD_4 and LTE_4 [102]. LTC_4 is a newly generated mediator of both mast cells and basophils, and is also present in various other cell types such as eosinophil [103].

Value as a marker

Increased urinary and serum levels of LTE_4, a product of LTC_4, has been detected in patients which came to A&E with suspected anaphylaxis [99]. Measurement of LTC_4 levels may thus be useful in anaphylaxis, but the extent to which raised levels may occur in non-allergic conditions is not known.

Platelet-activating factor (PAF)

The phospholipid platelet-activating factor (PAF) is synthesised by mast cells and basophils, and it is degraded rapidly by PAF acetylhydrolase to the biologically inactive lyso-PAF [104].

Value as a marker

Vadas et al. have found serum levels of PAF to be raised in patients who suffered from an acute allergic reaction, and low levels of PAF acetylhydrolase, an enzyme that plays a key role in PAF degradation were found in children who had had fatal peanut-induced anaphylaxis [104]. A very short half-life of PAF in biological fluids may argue against PAF as diagnostic marker, though in a research setting its measurement could yield information of value to an understanding of underlying mechanisms.

1.4 Identification of drug hypersensitivity

1.4.1 In vivo and in vitro tests for drug hypersensitivity

In vivo tests

Skin testing and oral challenge

Skin prick test (SPT) is a well-established means for the diagnosis of drug hypersensitivity. This involves the suspected trigger agent being applied directly to the skin and the skin surface is pricked with a lancet. The result can be interpreted and compared with a positive control (histamine) after 15-20 minutes [93].

The intradermal test (IDT) is performed if there is a negative skin prick test, and this is carried out by injecting 0.02–0.05 ml of the diluted medication into the skin [105]. Oral drug challenges involve administration of the drug in various dilutions in sterile water.

In vitro tests

Measurement of allergen specific IgE

Assays for the measurement of allergen-specific IgE in serum are available for several drugs implicated in hypersensitivity reactions. With drugs of small molecular weight, allergen-specific IgE may be measured with the drug coupled to a carrier molecule before it is bound to a solid support and incubated with the patient's serum [106]. Bound IgE may be detected using a secondary anti-human IgE antibody which is labelled with an enzyme and levels may be read by colorimetric or fluorescence based methods.

CD63 or CD203c up-regulation on basophils

The concept of the basophil activation test is to mimic the *in vivo* contact of basophils and allergen. IgE-induced basophil activation leads to expression of CD63 antigen on the cell surface and up-regulation of CD203c (Figure 1.5).

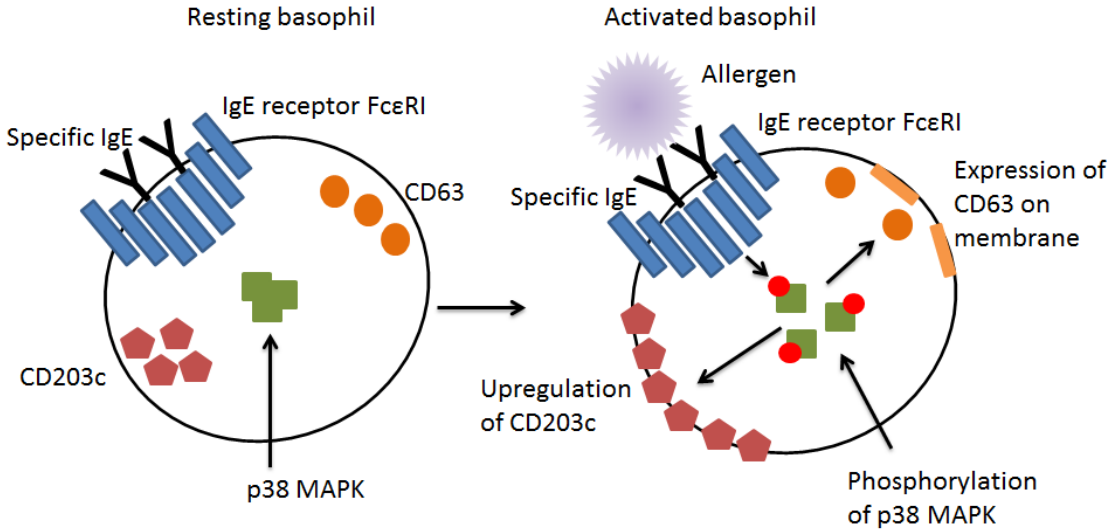

Figure 1.5: Up-regulation of CD203c and CD63 expression on the basophil. The allergen binds to the FcεRI receptor and induces phosphorylation of p38 MAPK which activates the expression of CD63 and CD203c on the membrane surface.

CD63 can be detected by a monoclonal antibody called MAB 435 [107]. In resting basophils CD63 is barely expressed on the cell surface. As an alternative to CD63, CD203c may also be used in basophil activation tests. CD203c up-regulation can be detected by the monoclonal antibody 97A6 on the basophil surface after stimulation [108]. This type II trans-membrane protein is also expressed on mast cells and their CD34-positive progenitors and is involved in cleavage of a number of molecules such as deoxynucleotides and nucleotide sugars [108-110].

The test is performed in whole blood and requires a flow cytometer to analyse basophil activation. The test is generally carried out using commercially available kits which include the buffer, optimised concentration of the allergen and basophil specific antibodies. The basophil specific antibodies can include CCR3 and CD63 or CD203c. CCR3 is a chemokine receptor and highly expressed on basophils but also on eosinophils [111]. Whole blood is incubated with the allergen and then basophil-specific antibodies are added into the tube. Red blood cells are lysed and the activation of basophils is analysed by flow cytometry. Anti-IgE and formyl-methionyl-leucyl-phenylalanine (fLMP) may be employed as positive control stimuli.

1.4.2 Limitations

Skin testing

The diagnosis of drug hypersensitivity is assessed mostly on the basis of clinical history. Skin testing is a safe procedure and can help to detect an allergy to a specific drug but the intradermal test can cause life-threatening allergic reactions. In addition, the test drug concentration can induce in some cases skin irritation which can lead to a false- positive result. Care should be taken to employ non-irritating test concentrations in all skin tests is available for the drug implicated. Patients with a false negative result are at risk of a possible anaphylactic reaction [112] (Table 1.5). Some allergens are not available for skin testing commercially [73], especially when the antigen is an unidentified breakdown product or a metabolite of a medication. The diagnosis of drug hypersensitivity with skin testing is not always straightforward to identify [112] and performance and interpretation of skin tests differs between allergy test centres in Europe [113] and elsewhere. False positive and false negative results are common. False positive results can restrict the patient's quality of life especially if no replacement medication is available for the drug implicated. Patients with a false negative result are at risk of a possible anaphylactic reaction [112].

Table 1.5: Test concentrations for selected drugs and drugs classes (selected as being below concentrations that could cause skin irritation) [113]

Drug	SPT	IDT
Benzylpenicillin	10,000 UI	10,000 UI
Amoxicillin	20 mg/ml	20 mg/ml
Ampicillin	20 mg/ml	20 mg/ml
Propofol	25 mg/ml	2.5 mg/ml
Entanyl	0.05 mg/ml	0.005 mg/ml
Cis-atracurium	2 mg/ml	0.02 mg/ml
Rocuronium	10 mg/ml	0.05 mg/ml
Suxamethonium	10 mg/ml	0.1 mg/ml
Adalimumab	50 mg/ml	50 mg/ml
Omalizumab	1.25 µg/ml	1.25 µg/ml
Local anaesthetics	Undiluted	1/10 diluted
Chlorhexidine	5 mg/ml	0.002 mg/ml

Measurement of allergen-specific IgE

Like skin testing, not all allergens are available for the measurement of allergen-specific IgE, especially some drugs that are too small or are unidentified breakdown products of the drug. Moreover, the sensitivity and specificity of the commercially available tests to measure specific IgE can show great variation. The sensitivity and specificity can differ between different allergens and between the different tests [114, 115]. Fontaine et al. investigated the commercially available ImmunoCap system and a homemade radioallergosorbent test and found that both tests had an unacceptable lack of sensitivity and specificity when tested with the same group of patients and drug [115]. Some drugs employed as haptens have been found to induce positive results in non-allergic people. For example, in NMBA allergy the quaternary ammonium cations (NR_4^+), cannot be used in drug-(hapten)-carriers for specific IgE detection as there is IgE specific for NR_4^+ in the general population and this would lead to false positive results [106].

Measurement of specific IgE may provide a guide, but does not necessarily indicate test outcome in skin testing or oral challenge. In some cases, the serum IgE levels may be below the cut-off value (set at 0.35 kU_A/L for the ImmunoCAP test), and the result may be negative when a skin test showed positive reaction.

Basophil activation test

Like the commercial test for measuring allergen specific IgE, the sensitivity of basophil activation tests can vary between different test kits and for different allergens. Boumiza et al. has reported that basophil activation tests that detect CD203c up-regulation were more sensitive than those measuring CD63 up-regulation in patients with latex allergy [116]. In contrast, Sudheer et al. has reported a lower sensitivity for CD203c in muscle relaxant drugs [117]. That study also investigated histamine release, and CD63 and CD203c up-regulation in patients undergoing an allergy test who had suffered from peri-anaesthetic anaphylaxis in the past. Both *in vitro* methods (measurement of specific IgE and basophil activation test) showed a big variation in sensitivity and specificity. In addition, the basophil activation test is limited by the allergen being available and it is important to employ an optimised allergen concentration which causes strong basophil activation but without cytotoxic or inhibitory components that could lead to a false positive results [118]. False negative results can develop through technical causes, improper handling or storage [118].

1.3 Tryptase and histamine as markers for anaphylaxis

At present, a clinical diagnosis of anaphylaxis is made on patient history and physical examination. The clinical diagnosis can be supported by a laboratory test which measures tryptase (pro, pro' and mature forms of α and β tryptase) concentrations in serum or plasma [93]. The timing is important for the test and the British Society for Allergy and Clinical Immunology (BSACI) guidelines for the investigation of suspected anaphylaxis during general anaesthesia suggest taking a sample directly after the event, at 1-2 h and at or > 24 h as a baseline sample [119].

Tryptase is the only serum marker which is measured after anaphylaxis by routine pathology laboratories [93]. A high tryptase level can be only measured within a limited time period after an anaphylactic shock. The tryptase levels measured in anaphylactic cases do not differentiate between pro and mature forms. This can lead to a false diagnosis because a high level of tryptase cannot allow an increased mast cell load and anaphylaxis to be distinguished. While other products of mast cell activation should be explored as clinical markers for anaphylaxis (including carboxypeptidase A3, chymase, platelet-activating factor, and certain cytokines), there remains a need for better tests for tryptase.

1.5 Aims

As mast cells and basophils play key roles in mediating allergic responses to medicines, our hypothesis is that investigation of drug-induced activation of these cells should provide information that could help in diagnosis.

The aim of these studies is to develop new tests for allergic sensitivity to drugs based on assessment of mast cell or basophil activation either in vitro or in vivo. To achieve this objective, the plan has been to:

1 Recruit patients suspected of having suffered an allergic reaction to a drug, and investigate clinical responses following skin testing or oral drug challenge with the drug implicated.

2 Investigate the ability of selected drugs to stimulate the activation of cells of a rat basophil leukaemia cell line (RBL-703-21) that had been transfected with the α chain of the human IgE receptor, by measuring release of β-hexosaminidase.

3 Examine activation in vitro of basophils from drug allergic patients in in response to the drugs by measuring release of histamine by immunoassay or up-regulation of CD63 by flow cytometry.

4 Prepare reagents and optimise procedures for the measurement of mast cell tryptase and the basophil product basogranulin as markers of allergic reaction in vivo.

2. Methods

2.1 Subjects and sample collection

Ethical approval for this study was provided by Isle of Wight, Portsmouth and South East Hampshire Local Research Ethics Committee (application 08-H0501-17). Patients (n=201) were recruited from the Adult Allergy Clinic at Southampton General Hospital (50/171 male/female; mean age 48 (17 – 88) years) who had suffered a suspected allergic reaction to drugs in the past. Direct drug challenge was performed when clinical history and measurements of specific IgE (where available) could not clearly establish diagnosis of specific drug sensitivity

Testing

The drug testing was carried out with skin prick testing (SPT), intradermal testing (IDT) and for some drugs the testing was performed by oral challenge. SPT was carried out with the trigger agent placed directly to the skin and a lancet employed to prick under the skin surface. Increasing concentrations of the drug were employed if there was no weal increase detected. The observational period between each test dose was 15 minutes. A result was considered positive if a weal with a diameter of 3 mm or greater than the saline negative control; and histamine dihydrochloride (10 mg/ml; Soluprick, ALK) was employed as the positive control. The procedure was stopped when the dose of drug elicited a positive reaction. If SPT was a negative or inconclusive, the IDT was carried out. This involved intradermal injection of 0.02 – 0.05 ml of diluted drug under the skin to form a small bleb. A test was considered positive test result when the bleb raised in size over a twenty minutes period. As with the SPT, an increasing dose of diluted allergen was employed at in the IDT until a positive or negative diagnosis was established. Oral challenge was carried out in some cases when the antigen was not available for skin testing or to confirm a negative outcome on IDT. As with skin testing, increasing doses of the allergen were employed.

In all drug challenges, the patients were observed for two hours following conclusion of testing. This was to check that delayed symptoms were not experienced by the patient (through in the present studies no delayed symptoms were observed).

Sample collection and recording of patient information

After consenting, patients' blood was collected in uncoated, heparinized and EDTA coated tubes (BD) through a cannula before and after drug challenge. In addition, saliva was collected using a saliva collection tube (Salivette, Sarstedt, Nürnberg, Germany) before every allergen dose, and 15, 30 and 60 minutes after conclusion of the drug challenge process.

A questionnaire was completed (Appendix A), and test results and clinical observations including temperature, peak expiratory flow rate (PEFR), heart rate and blood pressure were registered. The allergic history of the patients was recorded, enquiring specifically about previous reactions to drugs or other allergens, current medication and family history of allergy.

Sample processing and storage

Samples, were stored on ice after collection, though for preparation of serum, blood samples in uncoated tubes were left at room temperature for 30 to 40 minutes to allow clotting. Blood samples collected in heparinised or EDTA coated tubes were placed on a roller for 30 minutes to avoid clotting prior to collection of plasma or blood cells. All samples were centrifuged at 627 g for 15 minutes at 4°C. The serum (uncoated tube), plasma (heparin coated tube) and blood cells (heparin) were collected and separated into three aliquots. Saliva samples were aspirated using a pastette and separated into three aliquots. Serum, plasma, blood cells (in an EDTA coated tube) and saliva samples were stored at -80° C after processing immediately.

Sample collection from grass pollen allergic subjects

Serum samples from volunteers with suspected grass pollen allergy had been collected and processed previously (by a medical student). There was skin prick test for confirmation of the allergy.

2.2 Cell lines and cell culture conditions

The LAD2 human mast cell line (a gift from Dr A.S. Kirshenbaum [120]) was grown in in serum-free media (StemPro-34; Life Technologies, Paisley, UK) supplemented with penicillin/streptomycin and L-glutamine (Sigma–Aldrich, Poole, UK), 100 ng/ml stem cell factor (Invitrogen, Paisley, UK) and nutrient supplements (StemPro-34; Invitrogen, Paisley, UK). RS-ATL8 cells, a human IgE receptor (FcεRI) transfected rat mast cell line with NFAT-responsive luciferase reporter gene (generously provided by Dr Nakamura [121], Tokyo, Japan), was cultured in minimum essential medium (MEM; Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum (FCS; Sigma–Aldrich, Poole, UK), 100U/ml penicillin, 10 mg/ml streptomycin and 2mM/ml L-glutamine (Sigma–Aldrich, Poole, UK), 200 µg/ml hygromycin B (50 mg/ml; Invitrogen, Paisley, UK) and 0.5 mg/ml G418-sulfate (50 mg/ml; PAA, Cölbe, Germany). The α chain of the IgE receptor (FcεRI) transfected rat mast cell line RBL-703-21 was grown in minimum essential medium (MEM; Invitrogen, Paisley, UK) supplemented with 5% heat-inactivated foetal calf serum (FCS; Sigma–Aldrich, Poole, UK), 100U/ml penicillin, 10 mg/ml streptomycin and 2mM/ml L-

glutamine (Sigma–Aldrich, Poole, UK) and 0.5 mg/ml G418-sulfate (50 mg/ml; PAA, Cölbe, Germany).

2.3 Measuring β-hexosaminidase release from passively sensitised and non-sensitised cells

Figure 2.1 provides a schematic illustration of the protocol for studies with passively sensitised and non-sensitised cells of a basophil leukaemia cell line (RBL-703-21/RS-ATL8) and a human mast cell line (LAD2).

The direct actions of drugs were investigated with the human mast cell line in medium (90 µl per well) in V-bottomed, 96-well polystyrene cell culture microplates (Corning life Science, Amsterdam, Netherlands). Drugs were added to wells in triplicate over a range of concentrations (10 µl per well), and incubated for 45 minutes at 37°C. Calcium ionophore A23187 (Sigma-Aldrich, Poole, UK) was added as a positive control, and buffer alone to assess the degree of spontaneous release of β-hexosaminidase. So as to investigate the potential for cytotoxic release of β-hexosaminidase, the effect of adding the metabolic inhibitors 2-deoxy-D-glucose (10mM; Sigma-Aldrich, Poole, UK) and antimycin A (1µM; Sigma-Aldrich, Poole, UK) was investigated. Cell supernatants from each well were collected for measuring β-hexosaminidase release.

Cells of the rat basophil leukeamia cell lines RBL-703-21 and RS-ATL8 were passively sensitised with patient serum. Allergen challenge was carried out in triplicate wells of a sterile, flat-bottomed, 96-well polystyrene cell culture microplate (Greiner bio-one, Stonehouse, UK). Equal numbers (25,000 to 50,000 cells per well) of RBL-703-21 or RS-ATL8 cells (50µl/well) were plated and incubated overnight at 37°C, with 5% CO_2. After removing the medium, 1%, 3% or 5% of patient serum diluted in full growth medium or 5% patient serum diluted in serum free medium was applied to the plate and incubated overnight at 37°C, with 5% CO_2. The cells were washed with 1 x Tyrode's buffer (68.4 mM NaCl; Sigma-Aldrich, Poole, UK, 1.34 mM KCl; Fisher, Loughborough, UK, 235 µM NaH_2PO_4*$2H_2O$; VWR, Leics, UK, 1.36 mM $CaCl_2$*$2H_2O$; Sigma-Aldrich, Poole, UK, 245 µM $MgCl_2$ * $6H_2O$; Sigma-Aldrich, Poole, UK, 50.4 mM HEPES; Sigma-Aldrich, Poole, UK, 555µM D-(+)-glucose, VWR, Leics, UK, 0.1% BSA; PAA, Cölbe, Germany, pH 7.45) diluted in dH_2O. For each patient, serum and drug or pollen extract (prepared according to the manufacturer's instructions), calcium ionophore A23187 or purified IgG faction of polyclonal goat antiserum to human IgE, Fc specific (Nordic Immunological Laboratories, Susteren, Netherlands) were added as positive controls. A range of drug concentrations and positive controls were diluted with allergen challenge buffer (1:1 ratio of 1 x Tyrode's buffer and deuterium oxide (D_20, Sigma–Aldrich, Poole, UK)). The drug or control stimulus was added

diluted 1:10 with buffer (90 µl allergen challenge buffer plus 10 µl of diluted drug, calcium ionophore, anti-IgE antibody or buffer alone). Cells were lysed for later measurement of total β-hexosaminidase by adding 100 µl of 1% Triton X-100 (BDH Chemicals, VWR, Leics, UK) in PBS (Gibco Life Technologies, Paisley, UK). After incubation for 45 min at 37 °C, cell supernatant of each well was collected for measurement of β-hexosaminidase.

2.4 Measurement of β-hexosaminidase

Cell supernatant (30 µl) was incubated with 10 mM p-nitrophenyl N-acetyl-α-D-glucosaminide substrate (Sigma-Aldrich, Poole, UK) in 0.1 M Na_2HPO_4 (Sigma-Aldrich, Poole, UK) at 37°C for one hour. Colour development was stopped with 2 M glycine (Sigma-Aldrich, Poole, UK) and the plate read spectrophotometrically at 410 nm.

2.5 Release of histamine from whole blood basophils

The patient's blood was collected in a heparinized blood collection tube after obtaining written informed consent, and it was transferred immediately to a roller until it could be processed. The blood was diluted with histamine release buffer (Immunotech, Marseille, France) or 1 x Tyrode's buffer in dH_2O at a ratio of 1 to 5. A aliquot (90 µl)of diluted blood was mixed with 10 µl of different drug concentrations or positive controls (1% anti-IgE and 1µM of fLMP (Sigma-Aldrich, Poole, UK) in a 96-well V- bottom plate and incubated at 37°C for 45 min. The total histamine was performed by adding 50 µl of blood to 950 µl dH_2O in an Eppendorf tube and subjected to 3 cycle of freeze and thawing. After incubation, the 96-well plate and the Eppendorf tube was centrifuged at 800g, 10 min, 8°C and stored at -80 °C until histamine measurement. The measurement of the histamine levels was carried out with an enzyme immunoassay (EIA) histamine kit from Immunotech, Marseille, France according to the manufacturer's instructions. All solutions were provided by Immunotech, Marseille, France. Briefly, 100 µl of sample, calibration standard or control was mixed with 25 µl of acylation buffer and 25 µl acylation solution. The solution was mixed immediately for 10 min at room temperature and 50 µl of the acylated solution and 200 µl histamine-alkaline phosphatase conjugate added to the anti-histamine antibody-coated 96-well plate. The contents of the 96-well plate were mixed briefly and incubated at 4°C overnight. After removing the solution, the plate was washed 3 times with 200 µl 1x wash solution diluted in dH_2O and developed by adding and mixing of 200 µl paranitrophenyl phosphate dissolved in diethanolamine-HCL buffer. The reaction was stopped with 50 µl of 1M NaOH after approximately 30 min and read at 410 nm.

2.6 PBMC isolation

Blood from human volunteers were collected into EDTA tubes (BD, Oxford, UK). Non-diluted blood (15 ml) was carefully layered upon Ficoll-Paque Plus solution (GE Lifesciences,

Paisley, UK) and density gradient centrifugation was performed at 600 g, for 30 min at 20 °C (with no brake applied on the centrifuge) (Figure 2.2). After centrifugation, the PBMC layer was carefully aspirated and transferred to a new 50 ml tube. The cell suspension was topped up to 50 ml with phosphate-buffered saline (PBS), 2 mM EDTA (Sigma-Aldrich, Poole, UK), and pH 7.4. The suspension was centrifuged at 300 g, for 10 minutes at 20°C. The pellet was resuspended in 50 ml of buffer and centrifuged at 200 g, for 10 minutes at 20°C. These centrifugation steps ensured that platelets were removed from the cell suspension. After removing the supernatant, the cells were re-suspended in 5 ml of red blood cell lysate solution (0.15 mM NH_4Cl; Sigma-Aldrich, Poole, UK, 0.009 mM $KHCO_3$; Sigma-Aldrich, Poole, UK and 0.01 EDTA·Na_2·$2H_2O$; Sigma-Aldrich, Poole, UK) and incubated for 1 min and centrifuged at 200 g, for 10 minutes at 20°C. The pellet was re-suspended in buffer and the cell number determined, evaluating cell viability by incubating with 0.4% Trypan blue dye (Sigma–Aldrich, Poole, UK).

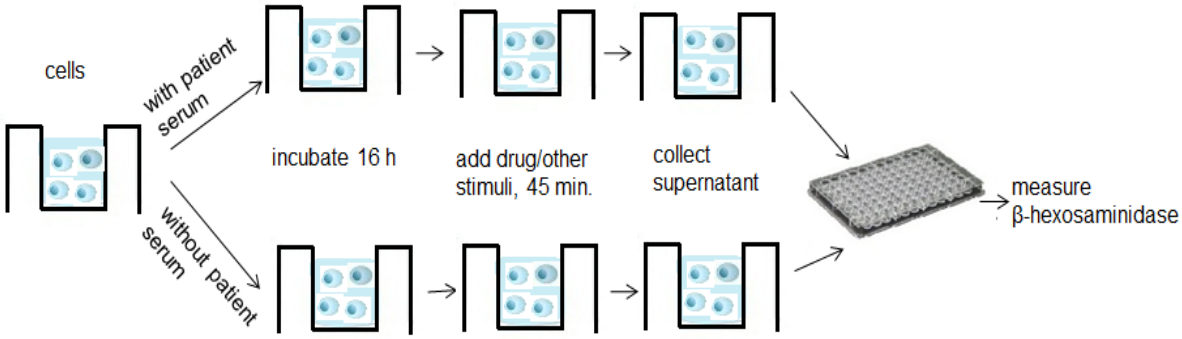

Figure 2.1: Measurement of β-hexosaminidase release from passively sensitise and non-sensitised cells.

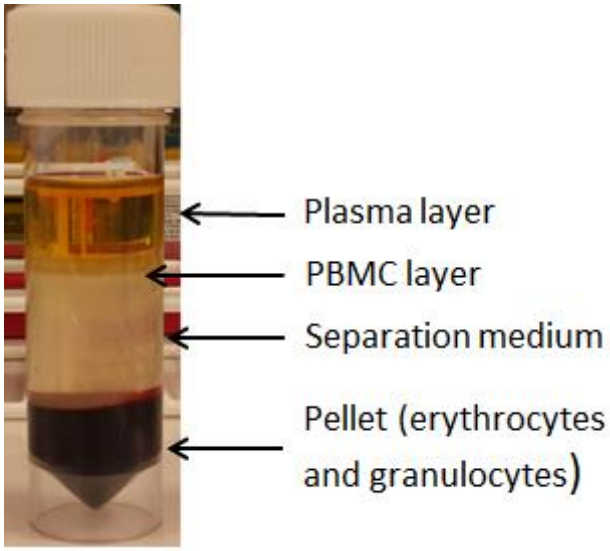

Figure 2.2: Separation of whole blood with Lymphoprep.

2.7 Basophil purification

Blood from human volunteers were collected into EDTA tubes. 25 mL of non-diluted blood was carefully layered upon 25 ml of Ficoll-Paque Plus solution (GE Lifesciences, Little Chalfont, UK) and density gradient centrifugation was performed at 600 g, for 15 min at 20 °C. After centrifugation, the PBMC layer and a third of the separation solution was carefully aspirated and transferred to a new 50 ml tube. The solution was centrifuged at 600 g, for 15 min at 20°C and the pellet was re-suspended in fresh chilled 1 x Tyrode's buffer for washing. An aliquot (10 µl) of the solution was collected before centrifugation for examination of cell purity. The supernatant was discarded and the cells were re-suspended in 2 ml of RoboSep Isolation buffer (DPBS without Ca^{2+}/Mg^{2+} with 2% FBS (v/v), 1mM EDTA) and transferred into a 5 ml tube. For negative selection, 30 µl of human basophil enrichment cocktail consisting of anti-CD2, anti-CD3, anti-CD14, anti-CD15, anti-CD16, anti-CD19, anti-CD24, anti-CD34, anti- CD36, anti-CD45RA, anti-CD56, and anti-CD235a (Glycophorin A) was added to the cell suspension and gently mixed. The suspension was incubated for 10 minutes at 4°C; and then were added 100µl of EasySep® nanoparticles. The suspension was mixed and incubated for 10 minutes at 4°C. After incubation, the tube was put into an Easy-Sep magnet and incubated for 5 min. at 20°C. The unbound cells were decanted into a fresh 5 ml tube and 10 µl of the solution was collected for examination of cell purity. The negative selection step was repeated if the purity was not satisfactory. Collected samples were examined using 0.4% Trypan blue dye (Sigma–Aldrich, Poole. UK) to determine cell viability and 3% alcid blue with methylene blue (kindly provided by Dr. Bernhard Gibbs) to identify basophils. After determination of purity, the cells were lysed by adding 2.7 ml 1% Triton-X 100 (BDH Chemicals Ltd, VWR, Leics, UK) in PBS and 300 µl of protease inhibitor (SIGMAFAST™ Protease Inhibitor, Sigma–Aldrich, Poole, UK)

2.8 Dot blotting for basogranulin

Basogranulin levels were measured using the dot blot technique. Polyvinylidene fluoride membrane (PVDF; Sigma-Aldrich, Poole, UK) was pre–wetted with 100% methanol (Sigma Aldrich, Poole, UK) for 2 minutes and equilibrated with PBS for 20 minutes. The PBMC lysate or basophil lysate were diluted with PBS and 100 µl were applied to a dot blot apparatus (Bio-Rad, Hemel Hempstead, UK) containing a PVDF membrane according to the manufacturer's instructions. The blot was removed and blocked with PBS containing 0.05% Tween 20 (Sigma Aldrich, Poole, UK), 3% blotting-grade blocker (Bio-Rad, Hemel Hempstead, UK), 0.3% H_2O_2 (Sigma Aldrich, Poole, UK) and 0.1% NaN_3 (Sigma Aldrich, Poole, UK), and probed with anti-human basogranulin antibodies BB5 (IgM isotype) or BB1 (IgG2a isotype [122]) in culture supernatant. The signal was visualized with an anti-mouse IgM (µ-chain specific)-peroxidase antibody (Sigma–Aldrich, Poole, UK) for BB5 and anti-

mouse IgG (γ-chain specific, Sigma-Aldrich, Poole, UK) peroxidase antibody for BB1 followed by Immun-Star HRP Substrate (Bio-Rad, Hemel Hempstead, UK). Spot detection and quantification were carried out by Genesnap apparatus with Gene tools software (Syngene, Cambridge, UK).

2.9 Basophil activation test

The basophil activation test was performed in collaboration with the Allergy and Immunology department. Blood from patients suspected of drug allergy was collected in EDTA tubes and stored at 4C. To achieve the best results, tests were carried out within 24 h after blood collection. The basophil activation test was performed by using the Flow-Cast® kit (Bühlmann, Schönenbuch, Switzerland) and carried out according to the manufacturer's instructions. Briefly, the blood sample was gently mixed by inverting several times. For each patient and allergen, 50 µl of allergen was transferred into a polystyrene tube. Two polystyrene tubes were set up as positive controls, monoclonal anti-FcεRI antibody (IgE) and N-formyl-methionyl-leucyl-phenylalanine (fMLP). The background was evaluated by adding 50 µl of stimulation buffer (containing calcium, heparin and IL-3 (2ng/ml)) into one of the four polystyrene tubes. A 100 µl aliquot of stimulation buffer was added into the four polystyrene tubes. Additionally, 50 µl of well-mixed whole patient blood and 20 µl of staining reagent containing a mixture of α-CD63-FITC and α-CCR3-PE were added to the tubes and mixed gently. The tubes were incubated for 15 min at 37ºC in a water bath. After incubation, 2 ml of pre-warmed lysing reagent was added to the tubes and incubated for 15 minutes at room temperature. The solution was centrifuged for 3 min at 1780 g after incubation and the supernatant decanted. The cells were washed with 2 ml wash buffer and centrifuged at 1780 g for 3 min. The supernatant was decanted and the cells were re-suspended by gently vortexing. The flow cytometric analysis was performed by Adnan Mani from the Allergy and Immunology department, and the up-regulation of CD63 expression on cells was measured using BD FACSCanto II and BDFACS Diva software. Basophil activation was measured using a commercially available basophil activation kit and a flow cytometer with a 488 nm argon laser diode. A gate was defined around the lymphocyte region by using the cell size (forward scatter (FSC)) and the cell's granularity (side scatter (SSC)) (Figure 2.3a). After identification of the lymphocytes, the basophils and eosinophils were identified with an anti-CCR3 PE. Cells which showed high-density fluorescence with anti-CCR3 PE were defined as eosinophils and basophils (Figure 2.3b). The identification of activated basophils was determined by using anti-CD63 FITC. Therefore, only cells which showed high-density fluorescence with anti-CCR3 PE and anti-CD63 FITC were activated basophils (Figure 2.3c). The up-regulation of CD63 expression was assessed as the percentage of the CD63-positive cells related to the total numbers of basophils identified. The experiments were

performed according to the instructions of the manufacturer, and the activation of basophils were analysed by flow cytometry gating strategy as described above. According to the kit manufacturer's instructions, CD63 up-regulation must exceed 5% of total basophil numbers, and the stimulation index (SI), which is generated by dividing the activated percentage of the allergen tube by the unstimulated tube should be >2.

2.10 Purification of tryptase from lung

The purification of tryptase was performed with Dr. Ahmed Abdelmotelb. All purification steps were carried out at 4 °C unless otherwise reported. Human lung or skin was finally chopped and blended with in a mixer in 1 l low salt buffer (0.1 NaCl; Sigma-Aldrich, Poole, UK, 0.05 M 2-(N-morpholino)ethanesulfonic acid (MES) ; Sigma-Aldrich, Poole, UK, 1 mM EDTA; Sigma-Aldrich, Poole, UK, pH 6.1). The mixture was centrifuged at 33,000 g for 60 min at 4 °C. The pellet was further homogenized in 2 x 1 l low salt buffer or 3 x 1 l high salt buffer (2 M NaCl, 0.05 M MES, 1 mM EDTA, pH 6.1) and centrifuged under the same conditions. Samples were taken from all supernatant to identify the enzyme activity. The first high salt buffer had the highest enzyme activity and was processed for tryptase purification. The solution of the high salt buffer was filtered and dialysed in low salt buffer using a dialysis tube with a 12,000 kDa (Sigma-Aldrich, Poole, UK) cut off. The dialysed solution was collected and applied to a HiPrep-butyl FF 16/10 column (GE Healthcare, Little Chalfont, UK) equilibrated with 2M $(NH_4)_2SO_4$ (Sigma-Aldrich, Poole, UK), 0.4 M NaCl, 1 mM MES, 10% v/v glycerol (Fisher Scientific, Loughborough, UK), pH 6.1. The column was washed with 2M $(NH_4)_2SO_4$, 0.4 M NaCl, 1 mM MES, 10% v/v glycerol, pH 6.1 and eluted with 0.4 M NaCl, 1 mM MES, 10% v/v glycerol, pH 6.1. The eluted fractions were tested for their tryptic activity. Active fractions were polled together and applied to a HiPrep-heparin FF 16/10 column (GE healthcare, Little Chalfont, UK) equilibrated with 0.4 M NaCl, 1 mM MES, 10% v/v glycerol, pH 6.1. The column was washed with one column volume of the equilibration buffer and proteins were eluted with a linear gradient of 0.2 M to 2.0 M NaCl, 1 mM MES, 10% v/v glycerol, pH 6.1. The collected fractions were examined for their tryptic activity and fractions with high enzyme activity were polled together. The solution was added to an e M uilibrated HiPrep 16/10 Sephacryl S200HR (GE healthcare, Little Chalfont, UK) column and the fractions were collected. All fractions were analysed for their tryptic activity and protein concentration, and those with high tryptic activity were pooled and examined on Coomassie blue or sliver stained (Plus BioRad Kit, Hemel Hempstead, UK) sodium dodecyl sulfate (SDS) page with western blotting.

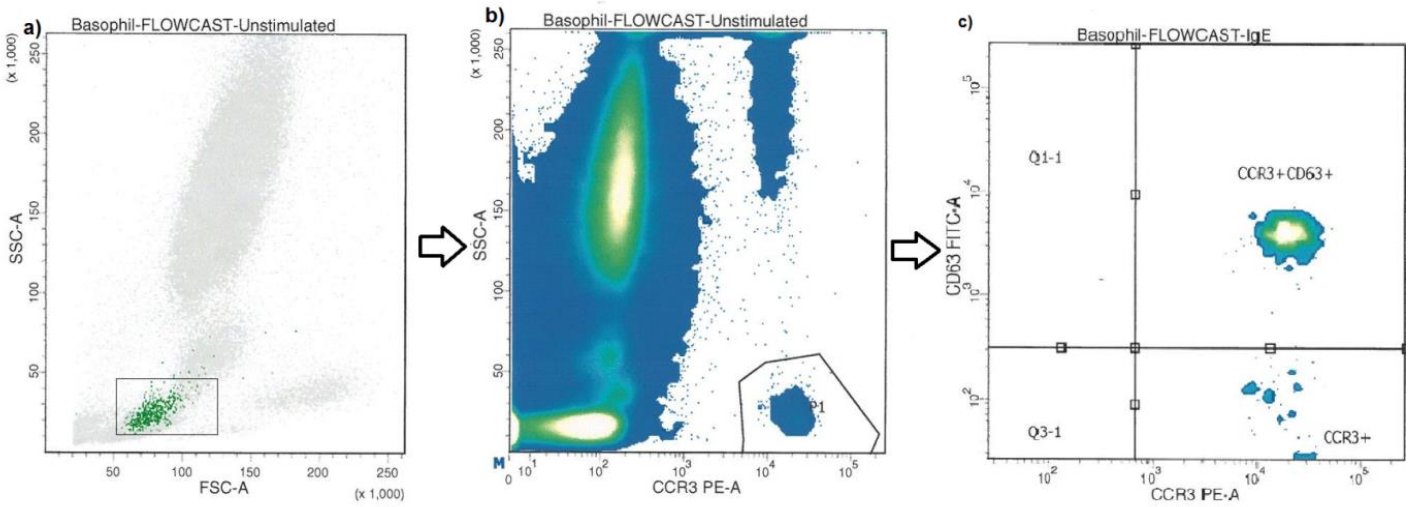

Figure 2.3: Flow cytometer gating strategy for human basophils from patient blood. (a) Singlet cells were identified by their size and granularity. (b) CCR3 positive cells were defined as basophils and eosinophil's. (c) Anti-IgE induced basophil activation were detected by up-regulation of CD63.

2.11 Sodium dodecyl sulfate (SDS)-PAGE

Samples of extracts in low salt and high salt buffers, pre butyl column, pre heparin column, post heparin column and after gel filtration were examined on 10% polyacrylamide gels (Table 2.1). The samples were diluted 1:3 with 3x Laemmli loading dye (Table 2.2) and heated at 95 °C for 5 min. Equal volumes of sample, Precision Plus Protein TM Dual color standards (Bio-Rad, Hemel Hempstead, UK) or magic markers (XP; Life Technology, Paisley, UK) for western blot were loaded on to the gel and run at a constant voltage in running buffer (120mM Tris base; Sigma-Aldrich, 960mM Glycine; Sigma-Aldrich, 17mM SDS; Sigma-Aldrich).

For Coomassie blue staining, the gel was removed and added to a container which contained the staining solution (0.1% Coomassie Blue; Bio-Rad, Hemel Hempstead, UK, dissolved in 10% acetic acid; Fisher Scientific, 40% methanol; Sigma Aldrich, Poole, UK and 60% H_2O). The container was heated for 10s in a microwave and left for 20 min on a shaker. After 20 min, the Coomassie stain solution was removed and the destaining solution (40% methanol, 10% glacial acetic acid, 60% distilled H_2O) was added to the container.

2.12 Western blot

The detection of the tryptase was carried out by western blotting. The enzymes were detected with specific monoclonal antibody AA5 and a secondary antibody with horseradish peroxidase (HRP).

First the PVDF membrane was activated in methanol (MetOH; Sigma-Aldrich, Poole, UK for 1 min and four pieces of filter paper per gel and the white sponges were soaked in transfer buffer (25 M Tris base; Sigma-Aldrich, Poole, UK, 200 mM Glycine; Sigma-Aldrich, Poole, UK, 0.04% SDS; Sigma-Aldrich, Poole, UK, 20% Methanol; Sigma-Aldrich, Poole, UK). Then white sponge and two pieces of the filter paper were placed on the transfer plate, followed by gel from the SDS-Page, the membrane and finally another two pieces of the soaked filter paper and white sponge. The transfer plates were put into the electrophorese machine which was filled with transfer buffer. The transfer of the protein on the gel on to the membrane was performed at 20mA overnight. ;

The membranes were removed and transferred to the 5% blocking buffer, which contained 2.5g milk powder; Bio-Rad, Hemel Hempstead, UK, in 50 ml of PBS-Tween (1xPBS, 0.05% Tween 20) for one hour at room temperature on the roller machine. After one hour, the blocking buffer was removed and the primary antibody was added for 30 min at room temperature on the roller machine. The membrane was probed with AA5 antibody specific

for tryptase. After 30 min the membrane was washed with PBS-Tween six times for 5 minutes. As secondary antibody, a 1/10,000 dilution of polyclonal rabbit anti-mouse immunoglobulins conjugated to HRP (Dako, Cambridgeshire, UK) was added and incubated for 30 min at room temperature on the roller machine. After 30 min of incubation, the secondary antibody was removed by further washing of the membrane with PBS-Tween six times for 5 minutes each time. The protein was detected using the chemiluminescent kit (Immuno-Star HRP Substrate, Bio-Rad, Hemel Hempstead, UK,) or colorimetric kit (DAB substrate kit, Vector laboratories, Cambridgeshire, UK).

2.13 Statistics

Statistical analysis of the β- hexosaminidase and basogranulin assay data was performed with GraphPad Prism 6 software. Comparison of net release of β-hexosaminidase induced by anti-IgE or calcium ionophore was carried out by the Mann-Whitney test. The basophil activation data was analysed by the BDFACS Diva software.

Table 2.1: Reagents employed for 10% SDS-PAGE.

Reagents	Resolving gel (10%)	Stacking gel (4%)
30:0.8% w/v acrylamide:bisacrylamide (Sigma-Aldrich, Poole, UK)	2 ml	660 µl
1 M Tris-HCl (Sigma-Aldrich, Poole, UK) pH 8.8	3 ml	-
1 M Tris-HCl pH 6.8	-	630 µl
20% SDS (Sigma-Aldrich, Poole, UK)	38 µl	25 µl
dH_2O	2.43 ml	3.6 ml
10% ammonium persulfate (APS) (Bio-Rad, Hemel Hempstead, UK)	36 µl	25 µl
TEMED (Bio-Rad, Hemel Hempstead, UK)	5 µl	5 µl

Table 2.2: Reagents for 3x Laemmli sample loading dye.

Reagents	Volume
1 M Tris-HCl pH 6.8	2.4 ml
20% SDS (Sigma-Aldrich, Poole, UK)	3 ml
100% Glycerol (Sigma-Aldrich, Poole, UK)	3 ml
B-mercaptoethanol (Sigma-Aldrich, Poole, UK)	1.6 ml
Bromophenol Blue (Sigma-Aldrich, Poole, UK)	5 Mg

3. Demographic features of study population

A cohort of 219 patients was recruited from the Adult Allergy Clinic at Southampton General Hospital. All underwent drug allergy testing, though four patients were excluded because they did not complete the process (Figure 3.1(. A positive reaction to a drug was provoked in 92 out of215 patients. More females)153(attended for allergy testing than males (62). There was no association between test outcome and having pre-existing hay fever or pre-existing asthma, but of those with eczema there were significantly higher numbers of patients who had no reaction (p=0.041, Pearson Chi Square).

Subjects were defined as being allergic when skin testing with the drug tested provoked an increase in weal size over that with saline of 3 mm x 3 mm, a rash, itchiness, angioedema or other symptoms, or when a reaction was elicited on oral challenge. Of 578 drug challenges performed there were 139 reactions that were positive, 413 negative and 26 for which the result was considered inconclusive (Figure 3.2a). Local anaesthetics, antibiotics, neuromuscular blocking agents (NMBA) and antiseptics were the most common drug classes tested in our study population. In almost all drug groups a negative test outcome was more common than a positive one, apart from in the case of antiseptics for which more positive reactions were induced than negative ones (Figure 3.2b). The drugs chlorhexidine (an antiseptic) and rocuronium (a NMBA) provoked a positive reaction in 50% of patients tested for these drugs. On benzylpenicillin challenge, positive reactions were triggered in around 40% of patients and on amoxicillin challenge around 30% (Figure 3.2c). While an increase in weal size following injection of drug was the most common symptom provoked, other indications of allergic reactions were observed including angioedema and oral tingling even in the absence of a positive weal (Figure 3.3).

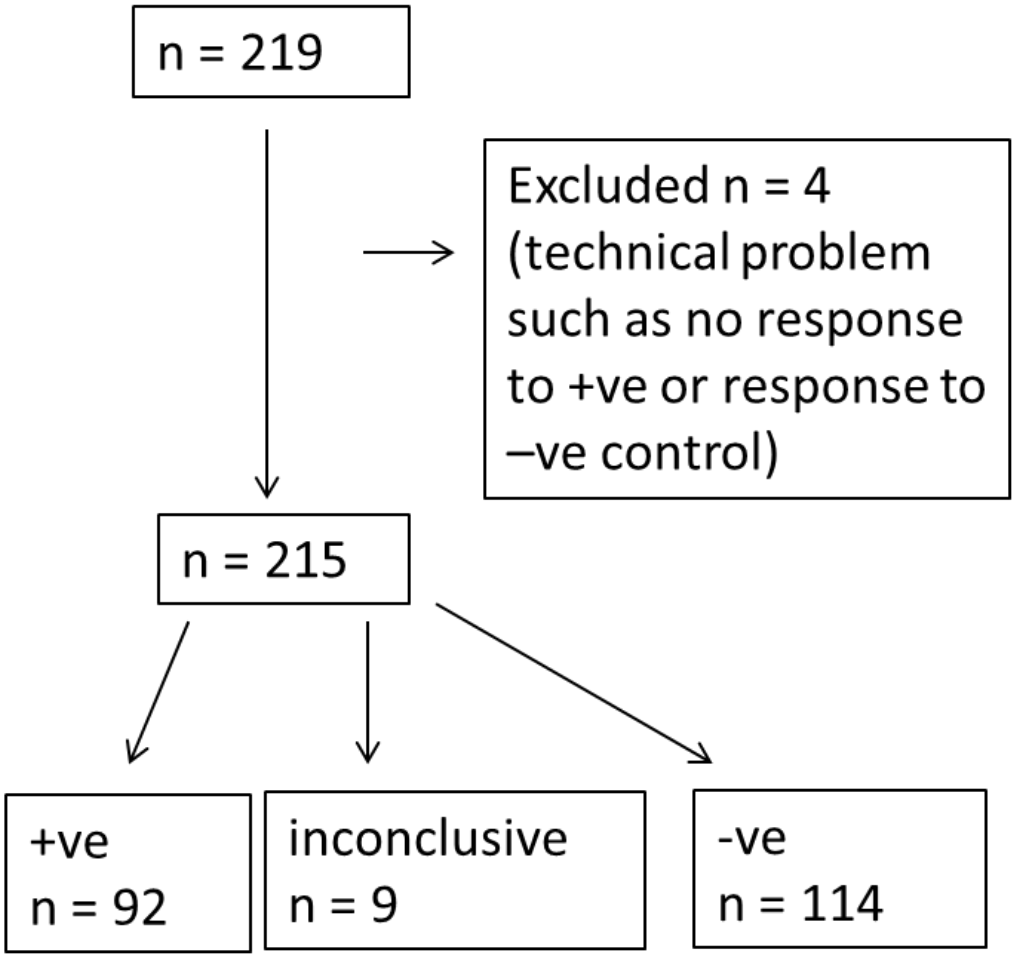

Figure 3.1: Outcome of drug challenge in 215 patients with suspected drug allergy. A positive reaction generally involved a skin reaction, but may have included other symptoms in the absence of this. Inconclusive reactions may have involved an increase in skin response below the size specified or subjective symptoms reported by the patients

Table 3.1: Clinical details as reported by patients recruited to the study

	Positive skin test outcome	Negative skin test outcome	Inconclusive skin test outcome
Gender F/M	67/25	80/34	6/3
Age median (range)	50 (17-76)	52 (21-82)	54 (37-88)
Asthma (yes/no)	14/78	26/88	2/7
Eczema (yes/no)	9/83	23/91	1/8
Hayfever (yes/no)	28/64	38/76	4/5

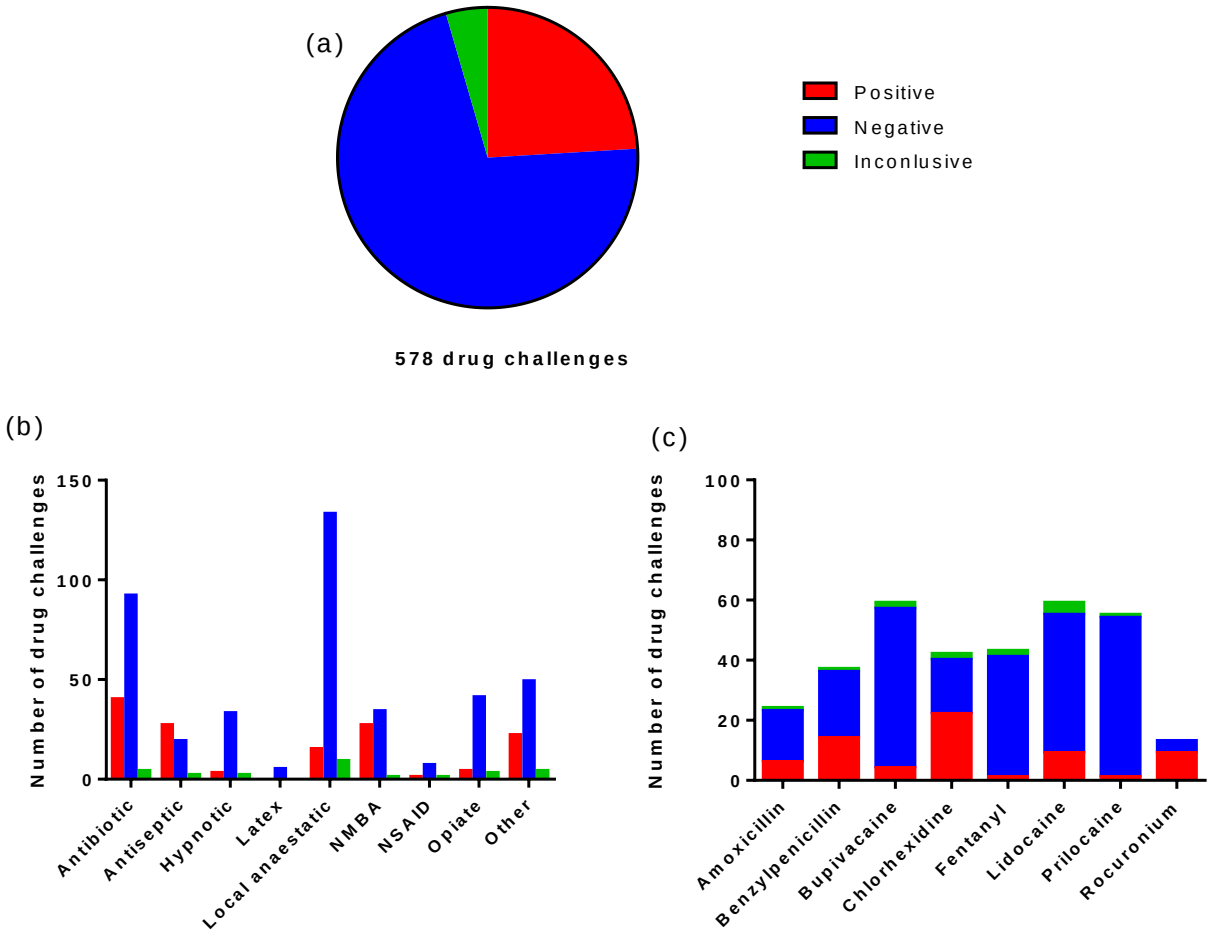

Figure 3.2: Outcomes of (a) 514 drug challenges performed shown together, and separately (b) for the major classes of tested, and (c) the most commonly tested drugs.

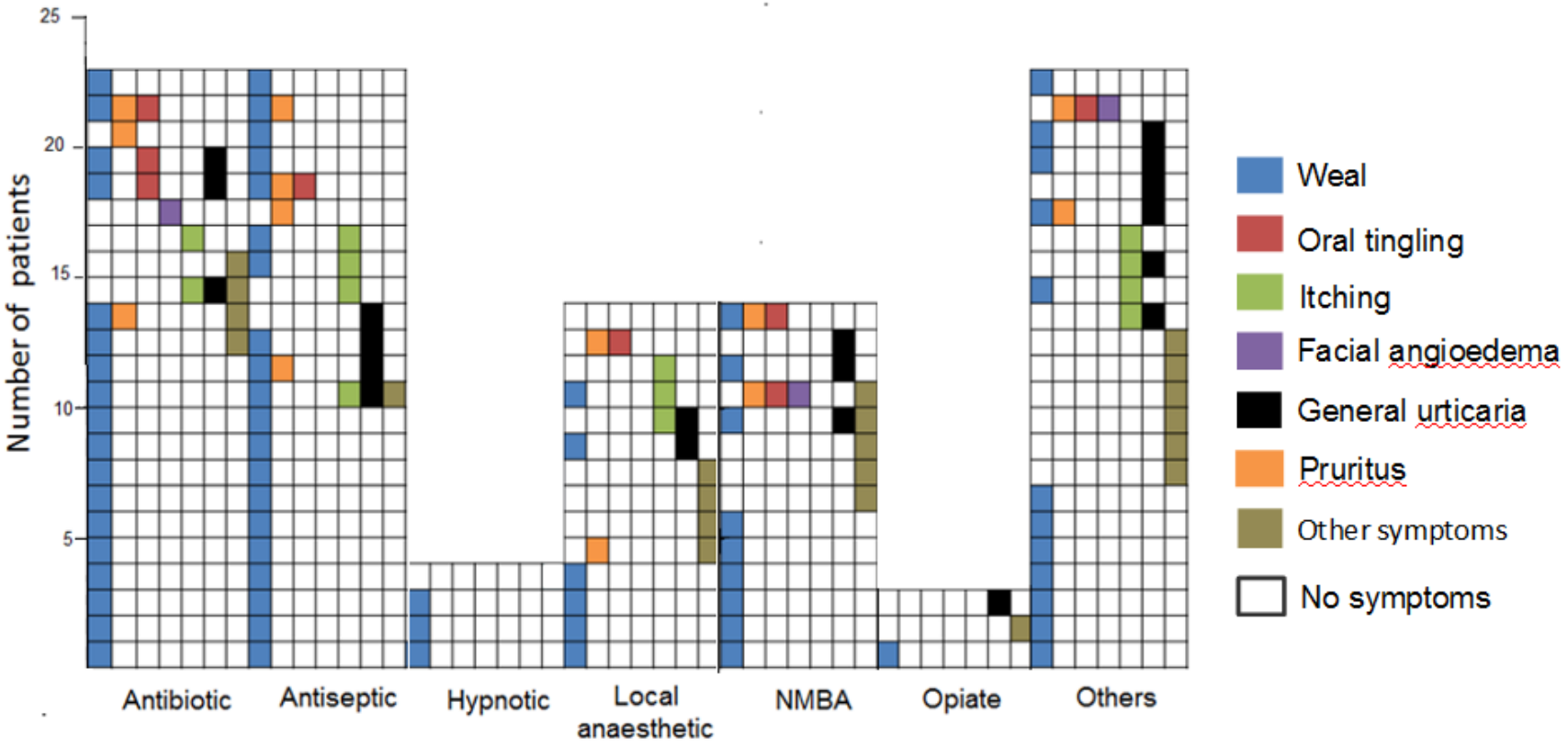

Figure 3.3: Symptoms provoked to drugs of different classes in each patient. Each squares represents one patient and the nature of the symptoms which the patients suffered after a positive reaction to the tested drug. Other symptoms noted included hives, post nasal drip, abdominal pain, vomiting, cough, shortness of breath, wheeze and stridor.

4. Identification of drug hypersensitivity

Methods were explored to investigate both IgE independent and dependent activation of mast cells and basophils by selected drugs. The LAD2, RBL-703-21 and RS-ATL8 mast cell lines were examined as well as activation of blood basophils.

4.1 Direct actions of drugs on mast cells

Addition of a range of drugs to LAD2 cells failed to stimulate β-hexosaminidase release directly. The drugs tested included prilocaine hydrochloride, paracetamol, benzylpenicillin, lidocaine, propofol, cefuroxime sodium, and rocuronium bromide and bupivacaine hydrochloride. However, with chlorhexidine there was a net release of around 10% of the total stored β-hexosaminidase when added at a concentration of 0.03 mM, and a net release of approximately 30% at 0.1 mM (Figure 4.1a).

When ability of isopropyl alcohol (employed as a solvent) to induce β-hexosaminidase release was investigated it was found to have little effect on β-hexosaminidase release at the concentrations employed (data not shown). When LAD2 cells were challenged with chlorhexidine following pre-treatment with the metabolic inhibitors 2-deoxy-D-glucose and antimycin A, β-hexosaminidase release was at levels approaching that when cells were not pre-treated in this way (Figure 4.1b). This suggests that much of the direct effect of chlorhexidine (at 0.1 mM and 0.03 mM) is the result of its cytotoxic actions.

Added as a positive control, calcium ionophore A23187 at 1 mM induced some 15% net release of β-hexosaminidase from cells (Figure 4.1c). Pre-treatment with metabolic inhibitors indicated that a proportion of this release was cytotoxic at this concentration, though there was little evidence for cytotoxicity with 0.1 mM calcium ionophore.

Preliminary attempts to passively sensitise LAD2 cells with IgE from patients or with myeloma IgE followed by stimulation with antibody specific for IgE or with allergen were not successful. Though the high affinity IgE receptor appeared to be present on the cells examined by flow cytometry with antibody specific for FcεRI (data not shown) it would appear that during culture cells had changed so that either intact FcεRI was not expressed or there was a defect in the coupling of the receptor to the degranulation process. For this reason further studies involving sensitisation with human serum were not performed. As with LAD2 cells there was no release of β-hexosaminidase in response to any drug tested with cells of the RBL-703-21 cell line, though the concentrations of chlorhexidine employed were below those that stimulated β-hexosaminidase release from LAD2 cells (data not shown)

4.2 Validation of the passively sensitised mast cell model

In order to determine the optimal concentration of serum for passive sensitisation, RBL-703-21 and RS-ATL8 cells were incubated with 1, 3 or 5% of a single serum sample with a high total IgE level (1820 kU/ml). When 1% anti-IgE was applied, β-hexosaminidase release was seen with all concentrations of serum tested. It was observed that a serum concentration of 3% induced the highest net release in RBL-703-21 cells whereas in RS-ATL8 cells it was highest at a 1% serum concentration. Both cell lines had decreased release of β-hexosaminidase at a concentration of 5%. At a concentration of 1%, the highest net release was induced by 3 μM calcium ionophore for both cell lines. However, the overall net release of β-hexosaminidase induced by 1% anti-IgE or 3 μM calcium ionophore was higher in the RBL-703-21 cells than in RS-ATL8 cells (Figure 4.2). For this reason, studies of allergen-induced cell activation were performed with RBL-703-21 cells only.

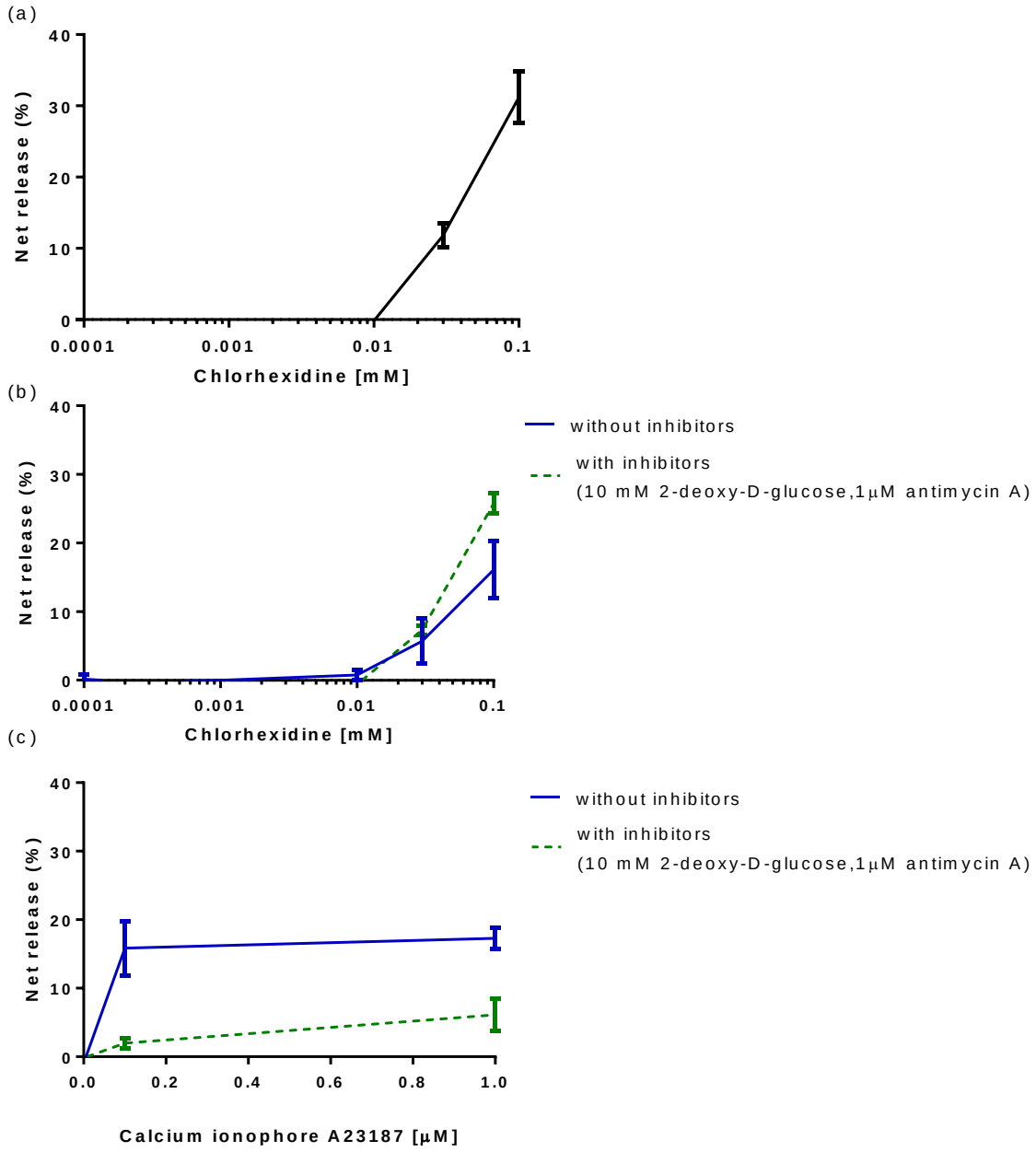

Figure 4.1: Direct activation of cells of the LAD2 mast cell line by chlorhexidine. (a) β-Hexosaminidase release from LAD2 cells induced by chlorhexidine (black line; mean ± SEM of four separate experiments in triplicate). β-Hexosaminidase release from LAD2 cells induced by (b) chlorhexidine following treatment with metabolic inhibitors (green dots) or without metabolic inhibitors (blue line; mean ± SEM from a single experiment performed in triplicate) and (c) calcium ionophore following treatment with (green dots) or without metabolic inhibitors (blue line mean ± SEM for a single experiment performed in triplicate).

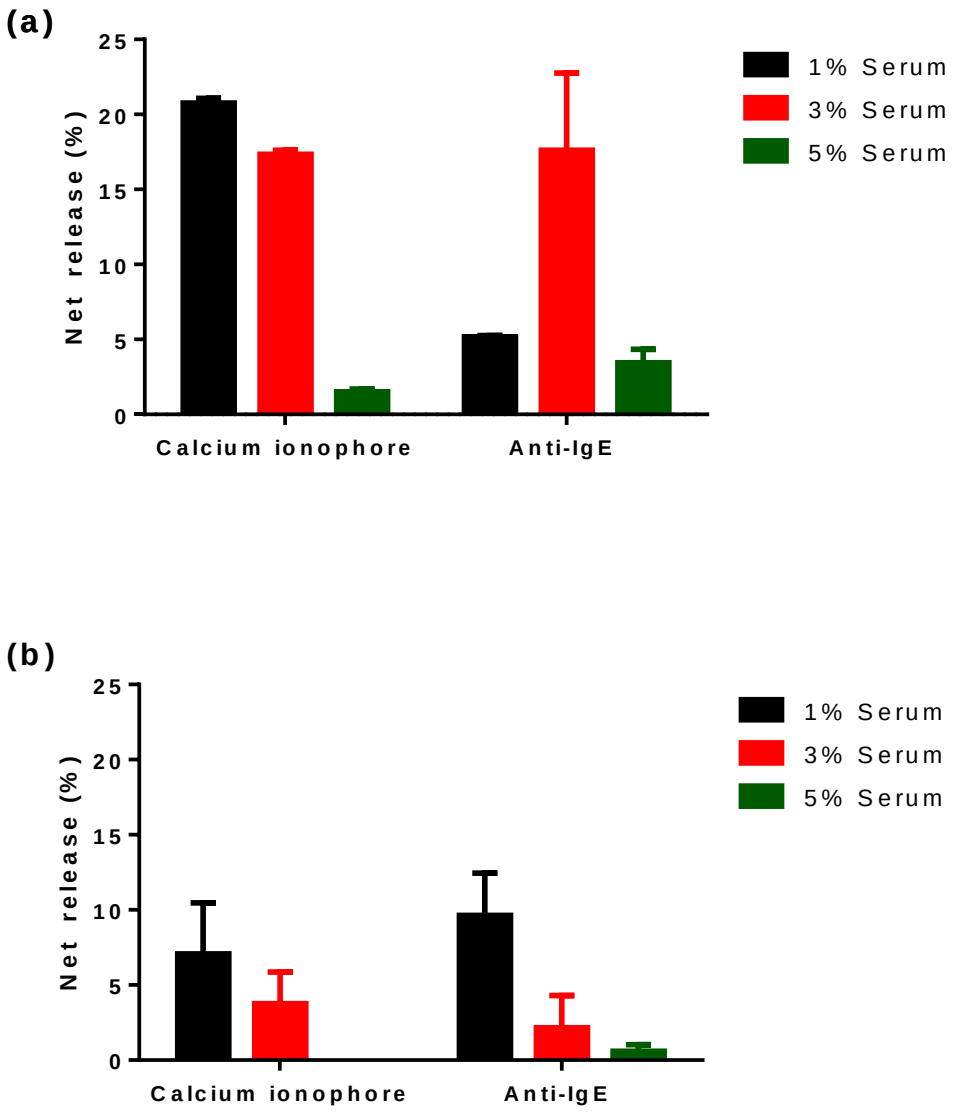

Figure 4.2: Anti-IgE and calcium ionophore-induced release of β-hexosaminidase from passively sensitised cells of the RBL-703-21 (a) and RS-ATL8 (b) cell line. Cells were sensitised with patient serum at 1% (black bar), 3% (red bar) and 5% (green bar). Data represents the mean ± SEM from a single experiment performed in triplicate.

Table 4.1: Characteristics of patients tested for reactions chlorhexidine, benzylpenicillin, lidocaine and rocuronium

Drug	Chlorhexidine		Benzylpenicillin		Lidocaine		Rocuronium	
Outcome of allergy testing	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
n	7	3	4	7	7	3	6	5
Age (mean and range)	57 (24-74)	49 (45-53)	48 (19-48)	35 (22-54)	52 (27-73)	57 (50-65)	61 (51-69)	58 (44-61)
Gender (female/ male)	4/3	2/1	4/0	6/1	4/3	2/1	2/3	3/2
Hay fever/ asthma/ eczema	1/1/0	1/0/0	1/0/0	4/3/2	1/2/1	1/0/1	0/0/0	0/0/1

4.3 Activation of passively sensitised mast cells

4.3.1 Drug induced activation

Having established and refined the *in vitro* cell challenge assay protocol, drug-induced release of β-hexosaminidase was examined in cells passively sensitised with serum (3%) from patients with positive or negative reactions on drug challenge. Details of the patients are shown in (Table 4.1). *In vitro* cell challenge was performed with one drug from each of the most commonly tested drug groups, benzylpenicillin (antibiotics), chlorhexidine (antiseptics), lidocaine (local anaesthetics) and rocuronium (NMBAs).

Benzylpenicillin

When RBL-703-21 cells were sensitised with serum samples from patients suspected of sensitivity to benzylpenicillin, addition of benzylpenicillin (at a concentration range of 0.001 to 84 mM) stimulated less than 2% net release of β-hexosaminidase (Figure 4.3). There was no difference seen between patients who had a positive reaction to the benzylpenicillin challenge and thoe who had no reaction.

Stimulation with anti-IgE resulted in a net release of β-hexosaminidase ranging from 0.1% to 12.7% with four benzylpenicillin skin test positive samples (Table 4.2). There was no significant difference in β-hexosaminidase release between patients who had a positive reaction on drug challenge and those who had no reaction (Table 4.2 and Table 4.3). All four patients who tested positive on drug challenge had an increase in weal size, and one showed increase in weal size but also urticaria and oral tingling.

Calcium ionophore employed as an IgE-independent control induced β-hexosaminidase release of 36.2 to 50.6%. There was no significant difference found between patients who had a positive reaction on benzylpenicillin challenge and those who had no reaction. There was no correlation between anti-IgE triggered β-hexosaminidase release and calcium ionophore-induced release. Spontaneous release (i.e. in the absence of any stimulus) ranged between 3.7 and 7.2% and did not differ between patients who responded to benzylpenicillin and those who did not.

Chlorhexidine

When RBL-703-21 cells were sensitised with serum samples from patients suspected of sensitivity to chlorhexidine, addition of chlorhexidine (in the concentration range 0.0003 to 0.01 mM) stimulated little β-hexosaminidase release (Figure 4.4). Release of β-hexosaminidase was generally less than 3% of that in the cells, and there were no

differences between cells incubated with serum from patients who had a positive reaction to chlorhexidine and those who had no reaction.

The degree to which anti-IgE could induce β-hexosaminidase release appeared to be strongly associated with the serum sample added, and between patients β-hexosaminidase release ranged from 0% to 21.3%. There was no overall difference in β-hexosaminidase release from cells sensitised with serum from patients who responded to skin testing with chlorhexidine and those who did not. However, there were only three non-responding patients included compared to seven for whom there was a response (Table 4.4 and Table 4.5); and while four of the seven chlorhexidine-positive patients had anti-IgE-induced β-hexosaminidase release greater than 12%, only one of the three chlorhexidine-negative patients had a net release above this level. All seven patients who tested positive on drug challenge had an increase in weal size and two showed additional symptoms.

β-Hexosaminidase release in response to calcium ionophore ranged between 9.4% and 66.3% for cells incubated with serum from different patients (Table 4.4 and Table 4.5). There was no significant difference in cell responses to calcium ionophore for cells incubated with serum from patients who had a positive reaction to chlorhexidine and those who had no reaction. There was no correlation between anti-IgE induced β-hexosaminidase release and calcium ionophore triggered release. Spontaneous β-hexosaminidase release ranged from 3.6 to 17.7%, and there was no apparent difference in release between patients who responded to chlorhexidine and those who did not.

Lidocaine

Lidocaine challenge of RBL-703-21 cells passively sensitised with serum from patients with suspected lidocaine hypersensitivity induced little release of β-hexosaminidase over a concentration range of 0.001 to 4.2 mM. Net release with serum from patients with a positive reaction on drug challenge was slightly higher than for patients with a negative reaction (Figure 4.5). However, the difference was quite small (1 to 2%), and there was no significant difference in β-hexosaminidase release between patients who experienced a positive reaction to the lidocaine challenge and those who did not.

In patients with a positive outcome on drug challenge, anti-IgE-induced β-hexosaminidase release varied from 0.8 to 25.8%, and in patients with negative test outcome from 0 to 21.5% (Table 4.6 and Table 4.7). There was no significant difference in release between those who had a positive reaction and those who did not (though there were only three patients with negative test outcome). Seven patients who tested positive on drug challenge had an increased weal size, and in one urticaria was provoked. In addition, there was one patient with no increase in weal size but who suffered hives and pruritus.

Net release of β-hexosaminidase triggered by calcium ionophore ranged from 50.7 to 68.3% with serum from patients with a positive test outcome, and from 42.8 to 54.9% from those with a negative test outcome (Table 4.6 and Table 4.7). The release of β-hexosaminidase was higher in patients with positive test outcomes. However, there were only three patients with a negative test outcome, and the difference in release did not reach significance. There was no apparent association between β-hexosaminidase release induced in response to calcium ionophore and that with anti-IgE as the stimulus. The spontaneous release ranged between 4.3 and 10.5%, but did not differ between patients who responded to lidocaine and those who did not.

Rocuronium

Following rocuronium challenge of RBL-2H2 cells passively sensitised with serum samples from patients who had undergone drug challenge to rocuronium, the net release of β-hexosaminidase was in all cases below 2% at drug concentrations of 0.001 to 0.1 mM. However, when rocuronium was added at a concentration of 1.89 mM, β-hexosaminidase release (3 (1.3-5.3)%; mean (range)%) was significantly greater for patients with positive test outcome than for those with a negative outcome (0.5; (0-2.3)%, p=0.02; Mann-Whitney test) (Figure 4.6). However, the net release of β-hexosaminidase was still less than 10%, which could be caused by assay variability.

Net release triggered by anti-IgE ranged from 1.1 to 47.5% and there was no significant difference observed between those with positive and negative test outcomes (Table 4.8 and Table 4.9). All five patients who tested positive on drug challenge had an increase weal size and one showed urticaria

Calcium ionophore induced higher net β-hexosaminidase release (76.7 (73.7-79.7)%; mean (range)%),for patients with a positive test outcome than for those who did not respond on rocuronium challenge (70.3 (63.3-75.7)%; p = 0.01; Mann-Whitney test) (Table 4.8 and Table 4.9). However, there was no correlation between calcium ionophore-induced β-hexosaminidase net release and anti-IgE-induced net release. Spontaneous net release was below 10% with all serum samples and there was no apparent difference in levels between serum samples from patients who responded to rocuronium and those who did not.

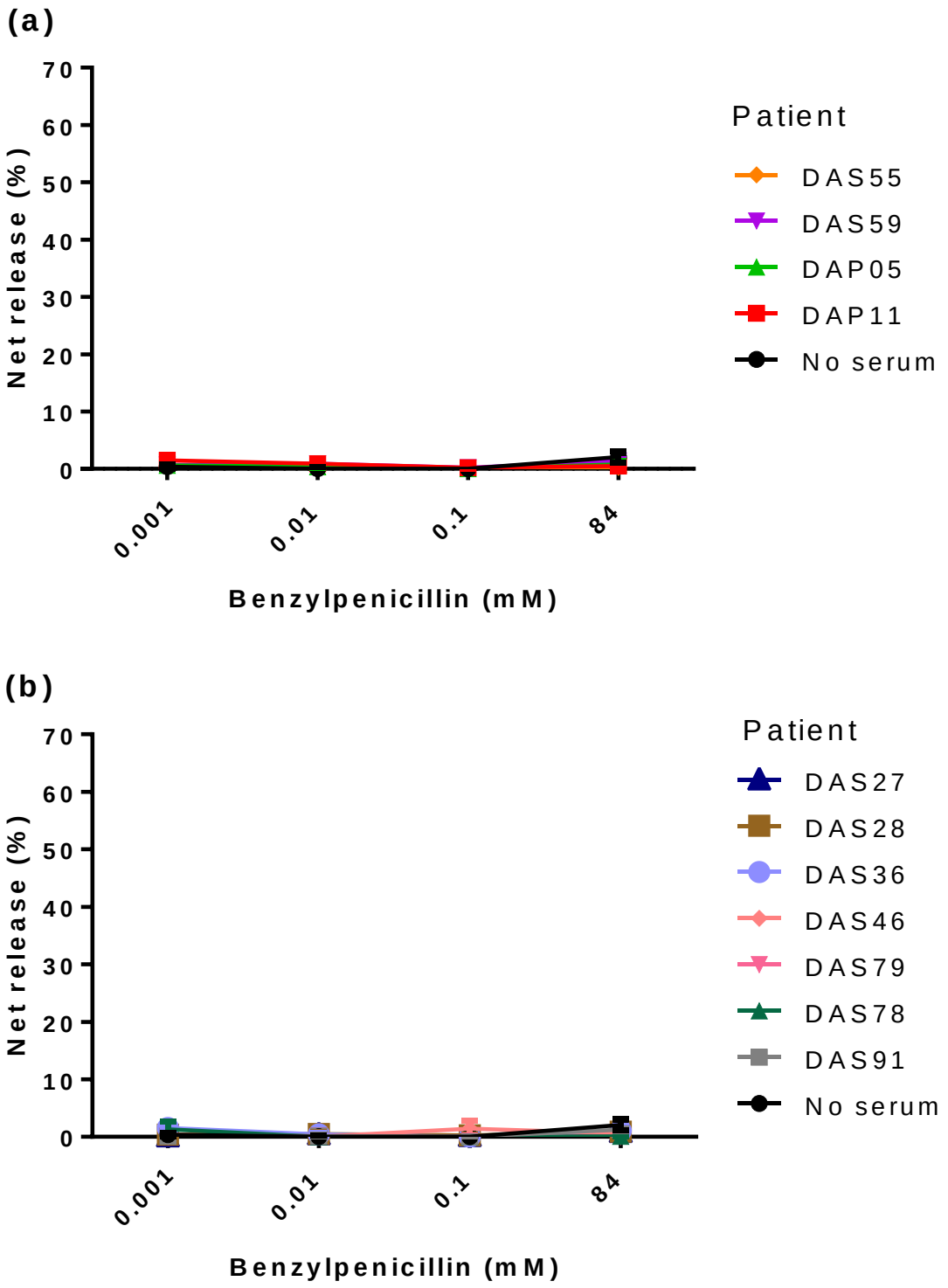

Figure 4.3: β-hexosaminidase release from passively sensitised RBL-703-21 cells induced by benzylpenicillin. RBL-703-21 cells were sensitised with serum samples collected from patients who had (a) a positive outcome or (b) a negative outcome when skin tested with this drug. Data represents the mean ± SEM from a single experiment performed in triplicate.

Table 4.2: β-Hexosaminidase release induced by 1 μM calcium ionophore and 1% anti-IgE from RBL-703-21 cells passively sensitised with serum from patients who reacted on skin testing with benzylpenicillin. The nature of symptoms provoked on challenge is indicated.

Patient sample	**β-hexosaminidase release (%)**		**Reaction on allergy testing**
	1% Anti-IgE	**1 μM calcium ionophore**	
No serum	0.5 ± 0.4	46 ± 5.4	-
DAS 55	0.1 ± 0.4	42 ± 3.9	Weal, urticaria and oral tingling
DAS 59	9.6 ± 1.3	44 ± 2.9	Weal
DAP 05	5.1 ± 0.8	45 ± 5.4	Weal
DAP 11	0.9 ± 0.9	47 ± 5.4	Weal

Table 4.3: β-Hexosaminidase release induced by 1 μM calcium ionophore and 1% anti-IgE from RBL-703-21 cells passively sensitised with serum from patients who did not reacted on skin testing with benzylpenicillin.

Patient sample	β-hexosaminidase release (%)	
	1% Anti-IgE	1 μM calcium ionophore
No serum	0.5 ± 0.4	46 ± 5.4
DAS 27	0.5 ± 0.2	39 ± 6.7
DAS 28	6.2 ± 0.4	37 ± 3
DAS 36	3.4 ± 0.6	36 ± 4.3
DAS 46	10 ± 0.6	41 ± 2.2
DAS 78	8.5 ± 2.1	42 ± 7.4
DAS 79	1.2 ± 0.3	44 ± 3.6
DAS 91	16 ± 4	51 ± 5.6

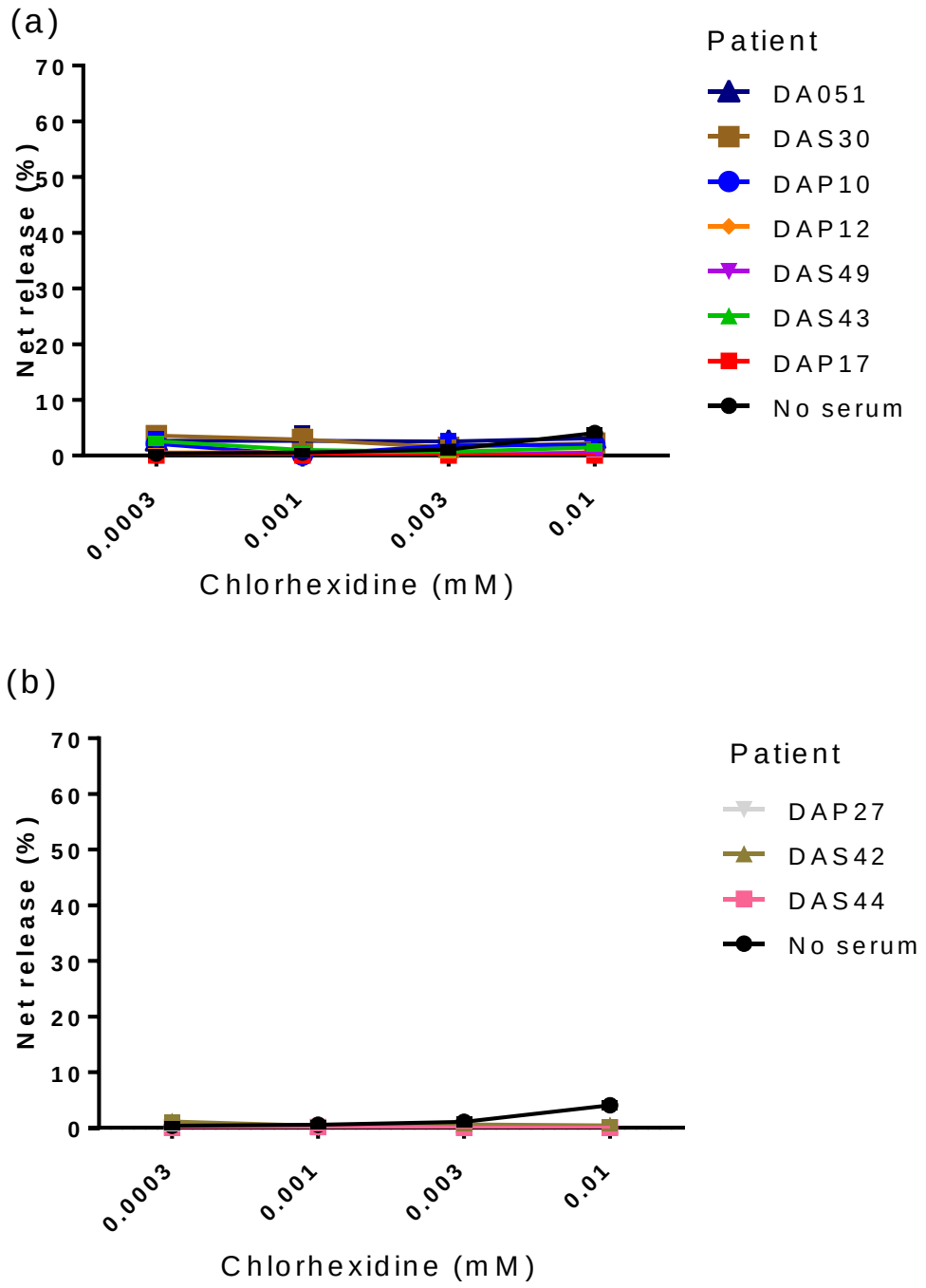

Figure 4.4: β-hexosaminidase release from passively sensitised RBL-703-21 cells induced by chlorhexidine. RBL-703-21 cells were sensitised with serum samples collected from patients who had (a) a positive outcome or (b) a negative outcome when skin tested with this drug. Data represents the mean ± SEM from a single experiment performed in triplicate.

Table 4.4: β-Hexosaminidase release induced by 1 μM calcium ionophore and 1% anti-IgE from RBL-703-21 cells passively sensitised with serum from patients who reacted on skin testing with chlorhexidine. The nature of symptoms provoked on challenge is indicated.

Patient	β-hexosaminidase release (%)		Reaction on allergy testing
	1% Anti-IgE	1 μM calcium ionophore	
No serum	0.3 ± 0.2	86 ± 1.7	-
DAP 12	5.9 ± 0.5	15 ± 0.5	Weal
DAP 17	0.3 ± 0.5	9.4 ± 3.8	Weal
DAS 49	1.5 ± 1	66 ± 3.9	Weal and urticarial
DAS 43	13 ± 4.7	53 ± 22	Weal
DAS 30	13 ± 2.3	38 ± 3.3	Weal, urticaria and pruritus
DAP 10	18 ± 1.3	29 ± 1.4	Weal
DA 051	19 ± 1.7	28 ± 4.4	Weal, pruritus and oral tingling

Table 4.5 β-Hexosaminidase release induced by 1 μM calcium ionophore and 1% anti-IgE from RBL-703-21 cells passively sensitised with serum from patients who did not reacted on skin testing with chlorhexidine.

Patient sample	β-hexosaminidase release (%)	
	1% Anti-IgE	1 μM calcium ionophore
No serum	0.3 ± 0.2	86 ± 1.8
DAS 42	2.2 ± 1.9	38 ± 1.1
DAS 44	13 ± 0.5	78 ± 3.1
DAP 27	2 ± 0.1	64 ± 6.2

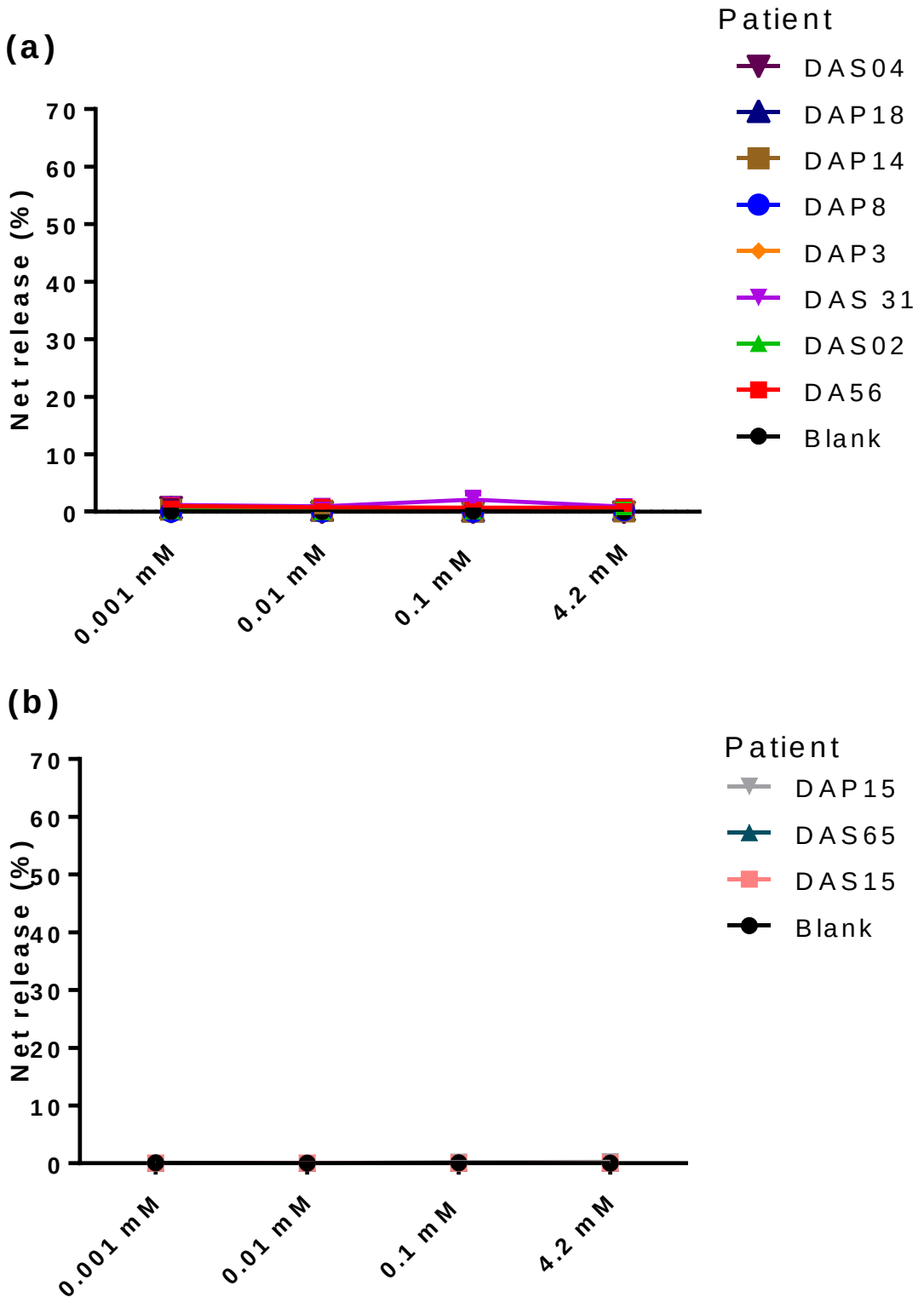

Figure 4.5: β-hexosaminidase release from passively sensitised RBL-703-21 cells induced by lidocaine. RBL-703-21 cells were sensitised with serum samples

collected from patients who had (a) a positive outcome or (b) a negative outcome when skin tested with this drug. Data represents the mean ± SEM from a single experiment performed in triplicate.

Table 4.6: β-Hexosaminidase release induced by 1 μM calcium ionophore and 1% anti-IgE from RBL-703-21 cells passively sensitised with serum from patients who reacted on skin testing with lidocaine. The nature of symptoms provoked on challenge is indicated.

Patient sample	**β-hexosaminidase release (%)**		**Reaction on allergy testing**
	1% Anti-IgE	**1 μM calcium ionophore**	
No serum	0 ± 0.18	69 ± 4.2	-
DA56	5.2 ± 0.4	68 ± 1.3	Hives, puritis
DAS02	4 ± 3.1	63 ± 7.3	Weal
DAS31	21 ± 0.7	51 ± 1.1	Weal, urticarial
DAP3	26 ± 2.7	54 ± 3.9	Weal
DAP8	18 ± 1.9	67 ± 10	Weal
DAP14	20 ± 2.4	67 ± 9.2	Weal
DAP18	2.4 ± 1.2	68 ± 4.3	Weal
DAS04	0.9 ± 0.4	66 ± 2.3	Weal

Table 4.7: β-Hexosaminidase release induced by 1 μM calcium ionophore and 1% anti-IgE from RBL-703-21 cells passively sensitised with serum from patients who did not reacted on skin testing with lidocaine.

Patient	β-hexosaminidase release (%)	
	1% Anti-IgE	1 μM calcium ionophore
No serum	0 ± 0.2	68 ± 4.2
DAS 15	0 ± 0.7	43 ± 7.3
DAS 65	0 ± 0.6	54 ± 3.2
DAP 15	21 ± 1.5	55 ± 1.7

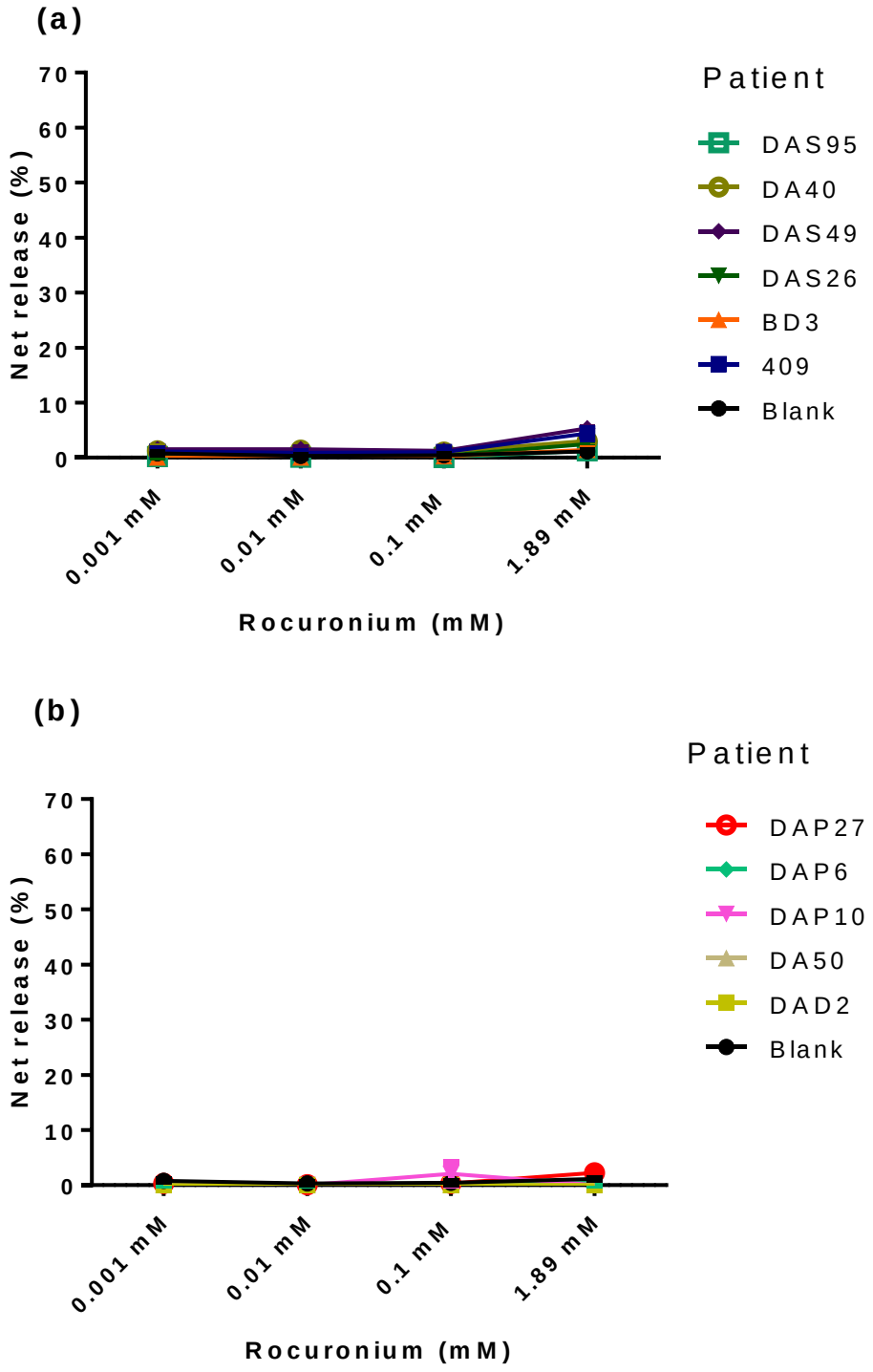

Figure 4.6: β-hexosaminidase release from passively sensitised RBL-703-21 cells induced by rocuronium. RBL-703-21 cells were sensitised with serum samples collected from patients who had (a) a positive outcome or (b) a negative outcome when skin tested with this drug. Data represents the mean ± SEM from a single experiment performed in triplicate.

Table 4.8: β-Hexosaminidase release induced by 1 μM calcium ionophore and 1% anti-IgE from RBL-703-21 cells passively sensitised with serum from patients who reacted on skin testing with rocuronium. The nature of symptoms provoked on challenge is indicated.

Patient sample	β-hexosaminidase release (%)		Reaction on allergy testing
	1% Anti-IgE	1 μM calcium ionophore	
No serum	1.8 ± 0.5	85 ± 1.2	-
409	47 ± 1.7	80 ± 1.4	ND
BD3	2.6 ± 0.5	75 ± 0.7	Weal
DAS26	29 ± 1.1	79 ± 3.6	Weal, urticaria
DAS49	7.9 ± 1.3	77 ± 0.4	Weal
DA40	1.1 ± 0.1	76 ± 0.8	Weal
DAS95	4. ± 0.6	74 ± 2.1	Weal

Table 4.9: β-Hexosaminidase release induced by 1 μM calcium ionophore and 1% anti-IgE from RBL-703-21 cells passively sensitised with serum from patients who did not reacted on skin testing with rocuronium.

Patient sample	**β-hexosaminidase release (%)**	
	1% Anti-IgE	**1 μM calcium ionophore**
No serum	1.8 ± 0.5	85 ± 1.2
DAP27	5.4 ± 0.1	74 ± 2.6
DAD2	11 ± 1.5	63 ± 4.8
DA50	28 ± 0	65 ± 2.5
DAP10	18 ± 1.1	73 ± 0.7
DAP6	15 ± 2.3	76 ± 11

4.3.2 Grass pollen-induced β-hexosaminidase release from RBL cells passively sensitised with sera from grass pollen-allergic subjects

Drug-induced release of β-hexosaminidase from RBL cells passively sensitised with serum from patients with suspected drug allergy was in all cases low. To validate the model employed, we sought to challenge RBL cells with a pollen extract following passive sensitisation with serum from subjects with pollen allergy (that had been confirmed by skin testing). Addition of the grass pollen extract at 0.3 to 300 BU/ml induced release of β-hexosaminidase with a bell-shaped curve (Figure 4.7(. The highest net release was with 30 BU/ nd it ranged fromml pollen extract a 0 to 6.2 .% Spontaneous release ranged from 2.5 to 10.0%.

Reproducibility of the *in vitro* test was assessed by repeating the same experiment on different plates on two or three separate occasions, and with the cell lines RBL-703-21 (Figure 4.8(. RBL-703-21 cells, the coefficient of variation (CV) was less than 15% at concentrations of 3, 30 and 300 BU/ml with samples from subjects N9 and N19.

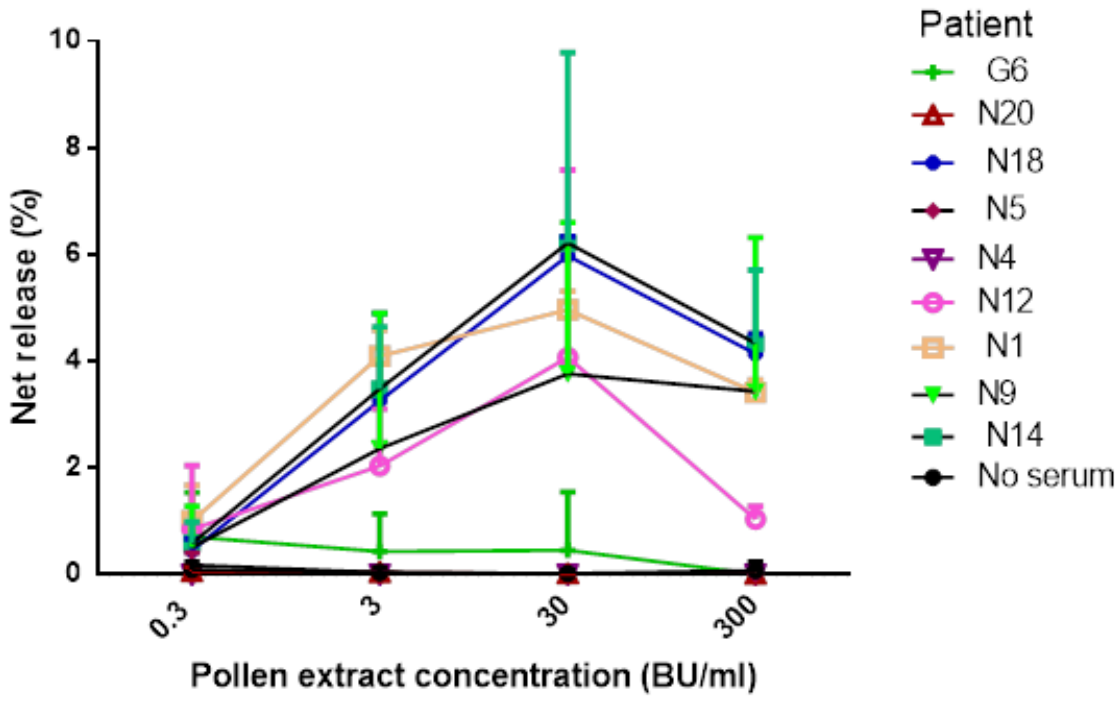

Figure 4.7: Grass pollen-induced net release of β-hexosaminidase from passively sensitised cells of the RBL-703-21 cell line. Cells were sensitised with 5% serum (diluted in serum free medium) from subjects with grass pollen allergy. Data represent the mean ± SEM from a single experiment performed in triplicate except of N9 and N14 which was performed three experiments and N5 and G6 with two experiments.

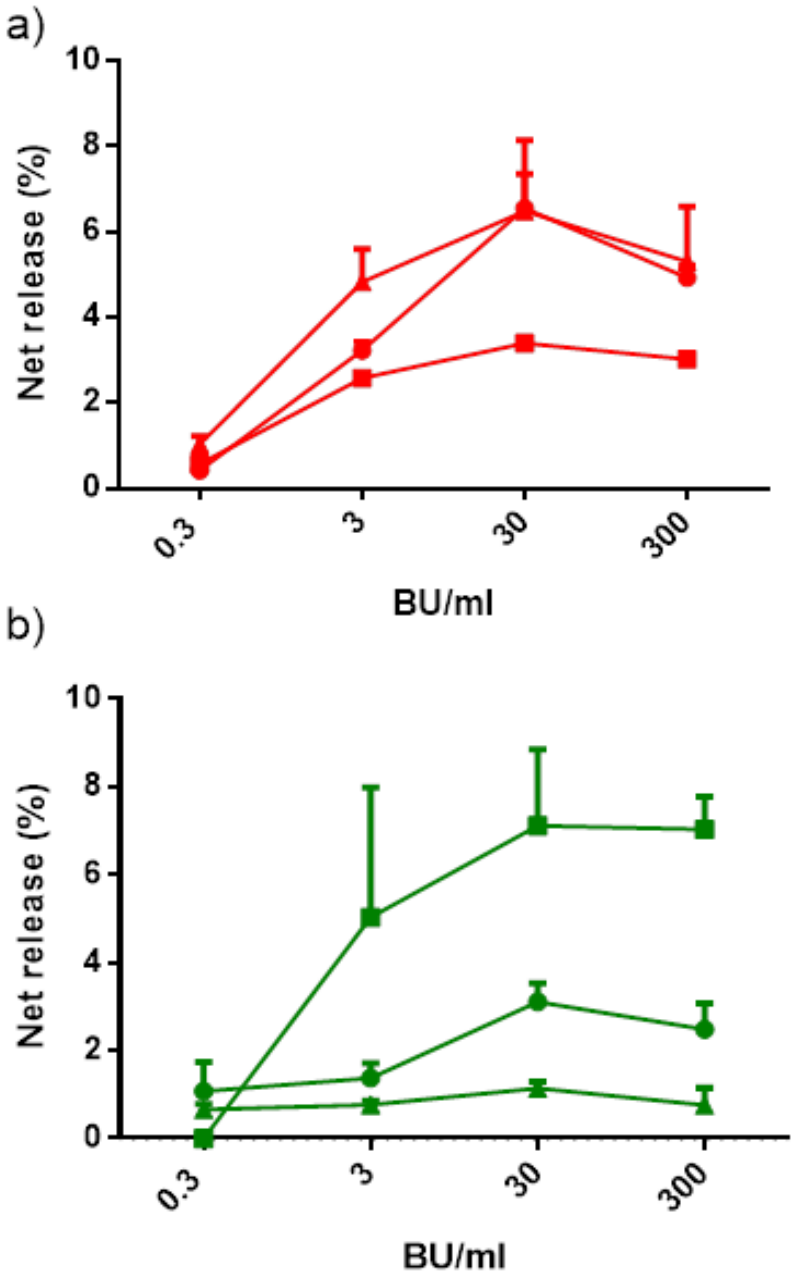

Figure 4.8: Reproducibility of *in vitro* challenge assay. Cells of the RBL-703-21 cell line were passively sensitised with serum from two grass pollen-allergic subjects (a) N14 and (b) N9 and the experiment was repeated on three separate occasions. Data represents the mean ± SEM for three experiments performed in triplicate.

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4.3.3 Activation of blood basophils by measuring histamine release of blood basophils

When peripheral blood basophils from 13 patients with confirmed drug hypersensitivity by skin testing were challenged with the drug implicated, histamine release was stimulated for only three patients (Table 4.10). Net histamine release greater than 10%, was provoked by 0.75 mg/ml meloxicam in two different patients and by 4 mg/ml gentamicin in one patient. Addition of fLMP as an independent stimulus did induce histamine release in cells from 9 of the patients and this varied from 3.0% to 107% (Figure 4.9 b). Anti-IgE at a concentration of 0.01 mg/ml stimulated histamine release in seven patients that varied from 3.31 to 106% (Figure 4.9 a). There was no correlation between the degree of histamine release in response to fLMP and anti-IgE (Figure 4.9 c). However, addition of anti-IgE over a concentration range from 0.03 to 1% induced histamine release which might be indicate a bell-shaped curve (Figure 4.10).

Table 4.10: Histamine release from basophils induced by fLMP, anti-IgE and selected drugs

Patient number	Histamine net release (%) induced by fLMP (1µM)	Histamine net release (%) induced by anti-IgE (0.01mg/ml)	Histamine net release (%) induced by drugs
DAD08	39.6 (35.8-43.4)	0	0 Diclofenac
DAD11	3 2.96 (2.9-3)	4 (4-4)	40.8 Meloxicam (0.75 mg/ml)
DAD 13	77.8 (77.8-77.8)	59.8 (42-72.6)	0 Chlorhexidine 0 Lidocaine 0 Ondansetron
DAD16	28.8 (28.5-29.1)	0	0 Panacuronium 0 Midazolam
DAD17	39.4 (32.9-45.9)	102 (101-103)	0 Chlorhexidine 0 Gentamycin
DAD20	81.2 (80.9-81.50)	0	90.4 Gentamycin (1/10) 0 Rocuronium 0 Fentanyl
DAD25	107 (100-113)	91.5 (89-94)	0 Chlorhexidine 0 Rocuronium
DAD27	18.7 (18-19.4)	45.2 (43.8-46.6)	0.6 Diclofenac (1.25 mg/ml) 33.3 Meloxicam (o.75 mg/ml) 2.9 Meloxicam (0.1 mg/ml) 2.7 Amoxicillin (0.1 mM)
DAD30	0	3.3 (0.8-5.8)	0 Rocuronium 0 Chlorhexidine 0 Propofol
DAD35	50.4 (43.8-57)	92.6 (90.3-94.9)	0 Chlorhexidine
DAD41	12.1 (10.4-13.7)	106 (105-106)	0 Amoxicillin
DAD45	1.3 (0-2.6)	52.1 (51.7-52.5)	0 Articurium 0 Rocuronium 0 Suxamethonium

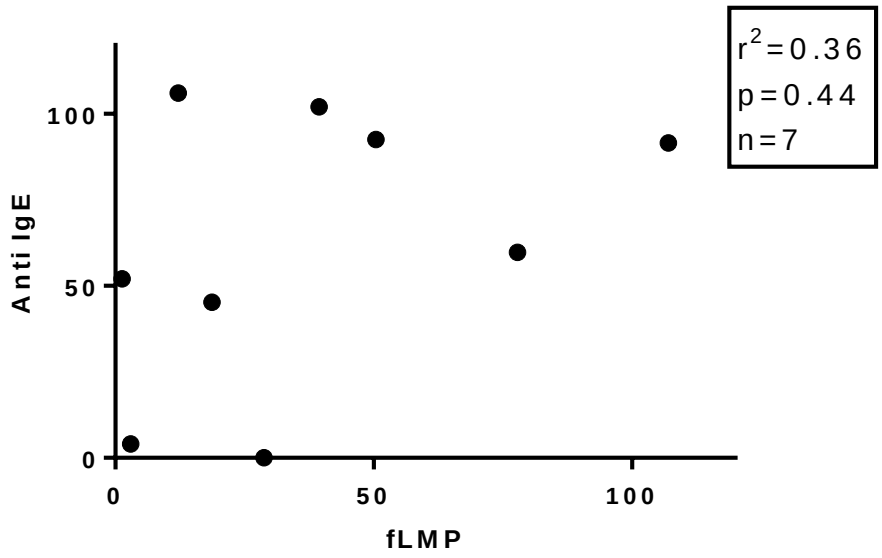

Figure 4.9: **Release of histamine from allergic patient blood basophils induced by anti-IgE and fLMP.** Anti-IgE induced histamine net release blotted against fLMP triggered histamine net release.

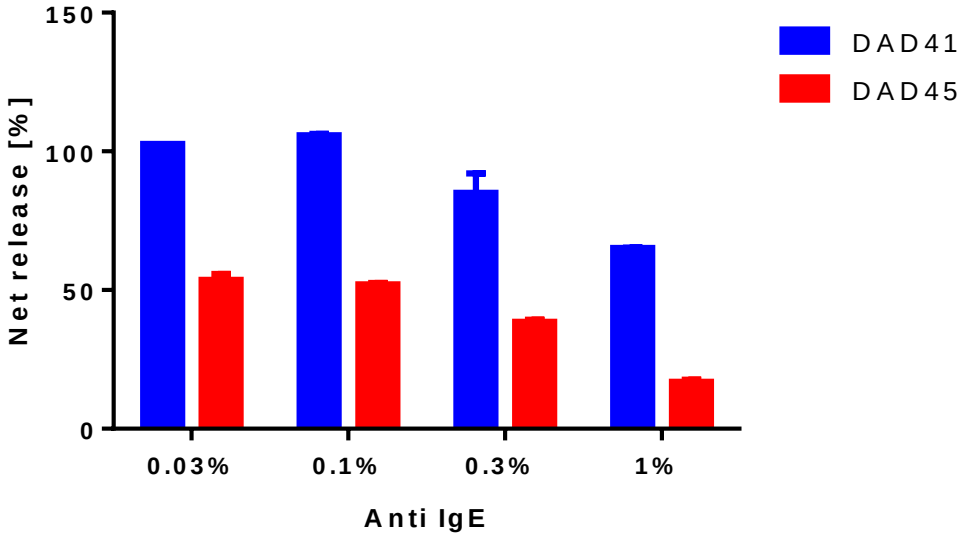

Figure 4.10: Basophil histamine release induced by anti-IgE. Data represent the mean ± SEM for two different patients

4.3.4 Expression of CD63

Validation of *in vitro* basophil activation

In order to investigate how long basophils may remain viable following collection, the basophil activation test was carried out 4, 24 and 48 hours after blood collection. Over the period studied, there was a decrease in the extent to which CD63 expression could be up-regulated. Numbers of activated basophils (taken as the numbers of CCR3 and CD63 positive 'events') were reduced from 299 at 4 hours to 151 at 24 hours after collection, and then dropped to just 29 at 48 hours after collection (Figure 4.11(.

Measurement of CD63 up-regulation after basophil activation by drugs

Ten patients with suspected drug hypersensitivity were tested for up-regulation of CD63 expression. In five patients there was an increase in CD63 expression of greater than 5% and a stimulation index (SI) higher than 2 which was taken as a positive reaction. In two of these five patients (patients ID DAD 13 and DAD 24) there were percentage increases of some 80 to 100%, and SI of greater than 10 representing a substantial response (Table 4.11). The other five patients with positive tests did not show a CD63 up-regulation according to the criteria employed. However, there were two patients who had either up-regulation of CD63 expression of greater than 5 or an SI greater or equal to 2%, but not both together. When cells from seven patients were stimulated with 3 ug/ml anti-IgE, CD63 expression in all seven patients was greater than 25%.

When 0.875 µg/ml fMLP was added to basophils from four patients, CD63 up-regulation greater than 80% was seen with basophils from one of them. With the other three patients CD63 expression following addition of fMLP was less than 10% (Table 4.11).

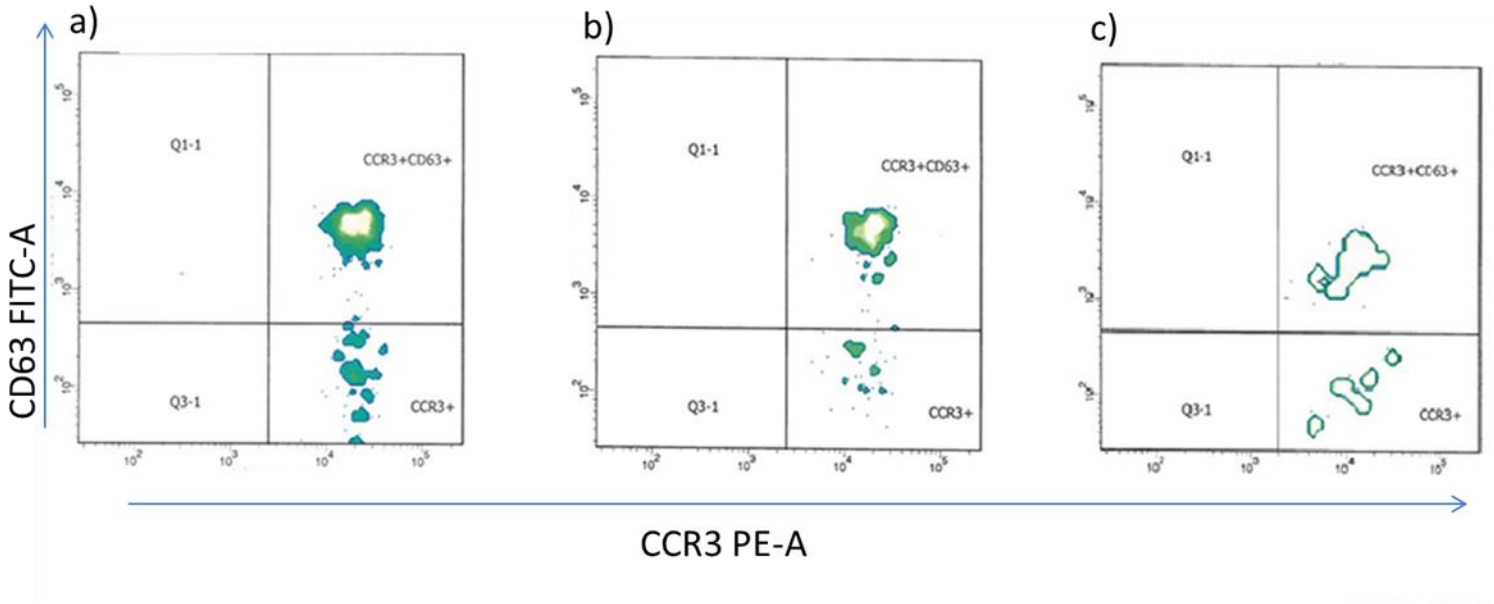

Figure 4.11: Basophil activation in cells at various periods following collection. Basophils were stimulated with chlorhexidine after they had been stored at 4 ºC for (a) 4 hours, (b) 24 hours and (c) 48 hours.

Table 4.11: Outcomes of basophil activation test, skin testing and drug-specific IgE determinations in patients with suspected of having had a drug reaction

Patient ID number	Drug	Outcome of skin test	Measurement of specific IgE kU_A/l[1]	Basophil activation test			
				CD63+ up-regulation[2]	SI[3]	Positive control-anti-IgE (3µg/ml)	Positive control-fLMP (0.875 µg/ml)
BD1	Chlorhexidine	Positive	< 0.35	7%	1.75	56%	ND
BD2	NMBMIX	ND	< 0.35	1%	1	43%	ND
	Atracurium	Positive	< 0.35	1%	1	43%	ND
	Vecuronium	Positive	< 0.35 l	1%	1	43%	ND
	Rocuronium	Positive	< 0.35	1%	1	43%	ND
BD3	Ibuprofen	ND	ND	8%	8	61%	ND
	PPL	ND	< 0.35 l	1%	1	61%	ND
	MDM	ND	< 0.35	3%	3	61%	ND
	Amoxicillin	ND	< 0.35	5%	5	61%	ND
BD4	Ibuprofen	ND	ND	0%	0	ND	4%
	1.25 mg/ml diclofenac	ND	ND	7%	7	ND	4%
	0.3 mg/ml diclofenac	ND	ND	1%	1	ND	4%
	0.06 mg/ml diclofenac	ND	ND	1%	1	ND	4%
	0.01 mg/ml diclofenac	ND	ND	1%	1	ND	4%
BD5	Patent blue	Positive	ND	11%	1.2	ND	9%
	NMBA mix	ND	ND	8%	2.67	ND	9%
	Rocuronium	Negative	ND	14%	7	ND	9%
	Articurium	Positive	ND	9%	3	ND	9%
	Vecuronium	Negative	ND	14%	7	ND	9%
BD6	Ibuprofen	ND	ND	2%	2	68%	ND
BD7	MDM	Positive	ND	1%	1	ND	8%
	Amoxicillin	Positive	ND	1%	1	ND	8%
DAD16	NMBAMIX	Positive	ND	2%	0.5	25%	ND
DAD13	Chlorhexidine	Positive	1.2	79%	79	85%	83%
DAD24	Rocuronium	Positive	12.5	98%	12.25	98%	ND
	Atracurium	ND	ND	97%	12.12	98%	ND
	NBMAMIX	ND	ND	100%	12.5	98%	ND

[1] kU_A/l is a unit which express the allergen-specific IgE level evaluated against a total IgE calibration curve. One kU/L is almost 2.5 ng/ml and should express the level of IgE in peripheral blood [123, 124].[2] CD63 + up regulation is the calculated percentage of the CD 63 positive cells compared to the total numbers of basophils. [3] The stimulation index (SI) has a lower cut-off for drugs and other chemical allergen to avoid false positive results and is calculated by dividing the allergen stimulation with the negative control (stimulation buffer).

4.3.5 Comparison of in vivo and in vitro assays of specificity

Clinical data for 17 patients is shown with the results of the *in vitro* basophil activation assays (Table 4.12). Of these, fourteen patients underwent either skin testing or oral challenge. One patient was negative on allergen challenge in the clinic, and thirteen patients had a positive test outcome. Measurements of specific IgE against the drug implicated were available for eight patients, and drug-induced alterations in expression of CD63 were determined in eight patients. Basophil activation as assessed by measurement of CD63 up-regulation following addition of the drug implicated was detected in three patients who had a positive outcome on challenge with the same drug. Two of these patients had suffered anaphylactic shock previously. Of thirteen patients with positive test outcome, three had also a high level of specific IgE and three patients of the thirteen had neither a high level of specific IgE nor up-regulation of CD63 on basophil challenge with the drug implicated. However, seven of the thirteen patients who had a positive outcome had suffered anaphylactic shock previously to the drug that was examined.

Drug induced up-regulation of CD63 on basophils was investigated in ten patients. In four patients the results of the pattern of CD63 up-regulation did correspond with specific IgE measurements. Histamine release from blood basophils after drug challenge was detected in one patient who had a negative outcome on *in vivo* challenge with the same drug, and one patient who had a positive outcome on challenge with the same

Table 4.12: Summary of laboratory measurements of allergic sensitivity to drugs considered against the nature of the reaction experienced and response on *in vivo* challenge of seventeen patients with suspected drug hypersensitivity

Patient number	Historical reaction	*In vivo* test outcome	Measurement of specific IgE	Basophil activation	
				CD63 up-regulation	Histamine release
DAD13	Anaphylaxis	+	+	+	-
DAD16	Anaphylaxis	+	-	-	-
DAD17	Anaphylaxis	+	-	ND	-
DAD20	Anaphylaxis	+	ND	ND	+
DAD24	Anaphylaxis	+	ND	+	ND
DAD30	Anaphylaxis	+	+	ND	-
DAD35	Sickness, coughing, tachycardia	+	ND	ND	-
DAD41	Swelling, rash, tight chest	+	ND	ND	-
DAD45	Severe bronchospasm, high ventilation	+	+	ND	-
BD1	Swelling of arm	+	-	-	ND
BD2	Anaphylaxis, rash	+	-	-	ND
BD5	ND	+	ND	+	ND
BD7	ND	+	ND	-	ND
BD6	ND	ND	ND	-	ND
BD3	Anaphylaxis	ND	-	+	ND
BD4	ND	ND	ND	+	ND
DAD27	Breathing problems, swelling	-	ND	ND	+

5. Purification of tryptase from human lung

When tryptase was extracted from chopped human lung with low and high salt buffers, the highest protein concentrations were found in the first of the low salt buffer extracts (LSB1) and in the first high salt buffer extract (HSB1). However, the tryptase activity was much greater in the first high salt buffer extract (81.4 ± 4.71 milliunits/ml (mU/ml)) than in the first low salt extract (15.5 ± 0.95 mU/ml) (Figure 5.1Error! Reference source not found.). Subsequent low and high salt buffer extracts had a much lower tryptase/mlivity and protein concentration. On the basis of these findings, the first high salt buffer extract was subjected to a further purification step. When the extract was added (after desalting) to the HiPrep-butyl FF 16/10 column (following equilibration with the loading buffer), tryptase activity was eluted by decreasing the ammonium sulphate concentration in the column as a sharp peak (Figure 5.2a). Highest tryptase activities appeared in Fractions 9 (577 mU/ml) and 10 (898 mU/ml), and were decreased in subsequent fractions. Fractions 9 to 30 were collected and transferred on to an equilibrated HiPrep-heparin FF 16/10 for affinity interaction chromatography. Tryptase-rich fractions were eluted over aNaCl gradient, with peak activity detected between Fractions 51 and 71 (Figure 5.2b). The highest tryptase activity was found in Fraction number 61 (436 mU/ml), and this was eluted at a NaCl concentration of 1.0 M. The fractions with tryptase activity were eluted at 0.845 to 1.175 M NaCl. Fractions with over 40 mU/ml tryptase activity were pooled (preparation A), and also those with activity between 5 mU/ml and 40 mU/ml (preparation B). The two preparations were concentrated and analysed with the samples from other stages of the purification process on a 10% gel by SDS-PAGE with Coomassie blue staining, and by western blotting with a tryptase-specific antibody. The western blot showed a band at approximately 30 kDa for which there was increased intensity with progressive tryptase purification (Figure 5.3a). On the Coomassie blue-stained gel, the majority of staining was of the 30 kDa band, though there were also minor bands which did not appear on the western blot (Figure 5.3b).

A gel filtration step undertaken to increase the purity of the tryptase in the partially purified preparation resulted in a single peak of tryptase activity (Figure 5.4). All fractions had a protein concentration greater than 8 ug/ml as determined by Bradford protein assay (Table 5.1). When fractions were investigated to assess the purity of fractions by Coomassie blue and silver staining, the presence of a major band of about 35 kDa was identified in fraction 10 and at a lower intensity in fraction 11 (Figure 5.5a). There was a high degree of purity for fraction 10 and little evidence for contaminating proteins of other molecular weights. With the more sensitive silver staining method, there was a band at 100 kDa in fraction 11, and also in fractions 12, 14 and 13 which also exhibited the 35 kDa band to some extent (Figure 5.5b).

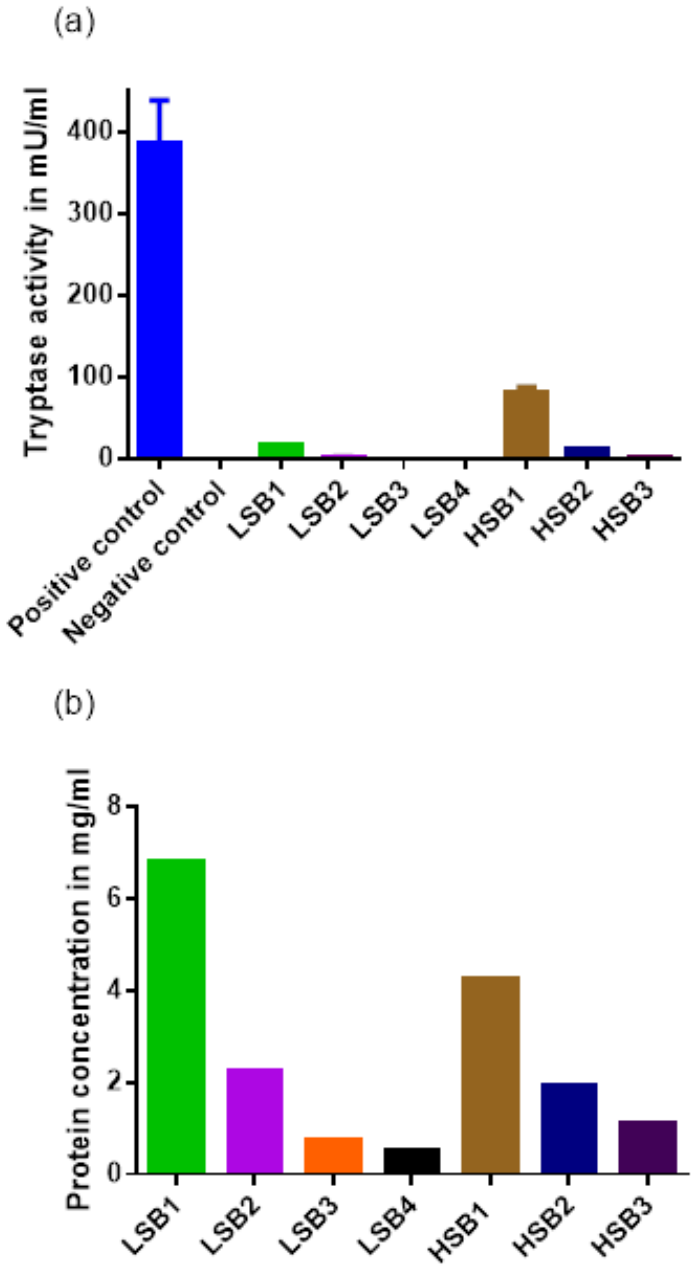

Figure 5.1: Tryptase activity and protein concentration of four low salt buffer extractions (LSB1-4) and three high salt extractions (HSB1-3). For determination of tryptase activity, a positive control consisted of a purified preparation of tryptase and the negative control of buffer alone.

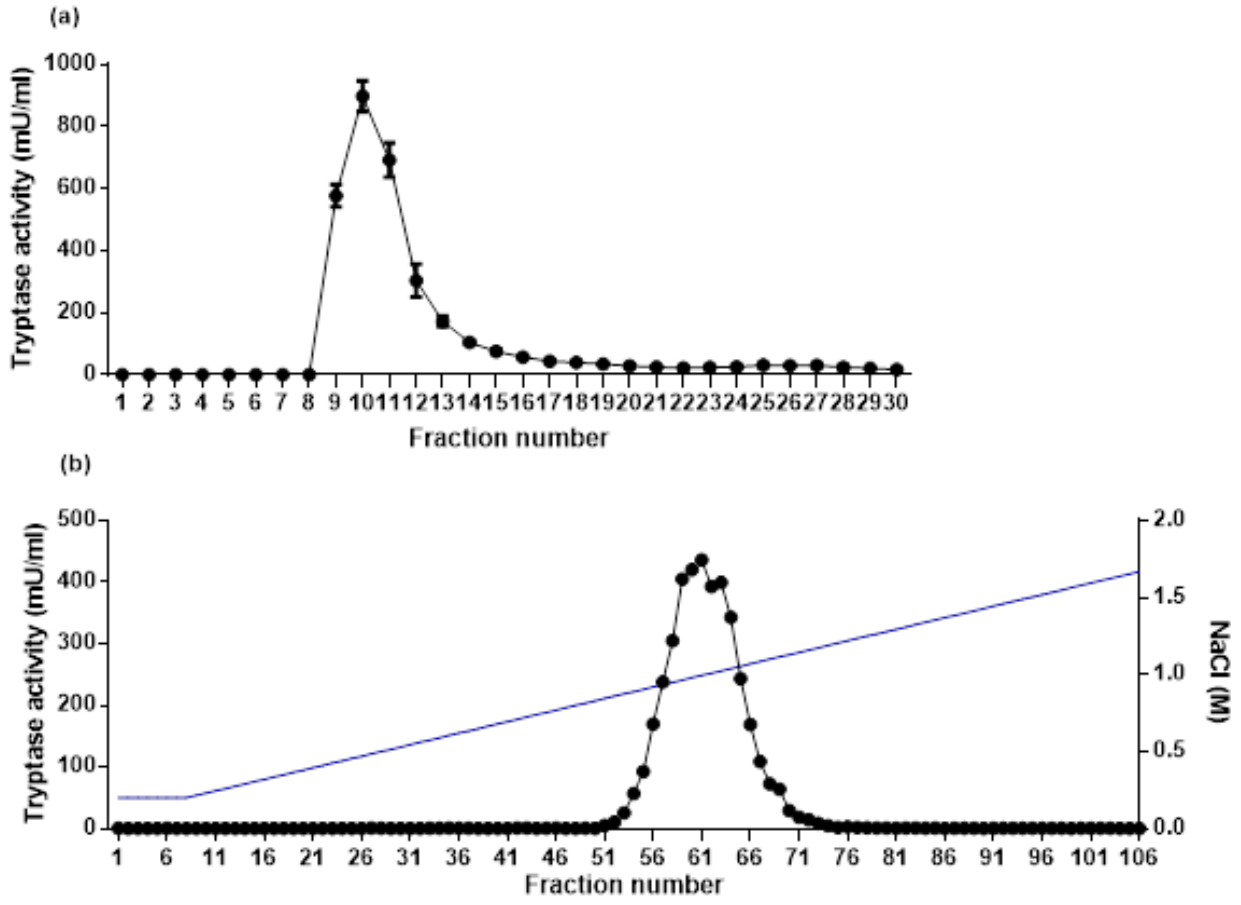

Figure 5.2: **Elution pattern for hydrophobic interaction and affinity chromatography.** Tryptase activity measured fractions collected from (a) a HiPrep-butyl FF 16/10 and (b) a HiPrep-heparin FF 16/10 column.

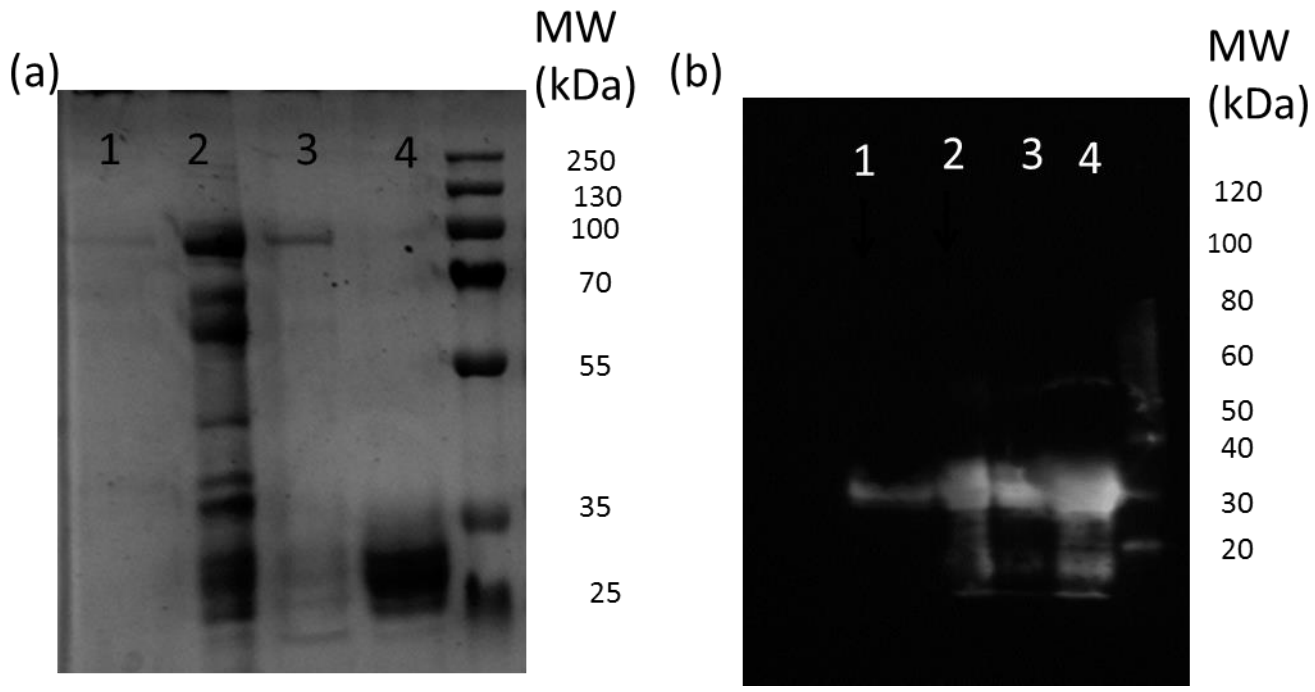

Figure 5.3: Analysis of partially purified fractions of tryptase by (a) 10% SDS-PAGE with Coomassie blue staining, and (b) Western blotting with tryptase-specific monoclonal antibody AA5. (1) First high salt extract of lung tissue, and fractions following separation on (2) butyl agarose, (3) heparin agarose purified fraction A, and (4) heparin agarose purified faction B. The size of the molecular weight markers are indicated

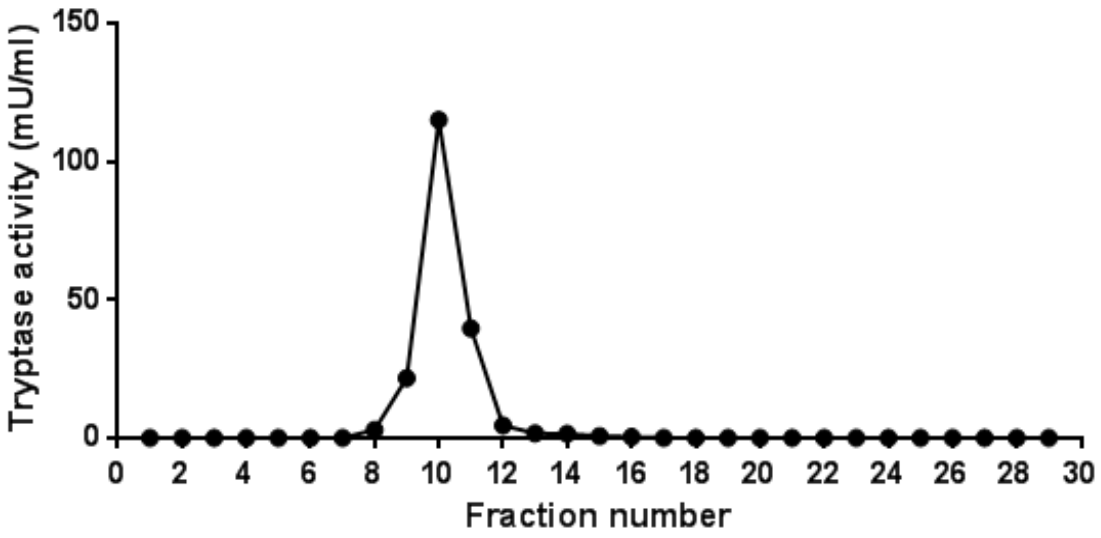

Figure 5.4: Tryptase activity in fractions of a partially purified preparation of tryptase subjected to S200HR gel filtration.

Table 5.1: Tryptase activity, protein concentration and specific activity of fractions from S-200HR gel filtration

Fraction number	Tryptase activity (mU/ml)	Protein concentration (mg/ml)	Specific activity (mU/mg)
8	2.92	0.013	226
9	21.7	0.012	1,808
10	115	0.018	6,389
11	39.7	0.017	2,335
12	4.55	0.013	350
13	1.67	0.01	167
14	1.52	0.008	190

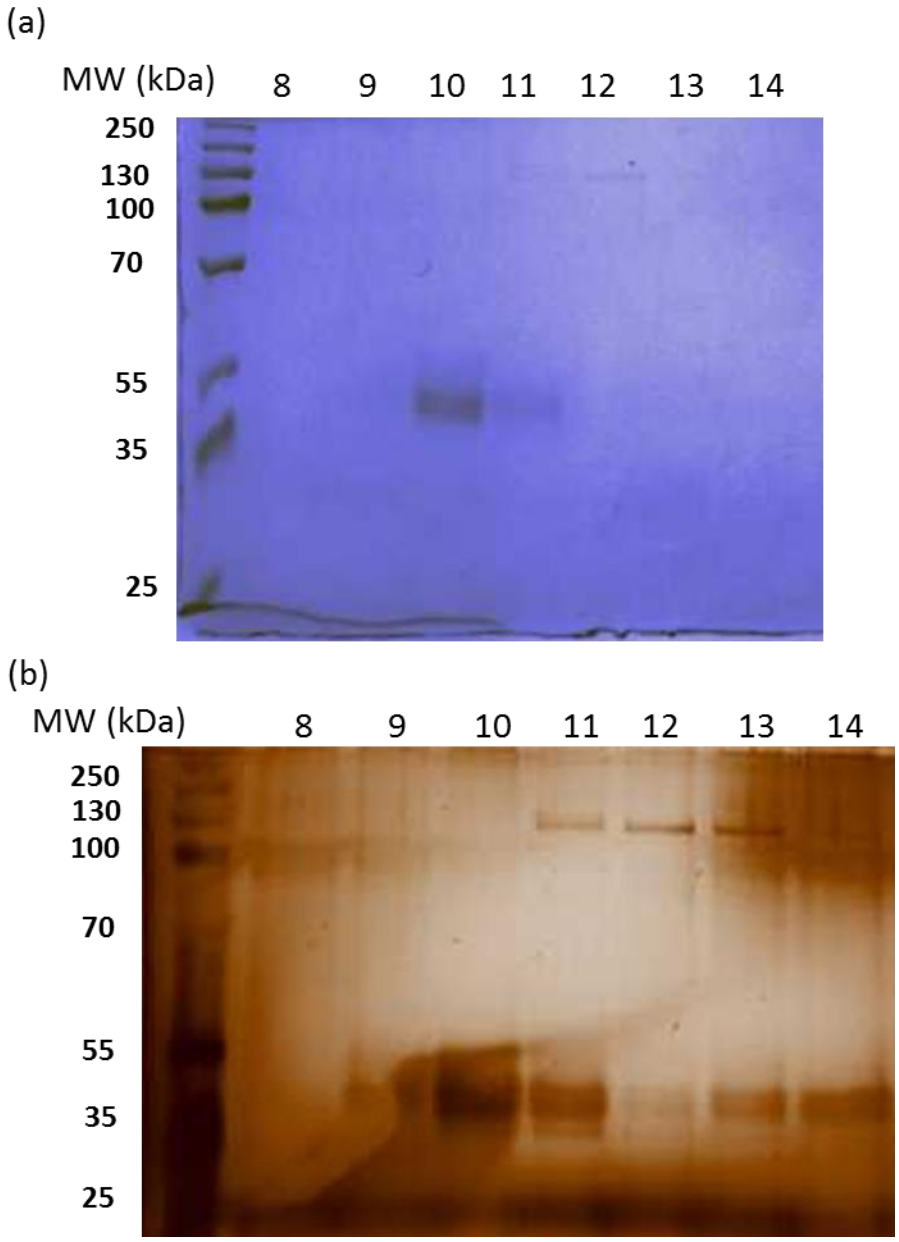

Figure 5.5: Analysis of fractions recovered on gel filtration by 10% SDS-PAGE with (a) Coomassie blue staining, and (b) sliver staining.

Table 5.2: Total volumes, tryptase activity, protein concentration, specific activity and degree of purification at different stages of purification from human lung tissue. Tryptase activity was determined by cleavage of the chromogenic substrate BAPNA. Protein concentration was determined by the Bradford protein assay and NanoDrop.

Step	Volume (ml)	Tryptase activity (mU/ml)	Protein concentration (mg/ml)	Specific activity (mU/mg)	Purification
High salt extraction 1	1,000	81.42	4.27	19.1	1
Post butyl agarose	110	1,434	4.49	319	16.7
Post heparin agarose	46.2	1,681	1.680	1,000	52.4
Gel filtration Fraction 10	5	115	0.018	6,389	335

6. Purification and optimisation of basogranulin detection

In evaluating means for detection of basogranulin, dot blotting procedures were evaluated using specific monoclonal antibodies BB1 and BB5. As purification of basogranulin has proved problematic, lysates of PBMC or purified basophils were employed as the standard source of this basophil marker in assays. Negative controls were performed at the involved use of cell culture medium or wash buffer (PBS-Tween) instead of 1/10 BB15 or BB5 antibody, dilution and 1/3 BB1 antibody dilution to exclude any un-specific binding of other controls included omitting the secondary antibody, cell culture medium or wash buffer (PBS-Tween) . It was found less than 16,000 arbitrary units as un-specific binding in all controls for BB1 and BB5 (Figure 6.1(. There was an increasing the PBMC concentration resulted in increasing signal detected over the range of dilutions of PBMC investigated, and with a stronger signal with a 1/3 than a 1/50 dilution of BB5 (Figure 6.2(. With both BB5 antibody dilutions, the PBMC lysate solution treated with red blood cell lysis buffer was slightly higher than the PBMC lysate without this treatment, and so was employed in subsequent studies.

Storage of the PBMC lysate for 14 days at -20ºC (and treated twice with red blood cell lysis buffer to make sure that all red blood cells were removed) was associated with substantial loss of signal in the dot blotting assay. This was observed, when findings were compared with those for assays (conducted under identical conditions) with the same PBMC lysate stored for just one day at -20ºC (Figure 6.3(. This was a consistent finding over a wide range of dilutions of PBMC lysate, and when the supernatant containing BB5 antibody was diluted either 1/3 or 1/10. On account of the apparent poor stability of basogranulin in PBMC lysates stored at -20ºC, it was investigated if this basophil product may be more stable in a lysate of purified basophils stored at -80ºC. A basophil lysate stored at -80ºC for 12 day old was found to have high amounts of detectable basogranulin, but not a lysate (same batch of purification) stored for 21 days. (Figure 6.4(.

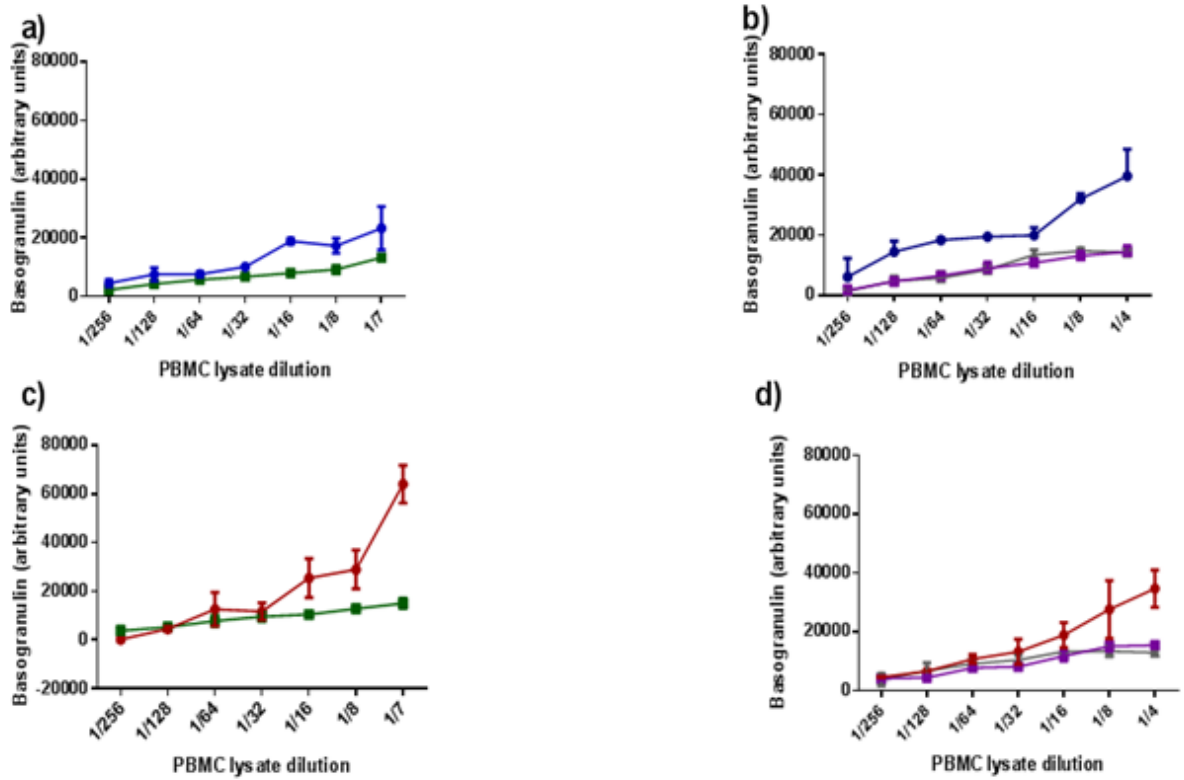

Figure 6.1: Comparison of nonspecific binding with secondary antibody, cell culture medium or wash buffer (PBS-Tween) in dot blot for basogranulin. Three different negative controls: no primary antibody but secondary antibody (purple line; b and d), only wash buffer (grey line; b and d) and cell culture medium (green line; a and c), were employed when investigating use of BB1 or BB5 to detect basogranulin in lysates of PBMC. In addition, BB5 (diluted 1/10; blue line; a and b) and BB1 (diluted 1/3; dark red line; c and d) were employed with secondary antibody against mouse IgM and IgG respectively. Results shown represent the mean ± SEM of triplicate determinations in one experiment.

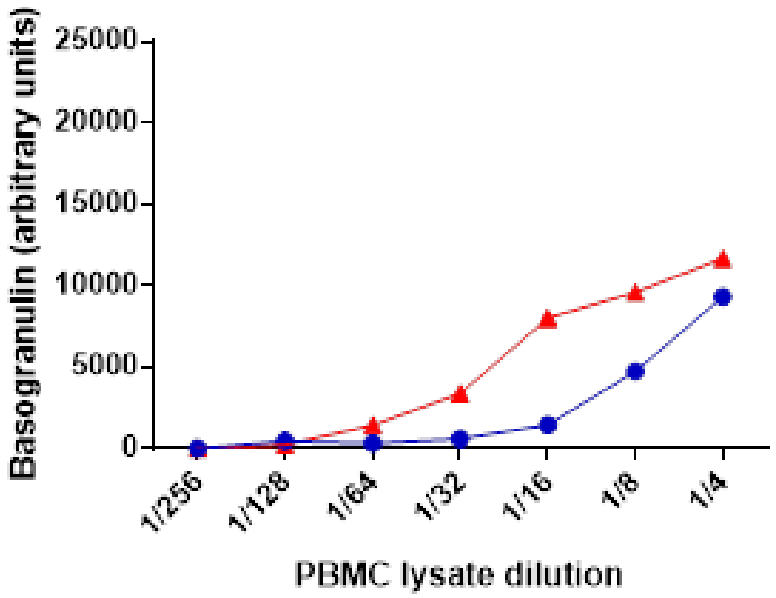

Figure 6.2: Relative levels of basogranulin detected by dot blotting with BB5 antibody diluted (a) 1/3 or (b) 1/50 dilution. The assay was performed with PBMC preparations which had been treated with (red) or without (blue) a red blood cell lysate buffer. Results shown are the mean of duplicate determinations in arbitrary units.

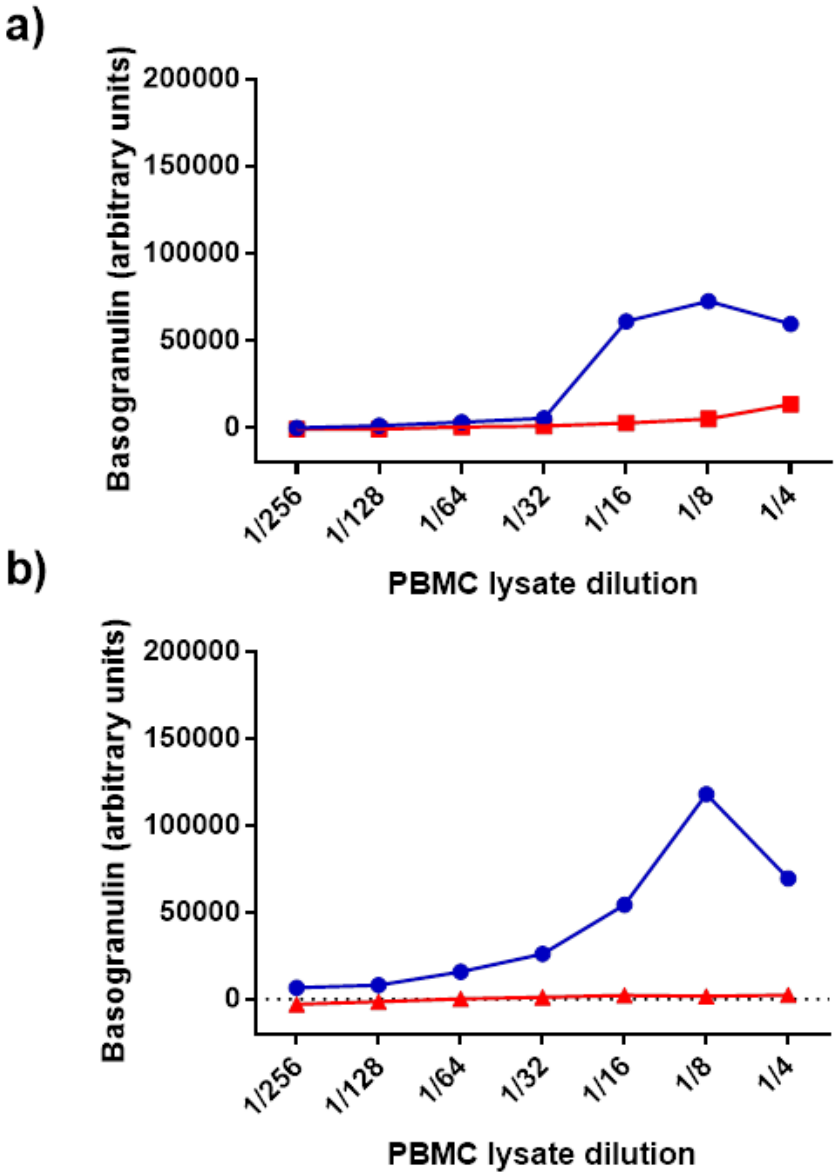

Figure 6.3: Relative levels of basogranulin detected in PBMC lysate stored for one (blue) or 14 days (red) at –20°C. Studies were performed by adding BB5 culture supernatant diluted (a) 1/3, or (b) 1/10 Data represents median of duplicate determinations.

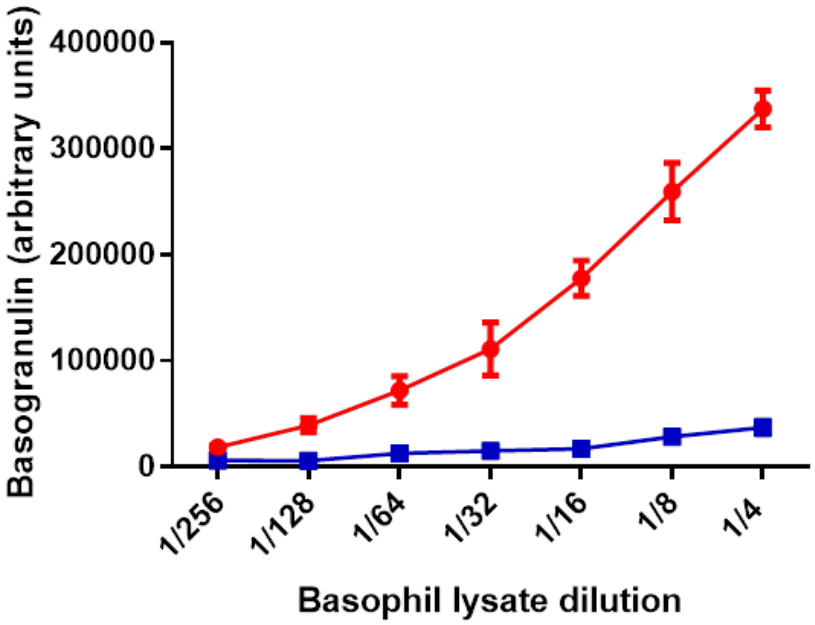

Figure 6.4: Stability of basogranulin in purified basophil lysate. A basophil lysate stored at -80°C for 12 days (red line) was compared with the same lysate when stored for 21 days (blue line). The experiment was performed with BB1 culture supernatant diluted1/3, and results are shown as the mean ± SEM triplicate experiments of one experiment

7. Discussion

The findings of the present study have indicated the diverse range of clinical responses to drugs, even in the carefully controlled environment of a diagnostic allergy clinic involving skin or oral challenge. Some light has been shed on the challenge of establishing a reliable diagnosis of specific hypersensitivity to drugs, with discrepancies between the results of drug challenge and those of laboratory tests involving in vitro challenge of basophils or of cells of a passively sensitised mast cell-like line, and measurements of drug-specific IgE.

Characteristics of recruited patients with suspected drug hypersensitivity

Of the 214 patients investigated in the present study, more had a negative test outcome than positive outcome. The reasons for this will be related the study population analysed. Some patients who were recruited for this study had a negative specific IgE test outcome and the skin testing was performed to confirm the negative outcome. The patients who attended the Adult Allergy Clinic at Southampton General Hospital were chosen by the physician and various criteria were applied, including the need for urgent surgery or medication.

There was no association between outcome of the drug challenge and the patient suffering from chronic allergic conditions such as hayfever or asthma. On the other hand, having a history of eczema was associated with a negative outcome on drug testing in our study population. There was little evidence in the present studies to support the idea that drug allergy is associated with atopic diseases. In contrast with our findings with drug hypersensitivity, it has been reported by Summers et al. that in patients with nut allergy there may be an association between having eczema and suffering systemic allergic symptoms [125]. That study involved examining a mixed population of children and adults in Manchester, UK. Another finding of that study was that the patients with asthma had more severe allergic symptoms of upper and lower airways in reactions to peanuts and tree nuts. A study of allergic reactions in children and adults attending 79 clinics in Germany, Austria and Switzerland, has indicated that atopic patients are more likely to develop respiratory symptoms than circulatory symptoms [29]. However, the suggestion that atopy may be associated with the severity of an allergic reaction was not in accord with the findings of Summers et al. who did not find any correlation between a history of either asthma or eczema and the size of the weal elicited on skin prick testing with food [125].

An explanation for the apparent discrepancy between the two studies could be that atopic conditions have only an influence of the severity of the allergic symptoms when the causal agent is already in the blood system of the patient and not when locally applied. Another explanation could be related to the age of the patients studied. Whereas the patients we

investigated were all over 18 years, the studies which have shown included both children and adults.

Of the 578 drug challenges for which data was collected, the greatest numbers were for antibiotics, followed by local anaesthetics, general anaesthetics and NMBA. The ranking of drug categories here are similar to those reported in a survey by the World Allergy Organisation. In questionnaires filled out by members of that organisation, antibiotics emerged as the most commonly tested drug group followed by local anaesthetics and general anaesthetics in Europe [126]. In our study, antibiotics provoked more positive test outcomes than any of the other drugs tested, followed by antiseptics and NMBA. Harboe et al who investigated triggers of anaphylaxis during anaesthesia found that NMBA caused the greatest number of cases of anaphylactic shock in Norway [31]. Similar findings were made independently by Chong et al. in London, UK and Laxenaire et al. in France when seeking to identify the most common elicitors of anaphylactic shock during anaesthesia. [32, 127]. It has to be pointed out that in all three studies the patients who had an allergic reaction during anaesthesia were in most cases receiving NMBAs as well as local anaesthetics and antiseptics. However, it seems that the antiseptics were not the allergen provoking the anaphylactic shock in these studies instead it was mostly NMBA's and local anaesthetics. In contrast to the findings in France, UK and Norway, we identified that in our recruited study population patients who were challenged with antiseptics had more positive test outcome than negative test outcome followed by NMBA and antibiotics. This suggests that actually antiseptics next to NMBA can also be a trigger for anaphylactic shock. In a survey of general anaphylactic reactions in Central Europe it has been found that analgesics, antibiotics and local anaesthetics are the drugs that are the most common triggers of anaphylactic reactions [29].

In our study, most patients tested for local anaesthetics gave negative reactions and only in rare cases was there a positive reaction. In an investigation of triggers of anaphylactic shock in a study population living in Central Europe, Worm et al. found that local anaesthetics were the eliciting agent in 43% of cases [29]. However, the study did not confirm the drug hypersensitivity by carrying out an *in vivo* drug challenge or *in vitro* laboratory tests such as measurement for specific IgE or basophil activation. Differences between studies could possibly be related to the local anaesthetic-induced reactions being mediated by processes of delayed hypersensitivity (Type IV) rather than immediate hypersensitivity (typeI). The work of Zanni et al. has indicated that in patients with an allergic reaction to the local anaesthetics, lidocaine can exhibit specific T cell reactivity to this drug [128]. This group collected PBMC from four patients who had suffered a previous reaction to lidocaine, and stimulated the T cells with lidocaine. They found that lidocaine can activate CD4+ T cells and this raises the

possibility that allergic reactions induced by lidocaine could involve type IV/delayed hypersensitivity mechanisms.

Analgesics including NSAID are being implicated as triggers for an increasing numbers of anaphylactic reactions. In the study of Brown et al. it was reported that 32 out of 250 patients were diagnosed with hypersensitivity to NSAID [30]. However, in our study we found only one patient who had a positive test outcome to a NSAID of the nine patients tested in this study. Moreover, the patients were not tested for the same preparation of drug which had provoked the allergic reaction, and instead an alternative NSAID was investigated to avoid a severe reaction occurring during testing.

The antiseptic chlorhexidine and the NMBA rocuronium triggered the most positive skin reactions in the present study. Skin reactions were the most common symptoms provoked by drug challenge as would be expected with the drug injected under the first layer of skin. However, there were symptoms provoked in tissues other than skin and even in the absence of a skin reaction altogether. It is possible that different patterns of symptoms could involve different mechanisms or cell types, and the release of different mediators, and this requires investigation in future studies.

Comparison of *in vivo* and *in vitro* assays to identify drug hypersensitivity in patients

Direct challenge of cells of the LAD2 human mast cell line with various drugs did not result in release of β-hexosaminidase. Of those tested, only chlorhexidine was found to stimulate the release β-hexosaminidase and this was at the highest concentrations employed. However, addition of metabolic inhibitors to the assay showed that at these concentrations chlorhexidine had a cytotoxic effect on the cells, suggesting that chlorhexidine had not activated the mast cells through a distinct non-IgE dependent process.

As RBL-703-21 or RS-ATL8 cells express the human IgE receptor and are designed to degranulate in response to allergen after sensitisation, they were sensitised with serum samples from patients suspected of sensitivity to benzylpenicillin, chlorhexidine, lidocaine and rocuronium. However, benzylpenicillin, chlorhexidine, and lidocaine elicited little net release of β-hexosamindase from RBL-703-21 cells incubated with patient serum, and no difference in drug-induced releasability was found between patients with positive and negative challenge outcomes. On the other hand, there was release of β-hexosaminidase from RBL-703-21 cells which had been sensitised with serum from rocuronium-allergic patients, and this was significantly higher than when incubated with serum from of non-

rocuronium allergic patients. Nevertheless, net release was below 10% and the difference observed between serum samples could thus be caused by assay variability.

High net release of β-hexosamindase from RBL cells after serum sensitisation and allergen challenge has been reported by Ladics et al. [107]. When they investigated three different RBL cell lines sensitised with serum from peanut-sensitive patients and challenged with peanut allergen, they found that the net release of β-hexosaminidase varied greatly between cell lines. It was found that the cell line RBL-30/25 (transfected with the α subunit of human FcεRI receptor) and RBL-SX-38 (transfected with the α, β, and γ subunits of human FcεRI receptor) released β-hexosaminidase after peanut challenge. The work of Dibbern et al. has shown release of radiolabeled serotonin from RBL-SX-38 cells after sensitisation with serum of peanut allergic patients collected following peanut challenge [129]. Neither study employed the RBL cell line used in the present study, but the potential for different releasability between cell preparations could be relevant.

Ladics et al. have reported that high release of β-hexosaminidase cannot be induced by allergen in RBL cells following incubation with serum samples from some patients [107]. These authors proposed that degranulation could be dependent on the levels of specific IgE present in serum samples. A study by Dibbern et al. indicated that the amount of total IgE and specific IgE may be important for the induction of degranulation. With levels of peanut-specific IgE lower than 30 kUA/L in serum samples the net release of β-hexosaminidase was below 10%. The findings of both groups suggested that specific IgE levels have to be greater than 15 kUA/ml to allow allergen-specific degranulation to occur with sensitised cells. In the present study it is possible that the patients' serum used for sensitisation had a low a level of specific IgE and that this could explain why there was little release of β-hexosaminidase.

Levels of specific or total IgE in the serum could possibly be altered by long term storage and the freeze/thaw process could also affect stability. As the aliquots of serum were available in limited quantities and were thus used multiple times, degradation can be not excluded. The group of Henderson et al. showed that serum samples collected and stored at –70 or –20 °C had still measurable total and specific IgE after 32-37 years [130]. However, the group did not compare the total and specific IgE level at the collection day and after 32-37 years. Loss of IgE during storage seems unlikely, but it cannot be completely ruled out that there might have been some degradation in the present study.

The RS-ATL8 cell line is not only transfected with the α, β, and γ subunits of human FcεRI receptor but also with a luciferase gene [121]. We investigated this cell line but did not see β-hexosaminidase release higher than seen with the other RBL line investigated. The cells

were easily detached from the wells of 96-well plates, and the lack of attachment could have affected the results. Nakamura et al. reported that this new cell line can be sensitised with serum from egg allergic patients, and that following allergen challenge luciferase expression could be measured [121]. However, that that study involved sensitisation of cells with serum samples with low levels of specific IgE that did not induce much luciferase expression.

The RBL-703-21 cell line employed in the present studies was transfected with the human α chain, which associates, with the rodent β and γ chains to form the FcεRI receptor [131, 132]. Loss of the human β and γ chains from FcεRI on the cell membrane could represent another possible reason for low levels of β-hexosamindase release. A study of Takagi et al. found that RBL-703-21 cells transfected with the human α chain not only released β-hexosamindase after anti-IgE challenge, but also after allergen challenge following sensitisation with mouse IgE against DNP [133]. These results suggest that the cells also express an intact rat FcεRI receptor, which has the rat α chain. Expression of rat FcεRI receptor on the RBL-703-21 cells can decrease the amount of human FcεRI receptor present, and therefore the number of cells which can be activated through this receptor may be reduced. Wilson et al. found also that RBL-703-21 cells transfected with the human α chain express FcεRI receptors that are functional for human IgE, andthat rat IgE can also bind to this receptor and induce degranulation [131].

Scatchard analysis of data from studies involving incubation of a range of concentrations of ^{125}I-labelled human or rat IgE in presence or absence of a 100-fold excess of non-labelled IgE has suggested that the highest degree of human IgE binding may be to just 13% of the FcεRI molecules expressed [131]. The degree of β-hexosaminidase release seen in our study is likely to depend on how many receptors are available for binding by specific IgE. Wiegand et al. have observed that RBL-SX38 are able to express approximately 100,000 receptors/cell whereas basophils can express up to 500,000 receptors/cell after culturing *in vitro* with IgE for seven days [134, 135]. Thus, cells of the cell line express five times fewer receptors than basophils, and this could be even less if the rat FcεRI receptor is also expressed. This could provide an explanation as to why the degree of β-hexosaminidase release triggered through the FcεRI receptor was so low in the present study. To determine how many functional human FcεRI receptors may be expressed on the cell surface, flow cytometry and other appropriate approaches should be employed. It will be important to examine to what extent cell passage number or cell culture conditions could influence expression of the FcεRI receptor.

To investigate if the small amounts of β-hexosaminidase released following drug challenge was related to the drug allergens employed, parallel experiments were performed involving cell challenge with grass pollen following sensitisation with serum from grass pollen-allergic patients. These experiments also resulted in only a small amount of β-hexosaminidase release. In all cases, net release was less than 10%. When the reproducibility of the assay was investigated by performing the experiment three times on different days, the average percentage coefficient of variation was below 15%, though there were some big differences between the individual experiments. Our findings highlight the importance of optimising the experimental setup and processes to decrease variation between experiments. In addition, it has to be considered that some drugs can bind to other proteins or can be metabolised *in vivo* to form a reactive antigen which is more liable to cross-linking. In future experiments, creation of a drug-protein complex should be considered as a standard allergen for *in vitro* cell challenge.

Association between results of *in vivo* and *in vitro* tests in the diagnosis of drug hypersensitivity

When measurement of histamine release from peripheral blood basophils incubated with various drugs was investigated as a potential *in vitro* test for allergic sensitivity, release was found to be provoked in just three of the 13 patients examined, two who were challenged with meloxicam and one with gentamicin. In the other 10 patients, no histamine release was detected. A factor in the failure to stimulate histamine release could be the need for drugs of low molecular weight to act as haptens on endogenous proteins to enable cross-linking of drug-specific IgE and the triggering of basophil degranulation. It also might be that other cells are absorbed the drug and therefore it should performed with purified basophils to rule out interference from other cell or cell fragments Koller et al. found also low release of histamine from peripheral blood basophils incubated with benzylpenicilloyl poly-L-lysine (PPL) [136]. Of the seven patients investigated in that study, all had a net histamine release below 10%. A similar finding was made by Demoly et al. who observed that only 8 out of 35 patients had net histamine release greater than 10% [137]. In that study it was calculated that the sensitivity of the basophil histamine release test (the proportion of patients with a positive response who had reacted to the drug on skin testing) was 51.4% when a positive test outcome was taken as having a net histamine release over 5%. When a positive test outcome was taken as having net histamine release over 10%, sensitivity of the procedure fell to just 22.8%.

With fLMP as an independent stimulus, histamine release ranged between 3 and 107% in basophils from the 9 patients examined. With anti-IgE at a concentration of 0.01 mg/ ml there was release of 3 to 106% in the seven patients tested. An explanation for a figure for histamine release being calculated at greater than 100% is likely to be related to underestimation of the total quantity of histamine present in basophils, with incomplete liberation of histamine following basophil lysis. Various ways were investigated for optimising the protocol for lysing the peripheral blood basophils, involving heating the cells or subjecting to freeze/thaw cycles, but there remains a need for further optimisation of procedures.

Addition of anti-IgE to cells induced histamine release in a bell-shaped concentration response curve with maximal histamine release triggered at 0.1% anti-IgE. This suggests that at this concentration all available FcεRI receptors are occupied with anti-IgE and it leads to maximum release of histamine. Higher and lower concentrations of anti-IgE caused lower net histamine release, and with such concentration response curves there is a particular need to avoid false evaluation of the results through inappropriate selection of stimuli for IgE cross-linking. This pattern of release has been reported also by Mochizuki et al. [59]. Comparison between histamine release and CD63 up-regulation to identify differences between these two methods of assessing basophil activation could be not performed because of too few patient samples.

Analysis of CD63 up-regulation after basophil activation indicated that results are dependent on the period after blood collection. It was found that the blood should not be stored for longer than 24 hours to ensure a sufficient signal on flow cytometry. Sturm et al. have suggested that measurement of CD63 up-regulation should be carried out only with fresh blood to avoid any reduction in the ability of basophils to respond to allergen [138]. In the present studies, five out of ten patients with suspected drug hypersensitivity had a positive reaction.

A detailed comparison of drug-induced basophil activation *in vitro*, clinical history, drug challenge *in vivo* and specific IgE measurements was rendered difficult by there being some missing data. However, only six out of 13 patients who responded on drug challenge had either a high level of IgE specific for that drug, or had basophil activation in response to that drug *in vitro* (as assessed by measurement of CD63 up-regulation or histamine release). In four patients, a positive response on drug challenge *in vivo* was not confirmed using an *in vitro* assay. Only one patient had a positive *in vitro* test (basophil activation by measuring C63 up-regulation) but was negative on *in vivo* testing.

Several factors could have contributed to the lack of agreement between the various tests for allergic sensitivity other than the nature of the tests themselves. Among these, it was not possible in all cases to employ similar drug concentrations or the same preparations of drug. Only the studies of histamine release from blood basophils were performed with the same drug that had been used in the clinical testing. The assays for basophil CD63 expression and for specific IgE levels employed commercially available preparations of the drugs that had been optimised for each of these tests. For a closer comparison in future experiments these *in vitro* assays should be performed with the same drug that was used in skin testing or oral challenge.

Drug metabolism in the human body could provide a further reason for discrepancies found between the *in vitro* and *in vivo* test results. Gill et al. have shown that in rats, the drug sulfamethozazole (an antibiotic used for urinary infections) is metabolised and the chemical structure changed. It was also shown that sulfamethaxazole-specific IgG antibodies were still present in the rat after sulfamethaxazole treatment was terminated [139]. It seems likely that several drugs may be metabolised to a compound that may be more allergenic, or which may bind more readily to a protein to become an allergenic hapten. In addition, it might be also that in some drug-induced allergic reactions, IgG or specific T cells play an important role rather than IgE and mast cells or basophils (as seen with the local anaesthetic lidocaine [128]). This could explain why a positive histamine release response was seen with blood basophils from just one patient.

The measurement of drug-induced CD63 up-regulation on basophils allowed four patients to be identified as allergic who had high levels of drug-specific IgE in serum. However, none of the three *in vitro* tests investigated in the present study showed much sensitivity. The findings are in accord with some previous studies that have failed to show good agreement between measurements of CD63 up-regulation and serum levels of allergen-specific IgE. The value of the tests also appear to depend on the drugs that are investigated. The sensitivity of CD63 up-regulation as a test for allergic sensitivity to NMBA (with skin test positivity taken as the 'gold standard') has been calculated to range between 36 and 92% [113, 117, 140]. In contrast with the drug beta-lactam, the sensitivity has varied between 49 and 50% [105, 141]. Measurement of allergen-specific IgE has also shown great variation in sensitivity and specificity (assessment of the proportion of true negative cases in the control groups to identify the false positive rate). In the study of Blanca et al. sensitivity of the assay for the antibiotics amoxicilloyl and benzylpenicilloyl ranged from 32 to 50% [114].

In the present study, there were only 17 patients, and it will be important to increase the number to get a better view of the potential value of tests for drug hypersensitivity and their sensitivity. However, measurement of histamine release from basophils using the methods employed did not seem to be a good choice of *in vitro* assay for identifying drug hypersensitivity.

Purification and detection of mast cell and basophil markers

Purification of human lung tryptase

Tryptase was successfully purified from human lung using butyl agarose hydrophobic interaction, heparin affinity interaction and size exclusion chromatography. The activation and protein concentration was monitored by using the substrate N-benzoyl-arginine p-nitroanilide (BAPNA) for the activity and the Bradford assay or NanoDrop for protein concentration determination. The high salt extract of lung tissue had the highest tryptase activity, indicating that tryptase had been solubilised under the conditions employed, and this extract was processed for purification. Tryptase activity per unit volume increased over the purification process until it reached a peak value after the heparin affinity chromatography step; and thereafter dropped following gel filtration. The protein concentration was highest at the high salt extraction stage and after the post butyl agarose column, and thereafter decreased until the gel filtration stage. However, the specific activity showed a steady rise until the final step when there was a tryptase activity of 6390 mU/mg determined after size exclusion chromatography. Similar results were also found in the study of Smith et al. who also isolated tryptase from human lung [142]. That group used a two-step purification strategy which included a different hydrophobic interaction step (an octyl-Sepharose column) and they also used a cellulose-phosphate column as the final step.

Analysis of the by SDS-PAGE did not reveal any band for tryptase in the high salt extract either on Coomassie blue staining or by Western blotting probably because tryptase represented a very small proportion of total protein present at that stage. However, pooled fractions eluted from the butyl agarose column showed various bands on SDS-PAGE but only one single band on the western blot at around 30 kDa. The size of the band would be consistent for that of tryptase in the monomeric form. Bands representing the contaminants on SDS-PAGE largely disappeared after the next purification step. After the heparin agarose column, two pooled fractions were analysed and of these fraction A showed various bands between 25 and 35 kDa and a single band at 100 kDa. On the western blot tryptase appeared as a single band between 30 and 40 kDa and no other bands were seen. The reason for this could be that other tryptase forms are detectable with the SDS-PAGE but not with the antibody which was used on the western blot. It is possible that the 100 kDa band on the SDS-PAGE could represent trimers of tryptase, as Perng et al. found that lung tryptase can appear on the gel as monomers (30-39 kDa), dimers (64-68 kDa), trimers (92-100 kDa) and tetramers (125-130 kDa) [143]. However, in the present study the band is more likely to be a protein constituent other than tryptase.

After concentration of pooled fractions A and B, the extract was subjected to gel filtration for further purification. Seven fractions were collected and analysed by 10% SDS-PAGE with Coomassie blue and silver staining, but only fractions 10 and 11 exhibited a single band between 35 and 55 kDa corresponding to monomeric tryptase. However, the more sensitive silver staining procedure indicated not only a band at approximately 35 kDa for fractions 10 to 13, but also bands between 130 and 100 kDa in fractions 11 to 13. On the basis of these results fraction 10 was taken as being the most pure preparation.

Shortage of time prevented use of the purified tryptase as a standard for measuring tryptase levels in samples of body fluids from patients, and in the present studies it was not possible to explore tryptase as a marker for allergic reactions to drugs. The group of Sala-Cunill investigated tryptase level in patients who had an acute anaphylactic shock and found that the tryptase level is higher in anaphylactic events induced by drugs in comparison to food [144]. Moreover, it was found that the severity of anaphylaxis correlated with serum tryptase levels. Similar observations were made by Vadas et al. who found that serum tryptase levels were higher in patients with a severe reaction than in those with moderate or mild reactions [104, 118].

Evidence that tryptase may be a predictive marker for severe allergic reactions has come from a study by Blum et al. in which it was found that baseline tryptase levels were correlated to the severity of allergic reactions to hymenoptera stings [145]. This pattern was seen also in children with food allergy for whom tryptase levels at baseline were greater in those with moderate to severe anaphylaxis than in those without anaphylactic shock. Therefore, measurement of tryptase levels could help to predict the severity of an allergic reaction. However, the levels of tryptase rapidly decrease after two hours and are not always raised when an anaphylactic event occurred. In the study of Sala-Cunill, a third of the patients, who had acute anaphylaxis, had a concentration of tryptase which was within the normal range (under 11.4 ug/l) highlighting the shortcomings of tryptase as a biomarker for identifying anaphylactic shock [144]. Other mediators which could also be increased during an allergic reaction such as chymase, carboxypeptidase, and PAF [85, 90, 104]. Measurement of different markers should advance understanding of the mechanisms of allergic reactions.

Optimisation of basogranulin detection

As basogranulin is released after basophil degranulation and is a unique biomarker for basophil activation, its measurement should provide a means for determining the involvement of basophils in allergic disease. When detection of basogranulin in PBMC lysates was investigated in the present studies by dot blotting, specific binding to basophil lysates was found with both of the basophil-specific antibodies employed, BB1 and BB5. There was negligible binding of the antibodies to cell culture medium or wash buffer (PBS-Tween). Optimisation of binding conditions for BB1 and BB5 involved treating a PBMC lysate with a red blood cell lysis buffer to remove haemoglobin that could possibly interfere with the detection of basogranulin. It was found that this treatment allowed slightly higher detection of basogranulin. Therefore, the PBMC lysate used as a standard was thereafter always treated with the red blood cell lysis buffer. The PBMC lysate was stored at –20 °C and only one aliquot was used per experiment. However, we wanted to investigate the influence of the period of storage and found that the detection of basogranulin was greater in aliquots of PBMC lysate stored only one day at -20°C than PBMC lysate stored for two weeks. The same result was obtained when a purified basophil lysate was used for basogranulin detection. Both results suggest that immunoreactive basogranulin levels decrease over time, and this has important implications when applying the assay, and in its present form it will be important to employ freshly prepared lysates. Moreover, it was found that basogranulin was better detected in a purified basophil lysate than a PBMC lysate, indicating the potential for interference by products of other cell types.

Dot blotting can be a challenging technique to employ in quantitation with issues of poor reproducibility requiring particular attention, but further optimisation of the assay performed in the present study should open the way for measurement of basogranulin in body fluids. Mochizuki et al. showed that basogranulin is released from basophils after anti-IgE stimulation [59], and adapting the assay to plasma samples should allow comparison of basogranulin levels in healthy individuals and allergic patients. In addition, the measuring levels of basogranulin before and after drug stimulation will advance understanding of the role of basophils in drug hypersensitivity and other allergic reactions.

7.1 Conclusions and future work

The findings of this study have highlighted the need for reliable laboratory tests for the diagnosis of allergic reactions provoked by drugs, but indicates some of the challenges that must be overcome in achieving this. All of the existing tests have limitations and can lead to false results. There were some major discrepancies in findings between drug challenge of patients (though this was taken as the 'gold standard' technique), and laboratory tests for the activation of passively sensitised RBL cells or basophils *in vitro*, or for allergen specific IgE.

Overall the results of *in vitro* challenge of RBL cells with drugs following passive sensitisation with patient serum were disappointing, with little release of β-hexosamindase observed. In future studies, other cells that can mimick mast cell or basophil activation should be considered, and optimisation should take account of the degree to which functional FcεRI receptors are expressed. Moreover, it will be important to confirm that the drugs investigated are able to crosslink FcεRI receptor-bound IgE molecules on the cell, a particular concern with drugs of small molecular weight that may act as haptens within the body. Drug challenge of blood cells from patients with drug allergy resulted in little histamine release in the present studies, indicating that that there are important issues to consider with ex vivo challenge of basophils as well as in mast cell or basophil-like cell lines.

Appendix A: New biomarkers of anaphylaxis drug challenge record

UNIVERSITY OF Southampton School of Medicine

New biomarkers of anaphylaxis drug challenge record

Subject number	DA
Date	/ /
Age	
Gender	☐ Male ☐ Female

Observations

Time		**° C**		**mmHg**		**BPM**		**%**		**L/min**
	Temp 1		**BP 1**		**HR 1**		**SATS 1**		**PEFR 1**	
	Temp 2		**BP 2**		**HR 2**		**SATS 2**		**PEFR 2**	
	Temp 3		**BP 3**		**HR 3**		**SATS 3**		**PEFR 3**	
	Temp 4		**BP 4**		**HR 4**		**SATS 4**		**PEFR 4**	
	Temp 5		**BP 5**		**HR 5**		**SATS 5**		**PEFR 5**	

Challenges

Drug 1…….

SPT	**Time**						☐ +ve control
	Dose	1/	1/	1/	1/	1/	☐-ve control
	Reaction	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No	
IDT	**Time**						☐-ve control
	Dose	1/	1/	1/	1/	1/	
	Reaction	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No	

Drug 2……..

SPT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No
IDT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No

Drug 3……..

SPT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No
IDT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No

Drug 4……

SPT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No
IDT	**Time**					
	Dose	1/	1/	1/	1/	1/
	Reaction	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No

Sample collection

Collection Point	**Sample**	**Time Collected**
Baseline	Saliva Blood Observations	…… …… ……
Post SPT 1	Saliva	
Post SPT 2	Saliva	
Post SPT 3	Saliva	
Pre IDT	Temp	

Post IDT 1	Saliva Temp	……... ……...
Post IDT 2	Saliva	
Post IDT 3	Saliva	
Post IDT 4	Saliva	
Post IDT 5	Saliva	
15 mins post last IDT test dose	Saliva Temp	…… ……
30 mins post last IDT test dose	Saliva	
60 mins post last IDT test dose	Bloods Saliva Urine Temp Observations	…… …… …… …… ……

Results

Reaction occurred	☐ Yes ☐ No
Drug	☐ Drug 1 ☐ Drug 2 ☐ Drug 3 ☐ Drug 4
Tet	☐ SPT ☐ IDT ☐ Oral ☐ Other
Time of onset of first symptoms	
Time of onset of evere symptoms*	
Wheal size drug 1	
Wheal size drug 2	
Wheal size drug 3	
Wheal size drug 4	

Symptoms

	Present		Present
Hives	☐	**Fever**	☐
Itchiness	☐	**Sneeze**	☐
Generalised angioedema	☐	**Cough***	☐
Headache	☐	**Shortness of breath***	☐
Postnasal drip	☐	**Wheeze (expiratory) ***	☐
Abdominal pain	☐	**Stridor (inspiratory) ***	☐
Vomiting	☐	**Faintness***	☐
Diarrhoea	☐	**Collapse***	☐

Hypotension (< 100/60)	☐	**Bradycardia (< 60)**	☐
Tachycardia (> 120)	☐	**Desaturation (<90-95)**	☐
Urticarial rash	☐		
Reaction observations i.e any additional symptoms or observations taken.			

Allergy history

Previous reaction	☐ Yes ☐ No ☐ Uncertain			
Presumed drug	☐ Drug 1 ☐ Drug 2 ☐ Drug 3 ☐ Drug 4 ☐ Other			
Reason for referral				
Existing allergies (number accordingly)	Drugs	Foods	Insts	Pollens
	Other			

Allergy Symptoms

	Allergen **1 2 3 4 5**		**Allergen** **1 2 3 4 5**
Hives	☐ ☐ ☐ ☐ ☐	**Fever**	☐ ☐ ☐ ☐ ☐
Itchiness	☐ ☐ ☐ ☐ ☐	**Sneeze**	☐ ☐ ☐ ☐ ☐
Generalised angioedema	☐ ☐ ☐ ☐ ☐	**Cough**	☐ ☐ ☐ ☐ ☐
Headache	☐ ☐ ☐ ☐ ☐	**Shortness of breath**	☐ ☐ ☐ ☐ ☐
Postnasal drip	☐ ☐ ☐ ☐ ☐	**Wheeze (expiratory)**	☐ ☐ ☐ ☐ ☐
Abdominal pain	☐ ☐ ☐ ☐ ☐	**Stridor (inspiratory)**	☐ ☐ ☐ ☐ ☐
Vomiting	☐ ☐ ☐ ☐ ☐	**Faintness**	☐ ☐ ☐ ☐ ☐
Diarrhoea	☐ ☐ ☐ ☐ ☐	**Collapse**	☐ ☐ ☐ ☐ ☐

Atopy	☐ Hayfever ☐ Asthma ☐ Eczema

Medical history

Current medication list.	

Family history of allergy	
Additional notes	

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