Dissertation

The basophil activation test in diagnostic practice of hymenoptera venom allergy

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Table of contents

Abstract

The basophil activation test (BAT) is a widely validated and reliable tool especially for the diagnosis of hymenoptera venom allergy. Nevertheless, several pitfalls have to be considered and outcomes may differ due to diverse in-house protocols and commercially available kits.

In the first step, we aimed to identify factors that may influence results of the CD63-based BAT. The effect of stimulating factors such as IL-3, cytochalasin B and pre-warming of the samples was investigated. Additionally, we compared two different flow cytometer systems and evaluated the influence of storage time, different staining protocols, and anti-allergic drugs on the test results.

IL-3 enhanced the reactivity of basophils at 300 pM, but not at 75 pM and 150 pM. Pre-warming of samples and reagents did not affect basophil reactivity. CD63 expression assayed after storage time of up to 48 hours showed that basophil reactivity already started to decline after 4 hours. Basophils stained with HLA-DR-PC5 and CD123-PE antibodies gated as HLA-DR^{neg}/$CD123^{pos}$ cells showed the highest reactivity. No effect on test outcomes was observed at therapeutic doses of dimetindene and desloratadine.

The second aim was to identify potent influencing factors of the CD203c-based BAT and to emphasize differences between CD63 and CD203c detection. The effects of IL-3 and degranulation enhancing substances were investigated and compared with CD63 up-regulation. Furthermore, the influence of different storage conditions and incubation times was evaluated and the impact of anti-allergic drugs on the test results was assessed.

CD203c and CD63 expression was rapidly upregulated reaching a maximum after 20 to 30 min. Basophil CD203c up-regulation assayed after storage times up to 48 h declined already after 4h. IL-3 treatment increased CD203c and CD63 baseline levels and decreased basophil CD203c responses in a dose-dependent manner. Finally, therapeutic concentrations of dimetindene and desloratadine did not affect CD203c up-regulation.

In the next step we evaluated the optimized BAT in patients with (clinically irrelevant) double sensitization to bee and wasp venom, a frequent problem in the diagnosis of hymenoptera venom allergy. Among 117 patients, double sensitization (DS) was observed in 63.7% by the Immulite, in 61.5% by the CAP, in 47.9% by the intradermal test (IDT), in 20.5% by the ADVIA, and in 17.1% by the BAT. In CAP double positive patients, western blot inhibition revealed cross-reactive carbohydrate determinant (CCD)-based DS in 50.8%, and the component resolved diagnosis (CRD) showed 41.7% of patients with true DS. BAT, CRD, and ADVIA showed the lowest rate of DS and were helpful in finding the culprit insect. However, the rate of DS was higher than expected by personal history, indicating that the matter of clinical relevance is still not solved even by novel tests.

Clinically irrelevant sensitization is frequently observed. Therefore, we initiated the next study to prove if sensitized subjects without a history of systemic sting reactions (SSR) tolerate sting challenges with the respective insect. In addition, BAT and routine diagnostic tools were correlated with the outcome of sting challenges.

In 94 subjects 131 sting challenges with bees and wasps were performed. As expected, only 5 of 94 (5.3%) subjects showed SSR after the sting. Large local reactions (LLR) occurred in 41 of 94 (43.6%) subjects. A telephone survey was conducted among 1,401 subjects to determine the prevalence of SSR and LLR with an accuracy of ±1% in the general population. Of these, 46 of 1,401 (3.3%, CI 2.4%- 4.4%) reported SSR and 64 of 1,401 (4.6%, CI 3.5%-5.8%) LLR. Compared to the general population, sensitized subjects without a history of SSR had a comparable risk of SSR ($p=0.247$), whereas the frequency of LLR was about 10 times higher ($p<000.1$). BAT and CRD correlated best with the outcome of the sting challenges.

Introduction

IgE-mediated allergies become more and more a devastating social and economic burden to most industrialized countries' health care systems, with millions of allergy-afflicted individuals worldwide.

Hymenoptera venom allergy is a classical IgE-mediated disease with a potentially fatal course. Depending on the degree and severity of the systemic reaction, the skin, the gastrointestinal, respiratory, and cardiovascular systems can be involved (Table 1).

Grade	Symptoms
I	Generalized skin symptoms: flush, generalized urticaria, angioedema
II	Mild to moderate pulmonary, cardiovascular, and/or gastrointestinal symptoms
III	Anaphylactic shock, loss of consciousness
VI	Cardiac arrest, apnea

Table 1. Classification of systemic reactions by Ring and Messmer[1]

Up to 7.5% of the general population is reported to have experienced systemic anaphylactic reactions after Hymenoptera stings. The prevalence of systemic reactions among beekeepers is high and falls between 14% and 43%. The incidence of insect sting mortality is ranging from 0.03 to 0.48 fatalities per 1 000 000 inhabitants per year[2]. However, the true number of life-threatening sting reactions is likely to be underestimated.

For most patients as well as for their dependants, an anaphylactic reaction after a sting is a very traumatic event. It has been demonstrated that patients with anaphylactic responses following yellow jacket stings experienced impairment in their quality of life especially because of emotional distress[3]. Severe reactions or a status after resuscitation may leave

patients with permanent disorders such as hypoxic brain damage with permanent neurological deficits, or myocardial infarction[4]. Thus, especially during the insect season, allergic patients should carry an emergency kit for self-administration, containing antihistamines, corticosteroids and adrenaline.

Currently, specific immunotherapy is the only causal treatment of hymenoptera venom allergy and results in an almost complete protection against allergic reactions in the majority of patients. In clinical routine, unspecific sensitization to multiple allergens due to in vitro cross-reactions render it difficult for the clinician to make an unequivocal diagnosis. In Hymenoptera allergy, cross-reactions due to cross-reactive carbohydrate determinants causing double-positive test results for honeybee and yellow jacket venom have been repeatedly described. Moreover, a high proportion of the population has detectable IgE to honeybee and yellow jacket venom, but tolerates stings well.

Thus, there is still urgent demand for improvement of diagnosis to verify clinically relevant Hymenoptera allergy and to determine the relevant venom for treatment.

Hymenoptera venom allergy

Worldwide, hymenoptera venom allergy is a common problem, and although most patients develop only local reactions after a sting, generalized life-threatening reactions may occur. Most deaths related to Hymenoptera stings are the result of allergen-induced immediate IgE-mediated hypersensitivity reactions, causing anaphylaxis. Risk factors influencing the outcome of an anaphylactic reaction include the insect type, the time period between stings, the number of stings, the patients' age, cardiovascular diseases, mastocytosis, and drug intake[2]. The medically important groups of Hymenoptera are the *apoidea* (bees), *vespoidea* (wasps, hornets and yellow jackets), and *formicidae* (ants).

Bee venom is a complex mixture of biologically active components, primarily consisting of proteins, enzymes, and amines. The major component is mellitin, which acts as a detergent to

disrupt cell membranes and liberate biogenic amines and potassium. Further components are phospholipase A, hyaluronidase, apamin, acid phosphatase, biogenic amines, and mast cell degranulating peptide. Wasp venom contains three major proteins that act as allergens, and also a broad variety of vasoactive amines and peptides[5]. Mellitin is not found in wasp venom. The intense pain of vespid stings is mainly because of serotonin, wasp kinins, and acetylcholine. The major allergen found in wasp venom is called antigen 5, but its biological activity has not been fully determined. Further components are phospholipase A, hyaluronidase, acid phosphatase, biogenic amines, mast cell degranulating peptide and kinins[6].

Cross-reactivity, double or even multiple positive tests can be caused by true double sensitization or by cross-reactive IgE antibodies which recognize similar epitopes of different allergens, especially carbohydrate-containing epitopes of venoms and common allergens[7]. However, the distinction between cross-reactivity and true double-sensitization is important for the choice of the culprit venom for immunotherapy.

The most effective drugs for dealing with systemic allergic reactions are antihistamines, corticosteroids, adrenaline and sympatomimetics. Cutaneous reactions require oral or injected antihistamines. In case of bronchoconstriction, treatment with inhaled β2-agonists may be necessary. Severe reactions, including those with marked respiratory difficulties or hypotension, should be treated with adrenaline followed by antihistamines and hydrocortisone[8].

Recent diagnostic procedures are mainly based on assessment of case history, skin testing (skin prick test, intradermal test) and the determination of allergen-specific serum IgE antibodies (RAST, CAP-FEIA). Additionally the determination of serum tryptase levels and several *in vitro* tests, such as basophil histamine release test and leukotriene release test can be performed[2].

In the majority of cases these tests allow an accurate diagnosis, but there is still concern about their sensitivity and specificity[7, 9, 10]. The sensitivity of skin testing was found to range from 63 to 100%, depending on the protocols and venoms used, and with specificity ranging from 80 to 97%[9, 11-13]. By comparison, the sensitivity of RAST is estimated as 70 to 76%, and specificity as 60 to 94[9, 14, 15].

As a major obstacle of these tests, positive responses in skin testing and high levels of specific IgE antibodies are not predictive for the occurrence and the severity of systemic reactions after a field sting[16, 17] or unwanted episodes during immunotherapy[18].

Basophils in allergy and related diseases

Until recently, basophils have been (dis-)regarded as "circulating" or "precursor" mast cells due to many phenotypic and biochemical similarities[19]. Therefore, a major part of our knowledge regarding the function and pharmacology of basophils has been derived from studies with mast cells. Both mast cells and basophils express high-affinity IgE receptors (FcεRI) that are cross-linked upon engagement of receptor-bound IgE with corresponding antigens with subsequent release of mediators, such as histamine and LTC_4, suggesting a key pathogenic role in the early phase of IgE-mediated allergic reactions. In addition to this effector function, basophils produce large amounts of the pro-allergenic cytokines interleukin (IL)-4 and IL-13[20, 21] which are crucial for the production of IgE and can *per se* establish asthma in animal models.

Another feature of basophils is their rapid recruitment from the blood to sites of allergen exposure, in contrast to mature mast cells, which are tissue resident. It is now well established that basophils, along with eosinophils and Th2 lymphocytes, are rapidly recruited to the skin, lung or nasal mucosa after allergen challenge[22, 23]. For instance, basophils are a constituent of the cellular infiltrate in asthma and, more prominently, basophils are a major constituent of the cellular infiltrate in cutaneous reaction to allergens[24, 25].

IgE-dependent basophil activation

IgE-dependent cell stimulation involves cross-linking of high-affinity IgE receptors (FcεRI) by engagement of receptor-bound IgE with corresponding allergens. In some diseases (e.g. autoimmune urticaria) additional activation can also occur by autoantibodies directed against IgE molecules or FcεRI receptors[26]. Furthermore, certain plant lectins[27], superantigens[28], as well as parasitic products[29] can cause IgE receptor triggering. FcεRI receptor activation mediates complex intracellular signal transduction events provoking basophil degranulation, mediator release and cytokine *de novo* synthesis.

The basophil activation test (BAT)

In an attempt to find more sensitive and specific diagnostic tools, functional *in vitro* tests based on basophil activation have been developed[30].

Flow cytometric analysis of activated peripheral blood basophils relies on determination of phenotypic alterations. Allergen-induced cross-linking of receptor-bound IgE molecules not only leads to synthesis and release of a variety of bioactive mediators but also upregulates the expression of different basophil activation markers. These changes can be detected on a single-cell basis by multi-color flow cytometry using specific antibodies. At present, the most commonly applied markers in flow-assisted allergy diagnosis are CD63[10, 31, 32] and CD203c[33-36]. The first CD63-based protocol for allergy diagnosis was developed in the mid-1990s. In 2000, an excellent sensitivity and specificity in the diagnosis of hymenoptera allergy was demonstrated and subsequently confirmed in several studies[10, 31, 32, 37, 38].

In the last years, several authors claimed the advantages of CD63 over CD203c[39-41], whereas others confirmed the pivotal role of CD203c as the most promising new activation marker for flow cytometry-based allergy diagnosis[35, 42]. In the meantime, additional studies addressing kinetics, regulation, and activation of both markers have revealed a number of striking

differences. Expression of CD63 is closely related to the phenomenon of basophil IgE-dependent degranulation[43], while expression of CD203c has different kinetics, partially different enzymatic regulation[44], and also seems to be more easily upregulated in a non-specific manner[45, 46]. Although it has been claimed that CD203c yields a 3 to 8-fold higher fluorescent signal than CD63[47], others report exactly the opposite[48]. It has also been objected that basophil activation results in a continuous increase in fluorescent CD203c cells while expression of CD63 is an all-or-nothing phenomenon[49].

Flow cytometric gating and staining strategies in BAT

Several staining strategies for the flow-cytometric gating of basophils have been described. Staining with fluorescent labeled polyclonal anti-IgE antibodies has been questioned, since IgE is also found on other cells such as monocytes or eosinophils[50-52]. Moreover, the density of IgE and FcεRI receptors may vary considerably among individuals and also among single cells in the same individual[47], or IgE-antibodies could potentially activate the cells, which may confound the results in BAT assays[53]. In 2004 we published a very consistent basophil gating strategy based on anti-CD123 and anti-HLA-DR labeling and assessment of basophil activation by anti-CD63[31]. Alternative approaches to basophil labeling include antibodies against CD203c, which is exclusively expressed on basophils among blood cells, a combination of anti-CRTH2 and anti-CD3 (exclusion of T cells)[47], a combination of anti-CCR3 and anti-CD45[54], or anti-CCR3 alone (Flow2 CAST®, Bühlmann Laboratories). Until now, only one study directly compared two different staining strategies in terms of overall clinical diagnostic efficiency[54]. Therefore it remains to be investigated whether modifications of the basophil gating lead to increased sensitivities.

Surface marker phenotype of the basophil

Human basophils express several cell surface antigens which can be related to their immunological responsiveness. Challenging basophils with allergens or cross-linking substances results in a modified surface expression profile, a mechanism that can be evaluated by flow cytometry. Basophils express a broad spectrum of cell surface molecules such as cytokine receptors (CD123; IL-3 receptor), immunoglobulin (Ig) receptors (CD23; FcεRI), complement related antigens (CD11b; CR3), adhesion receptors (CD50, CD54, CD102; ICAMs), cell surface enzymes (CD13; aminopeptidase N), surface gangliosides (CD17; lactosyl-ceramide) and glycolipids as well as virus binding sites (CD46; measles virus binding site)[55].

Biology of recent basophil activation markers

CD63

Also known as LIMP-1, MLA1, PTLGP40, gp55, granulophysine, LAMP-3, ME491 or NGA, CD63 is a member of the tetraspanin superfamily which comprises a group of cell-surface proteins with four transmembrane domains. The tetraspanin superfamily also includes CD9, CD37, CD53, CD81, and CD82[56, 57]. Although the precise biological functions of these proteins are still unclear, several studies have implicated members of the tetraspanin superfamily in cell proliferation, activation, adhesion, and cell motility. In particular, CD63 is found in intracellular granules and becomes upregulated on the cell surface upon degranulation of the cell. Therefore, cell-surface expression of CD63 has been used to monitor degranulation[58, 59].

CD203c

The ectoenzyme CD203c (E-NPP3; pyrophosphatase/phosphodiesterase 3) was found to be expressed on blood basophils, tissue mast cells and their CD34+ progenitor cells, but not on other leukocytes[60, 61]. It is assumed that basophil expression of CD203c and CD63 is regulated

by different pathways with different time kinetics of upregulation[44]. In contrast to CD63, resting basophils show constitutive CD203c expression on their plasma membrane, whereas CD63 expression is upregulated upon degranulation[43]. Similarly to CD63, basophil CD203c expression increases after allergen challenge in sensitized individuals[36, 62, 63]. With regard to BAT, there is still controversy about advantages and disadvantages of CD63 over CD203c[33, 34, 40-42].

Current problems in the diagnosis of hymenoptera venom allergy

Diagnosis of hymenoptera venom allergy is not always straightforward and finding the relevant venom for specific immunotherapy can be sophisticated. Currently, two major problems arise in the diagnostic procedure: No diagnostic tool is able to give information about the clinical relevance of the test result. Many individuals have positive test results to bee or wasp venom although they tolerate stings well; the frequency of asymptomatic sensitization ranges from 23.1 to 66.7% depending on total IgE levels[64]. Moreover, sensitization to both, bee and wasp venom, is observed in up to 59% of patients[65], but clinically relevant double sensitization is rare and patients usually react either to bee or to wasp stings.

Current problems of the BAT

Usually, the BAT works well with all kinds of protein allergens like hymenoptera venom allergens. However, BAT protocols are not standardized and many research groups use their own protocols and different sources of allergens: Flow cytometric quantification of activated basophils can be used either on whole blood or on basophils separated by buffy coat centrifugation or sedimentation over dextran. Currently, there is a clear preference for whole-blood assays, which preserve basophils to a greater extent and can be performed more efficiently. Moreover, a large number of gating strategies to characterize basophils are in use

(e.g. IgE^{pos}, $CD123^{pos}$/$HLA\text{-}DR^{neg}$, $CD3^{neg}$/$CRTH2^{pos}$, $CCR3^{pos}$). Additionally, is controversially discussed whether the BAT can be performed with samples stored for several hours or even overnight. Therefore, it would be important to determine potential factors influencing the test results and to establish an optimal protocol of BAT for the diagnosis of hymenoptera venom allergy.

Aims

The basophil activation test is a widely validated and reliable tool especially for the diagnosis of hymenoptera venom allergy. Nevertheless, several pitfalls have to be considered and outcomes may differ due to diverse in-house protocols and commercially available kits.

1. In the first step, we aimed to identify factors that may influence results of the CD63-based BAT. Basophil responses to monoclonal anti-IgE and bee and wasp venom were determined by BAT based on CD63. The effect of stimulating factors such as IL-3, cytochalasin B and pre-warming of the samples was investigated. Additionally, we compared two different flow cytometer systems and evaluated the influence of storage time, different staining protocols, and anti-allergic drugs on the test results.

BAT based on CD203c upregulation, has been validated as a reliable tool for the diagnosis of IgE-mediated allergies. Nevertheless, CD203c-based BAT is hardly comparable to CD63-based tests, since the mechanisms of CD203c versus CD63 induction differ considerably.

2. The second aim was to identify potent influencing factors of the CD203c-based BAT and to emphasize differences between CD63 and CD203c detection. CD203c-based BAT was performed in 82 healthy controls and in 79 allergic patients. The effects of interleukin IL-3 and degranulation enhancing substances were investigated and compared with CD63 up-regulation. Furthermore, the influence of different storage conditions and incubation times was evaluated and the impact of anti-allergic drugs on the test results was assessed.

Double sensitization to bee and vespid venom is frequently observed in the diagnosis of hymenoptera venom allergy, but clinically relevant DS is rare. Thus it is sophisticated to choose the relevant venom for specific immunotherapy and overtreatment with both venoms may occur.

3. Therefore, we aimed to compare currently available routine diagnostic tests as well as experimental tests including BAT to identify the most accurate diagnostic tool.

   117 patients with a history of a bee or vespid allergy were included in the study. Initially, IgE determination by the ImmunoCAP, by the Immulite, and by the ADVIA Centaur, as well as the intradermal test, and the BAT were performed. In 72 CAP double positive patients, individual IgE patterns were determined by western blot inhibition and component resolved diagnosis with rApi m 1, nVes v 1, and nVes v 5.

Clinically irrelevant sensitization is a frequent problem in the diagnosis of Hymenoptera allergy. Recently, we observed that the frequency of asymptomatic sensitization depends on total IgE levels; up to 66.7% of healthy subjects had detectable specific IgE to bee- and/or wasp venom.

4. As these data only rely on personal history, we initiated a prospective study to prove if sensitized subjects without a history of systemic sting reactions tolerate sting challenges with the respective insect. In addition, BAT and routine diagnostic tools were correlated with the outcome of sting challenges.

   Ninety-four subjects with detectable specific IgE to bee- and wasp venom in the CAP system were prospectively enrolled in the study. Additionally, intradermal testing, and BAT were performed and correlated to the outcome of the sting challenges. Sting challenges were carried out after a complete health check in an intensive care setting with wasps (Vespula vulgaris) and/or honey bees (Apis mellifera).

The above mentioned goals were addressed step by step and published separately:

1. Sturm GJ, Kranzelbinder B, Sturm EM, Heinemann A, Groselj-Strele A, Aberer W. The basophil activation test in the diagnosis of allergy: technical issues and critical factors. Allergy 2009; 64:1319-26.

2. Sturm EM, Kranzelbinder B, Heinemann A, Groselj-Strele A, Aberer W, Sturm GJ. CD203c-based basophil activation test in allergy diagnosis: Characteristics and differences to CD63 upregulation. Cytometry B Clin Cytom 2010; 78B:308-18.

3. Sturm GJ, Jin C, Kranzelbinder B, Hemmer W, Sturm EM, Griesbacher A, et al. Inconsistent Results of Diagnostic Tools Hamper the Differentiation between Bee and Vespid Venom Allergy. PLoS One 2011; 6:e20842.

4. Bokanovic D, Aberer W, Griesbacher A, Sturm GJ. Prevalence of hymenoptera venom allergy and poor adherence to immunotherapy in Austria. Allergy 2011, doi: 10.1111/j.1398-9995.2011.02659.x

1. The CD63-based basophil activation test in the diagnosis of allergy: technical issues and critical factors

Introduction

Up to 7.5 % of the general population is reported to have experienced systemic anaphylactic reactions after hymenoptera stings[2]. Personal history, skin testing, and detection of allergen-specific IgE serum antibodies are the mainstays of the diagnostic procedure in cases of hymenoptera venom hypersensitivity, but are only of limited value in many instances. The basophil activation test (BAT) is now generally accepted as an additional and reliable diagnostic tool. Compared with the determination of IgE in serum, basophil activation tests offer the additional advantage of being able to demonstrate functional responses as positive test results will only occur after successful cross-linking and not by monovalent binding as seen in IgE assays.

The first CD63-based protocol for allergy diagnosis was developed by Sainte-Laudy and Sabbah in the mid-1990's, and the first English description of this application was published in 1996 [66]. In 2000, an excellent sensitivity and specificity in the diagnosis of hymenoptera allergy was described [67] and subsequently confirmed in several studies [31-33, 37, 38]. The first CD203c-based protocol for hymenoptera allergy was published in 2001 [63]; these data were recently validated by another research group [33]. Currently, BAT with CD63 is the best clinically validated test [49] while BAT based on CD203c still requires further investigation. Generally, the potentials and pitfalls of BAT are still a point at issue. Whether BAT should only be performed by experienced and validated laboratories is a debated question [68, 69]. However, pitfalls are scarcely reported in the published literature; most reports are based on personal communication and small numbers of patients [70]. Furthermore, BAT protocols are generally not standardized; diverse protocols are prone to yield different results. Flow cytometric quantification of activated basophils can be used either on whole blood or on

basophils separated by buffy coat centrifugation or sedimentation over dextran. Nevertheless, there is a clear preference for whole-blood assays, which preserve basophils to a greater extent and can be performed more efficiently. Moreover, a large number of gating strategies to characterize basophils are currently in use (e.g. IgE^{pos}, $CD123^{pos}$/$HLA\text{-}DR^{neg}$, $CD3^{neg}$/$CRTH2^{pos}$, $CCR3^{pos}$). Additionally, different antibodies are coupled with fluorochromes of varying signal intensity, which possibly results in poor separation of the target cells. As a consequence, the obtained percentage of activated basophils could be inaccurate. In addition, the accuracy of results when BAT is performed with samples stored for several hours or even overnight is controversially discussed. The influence of antihistamines and glucocorticoids on the activation of basophils also needs to be clarified. No extensive study has been performed yet on technical issues regarding BAT. The present study was initiated to establish an optimal protocol of BAT for the diagnosis of hymenoptera allergy and also to determine potential factors influencing the test results.

Methods

Reagents

All laboratory reagents were obtained from Merck or Sigma-Aldrich unless otherwise specified. Dulbecco's modified phosphate-buffered saline (PBS; with or without Ca^{2+} and Mg^{2+}) was purchased from Gibco-Invitrogen (Carlsbad, USA). CellFix and antibodies to CD123 (PE-conjugated) were supplied by Becton Dickinson (New Jersey, USA). Dimetindene and desloratadine were purchased from Novartis (Basel, Switzerland) and Schering-Plough (New Jersey, USA), respectively. Fixative solution was prepared by diluting 1:80 in PBS (without Ca^{2+} and Mg^{2+}). Antibodies to HLA-DR (PC5-conjugated), CD3 (PC7-conjugated) and CD63 (PE- and FITC-conjugated), and monoclonal antibodies to IgE (clone E124.2.8) were purchased from Beckman Coulter (Fullerton, USA). Antibodies to CRTH2 (FITC-conjugated) were obtained from Santa Cruz Biotechnology (Santa Cruz, USA) and antibodies to CD123 (FITC-conjugated) from Miltenyi Biotec (Bergisch Gladbach, Germany). Cytochalasin B and interleukin (IL)-3 were supplied by Sigma-Aldrich (St. Louis, USA). Aqueous bee and wasp venom (ALK wässerig SQ, corresponding to raw venom after microfiltration) were purchased from ALK-Abelló (Hørsholm, Denmark).

The composition of the lysis solution used for erythrocyte elimination from whole blood samples was 155 mM NH_4Cl, 10 mM $KHCO_3$, 100 μM Na_2-EDTA.

Experimental procedure

Basophil activation test

Blood was collected from healthy volunteers as well as from bee- or wasp venom-allergic patients according to a protocol approved by the local ethics committee.

Six milliliters of EDTA whole blood were stained with anti-CD123 PE-conjugated antibody (1:50), anti-HLA-DR PC5-conjugated antibody (1:50) and anti-CD63 FITC-conjugated antibody (1:50) for 10 minutes at room temperature. In all tests, EDTA whole-blood samples

were pre-incubated with 300 pM IL-3 simultaneously with the staining step, except those preliminary experiments in which the optimal IL-3 concentration was determined. Basophil reactivity was measured using serial dilutions of bee or wasp venom (1000, 100, 10, 1 ng/mL) or serial dilutions of anti-IgE antibody (1:10-1:1000 dilution; in the experiment with desloratadine from 1:100-1.12500). Hundred µL aliquots of whole blood were mixed with 100 µL of the respective stimulant or buffer and incubated for 40 minutes in a 37°C water bath; these conditions had been initially found to yield optimal responses in CD63 up-regulation. Subsequently, the reaction was terminated by adding 100 µL of a 3.8% EDTA stop solution. In order to achieve erythrocyte lysis, 2 mL of lysis solution were added to each sample. After 15 minutes incubation at room temperature in the dark, the samples were centrifuged (5 minutes, 200 x g) and the supernatants discarded. The resulting cell pellets were washed in 3 mL PBS (without Ca^{2+} and Mg^{2+}) and resuspended in 400 µL ice-cold fixative solution.

Finally, cell samples were analyzed as described previously [31] by three-color flow cytometry (FC 500, Beckman Coulter). Basophils were identified as a single population of cells that stained positive for CD123 (FL-2) and negative for HLA-DR (FL-4). Up-regulation of CD63 expression was indicated by an increase in fluorescence in the FL-1 channel. Acquisition was terminated after 200 basophil target events. Responses were quantified as percentages of CD63 expressing basophils in a higher FL-1 region, which had been adjusted to contain 10% of basophils (i.e. activated basophils), in an unstimulated sample (i.e. negative control).

Stimulation factors

As IL-3 is known to enhance basophil reactivity, the effect of IL-3 pre-treatment on BAT results was tested. For this purpose EDTA whole blood samples of 15 subjects (7 female, 8 male; mean age 40.8 ±13.8) were incubated with vehicle or three different concentrations of IL-3 (300 pM, 150 pM, 75 pM) for 10 minutes in conjunction with the staining procedure.

Similarly, the influence of cytochalasin B (5 µg/mL) was investigated because it is known to amplify degranulation. Since pre-warming of whole blood samples is suggested to increase basophil reactivity, EDTA whole blood from healthy volunteers and reagents were warmed for 10 minutes to 37°C prior to the assay. In all three cases, serial dilutions of anti-IgE were used for stimulation as described above.

Storage time

Samples of EDTA anti-coagulated whole blood derived from healthy subjects (n = 19; 10 female, 9 male; mean age 39.2 ±14.2) or patients allergic to bee (n = 15; 8 female, 7 male; mean age 39.9 ±14.9) or wasp venom (n = 20; 12 female, 8 male; mean age 43.0 ±16.4) were investigated at different points of time after blood withdrawal. In Hymenoptera venom allergic patients the personal history of a systemic sting reaction was confirmed by intradermal testing and IgE determination (Phadia, Uppsala, Sweden). BAT was performed immediately after blood sampling. Additionally, samples from healthy volunteers were stored for 4, 24, 28 and 48 hours at 4°C; samples from allergic patients were stored for 18 hours at 4°C before BAT was performed anew. For stimulation, serial dilutions of anti-IgE as well as bee or wasp venom were used as described above.

Staining strategies

BAT was performed using three different triple staining protocols. Therefore, EDTA whole blood from healthy volunteers (n = 16; 9 female, 7 male; mean age 42.0 ±13.2) was stimulated with serial dilutions of anti-IgE and processed as described above. For staining 1 (standard staining strategy), antibodies to HLA-DR (PC5-conjugated), CD123 (PE-conjugated) and CD63 (FITC-conjugated) were used. For staining 2, blood samples were stained with antibodies to HLA-DR (PC5-conjugated), CD123 (FITC-conjugated) and CD63 (PE-conjugated). Additionally, a protocol originally designed for determination of CD203c

was tested. Therefore, staining 3 was carried out with antibodies to CD3 (PC7-conjugated), CRTH2 (FITC-conjugated) and CD63 (PE-conjugated). All antibodies were added at a dilution of 1:50.

Flow cytometer systems

BAT was performed with EDTA whole blood from 15 healthy volunteers (7 female, 8 male; mean age 39.5 ±16.0) after stimulation with serial dilutions of anti-IgE in accordance with the standard protocol ($CD123^{pos}$/$HLA\text{-}DR^{neg}$) as described above. All tests were performed in duplicate, and up-regulation of CD63 expression was measured and analyzed in parallel with the Beckman Coulter FC 500 and the BD FACSCalibur.

Influence of anti-allergic drugs

To investigate the effect of prednisolone and dimetindene on the up-regulation of basophil CD63 expression, EDTA whole-blood samples derived from healthy subjects (n = 17; 8 female, 9 male; mean age 40.2 ±14.6) were pre-treated with vehicle, 50, 500 and 2500 µg/mL (108.7 µM, 1.1 mM, and 5.4mM) of prednisolone or vehicle, 1, 10 and 50 µg/mL (3.4 µM, 34.2 µM, and 171.0 µM) of dimetindene for 30 minutes at 37°C. Subsequently, BAT was performed using serial dilutions of anti-IgE in accordance with the standard protocol.

To examine the influence of desloratadine on the results of BAT, the assay was performed in 10 subjects (5 female, 5 male; mean age 36.4 ±6.0) before and three hours after the intake of the two-fold daily dose (10 mg) of desloratadine.

Statistics

Patients characteristics are expressed as mean ± SD. Data are shown as means ± SEM for *n* observations. Comparisons of groups of data were performed using repeated measures

ANOVA. Probability values of $p < 0.05$ were considered to be statistically significant. Data were analyzed using the SPSS 15.0 software (SPSS Inc., Chicago, Illinois, USA)

Results

Stimulation factors for basophil activation

Initially we tested different concentrations of IL-3, cytochalasin B and pre-warming of samples and reagents in terms of enhancing basophil reactivity. As shown in Figure 1A, IL-3 significantly enhanced basophil responsiveness to cross-linking of IgE when compared to controls; the optimal concentration was 300 pM of IL-3. In contrast, cytochalasin B reduced basophil reactivity to anti-IgE while pre-warming of the samples had no effect (Figure 1B). Therefore, further experiments were conducted in the presence of IL-3 (300 pM) but without cytochalasin B and without pre-warming.

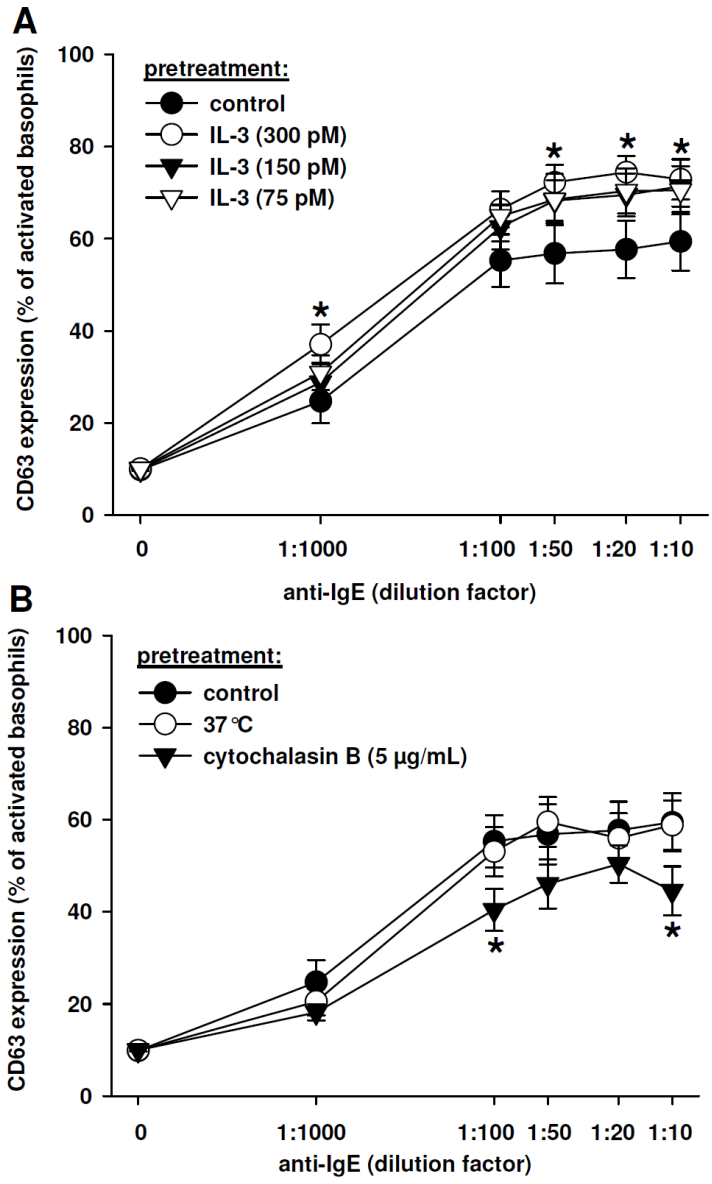

Figure 1. Enhanced basophil reactivity by IL-3, but not by cytochalasin B and pre-warming of samples and reagents.
(A) Only incubation for 10 min with 300 pM IL-3 increased reactivity of basophils significantly at nearly all tested anti-IgE dilutions.
(B) Pre-warming of samples and reagents at 37°C for 10 min had no effect on reactivity, whereas incubation for 10 min with cytochalasin B even reduced the percentage of activated basophils.
Data are expressed as means ± SEM, $n = 15$, * $p < 0.05$ *versus* control.

Experiments performed by Ines Freistätter

Storage time and basophil activation

The degree to which different storage times of blood samples interfered with basophil responsiveness was investigated. CD63 expression of the same blood sample was stimulated with different dilutions of anti-IgE repeatedly at given times. As shown in Figure 2, basophil activation decreased in a time-dependent manner: CD63 expression assayed after a storage time of 24 hours resulted in a loss of basophil reactivity, depending on the anti-IgE dilution, by 22.3 to 47.9%. After 48 hours the basophil responses were reduced by 42.3% to 58.5%. Even after 4 hours a reduction at usual dilutions of anti-IgE was observed, but only with a statistical trend (p =0.08).

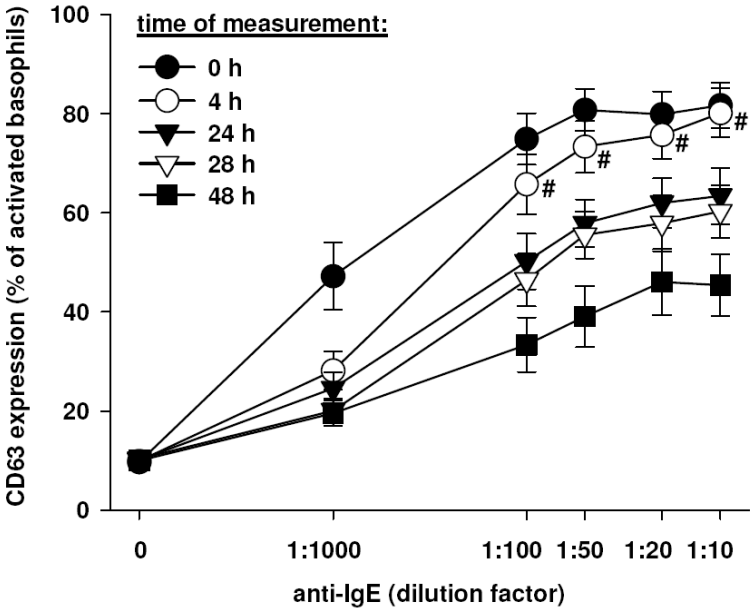

Figure 2. Time dependent decrease in basophil responses to stimulation with anti-IgE
Blood samples were stored for up to 48 h and the basophil activation test with stimulation of anti-IgE for 40 min was performed at different points of time. Best results were obtained when blood samples were processed immediately. A storage time from 24 to 48 hours resulted in significantly reduced basophil activation.
Data are expressed as means ± SEM, *n* = 19, # p > 0.05 *versus* 0 h (not significant).

Similar results were obtained on BAT with regard to bee and wasp venom: of initially 15 bee-venom-allergic and BAT-positive patients, only 2 were positive after 18 hours; of 20 wasp-venom-allergic patients, only 9 remained positive after 18 hours. Again, a loss of reactivity by up to 59.7 % was found after 18 hours (Figure 3).

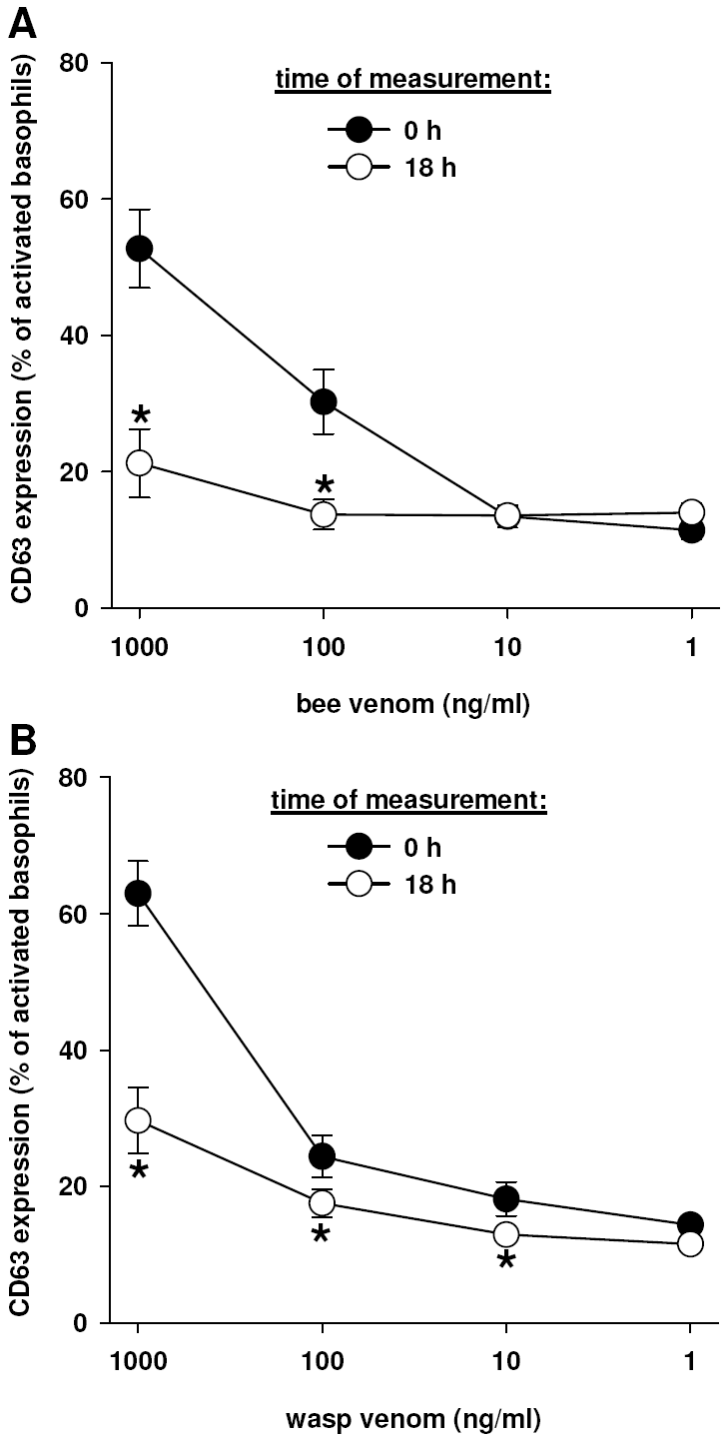

Figure 3. Time-dependent decrease in basophil activation to bee and wasp venom
Blood samples were stored for 18 h and basophil responses to stimulation for 40 min with (A) bee or (B) wasp venoms at the indicated concentrations were recorded at 0 and 18 h. Data are expressed as means ± SEM, n = 15-20, * $p < 0.05$ *versus* 0 h.

Experiments performed by Ines Freistätter

Staining strategies

To examine the role of fluorochromes and different staining strategies, we compared our standard staining (staining 1; HLA-DR-PC5, CD123-PE, CD63-FITC, Figure 4A) with the same staining strategy, but switched fluorochromes (staining 2; HLA-DR-PC5, CD123-FITC, CD63-PE, Figure 4B), and also used a completely different strategy which had been originally designed for quantification of CD203c expression (staining 3; CD3-PC7, CRTH2-FITC, CD63-PE, Figure 4C).

Basophils gated with the brighter fluorochrome PE resulted in better separation of the target cells as compared to FITC (Figure 4).

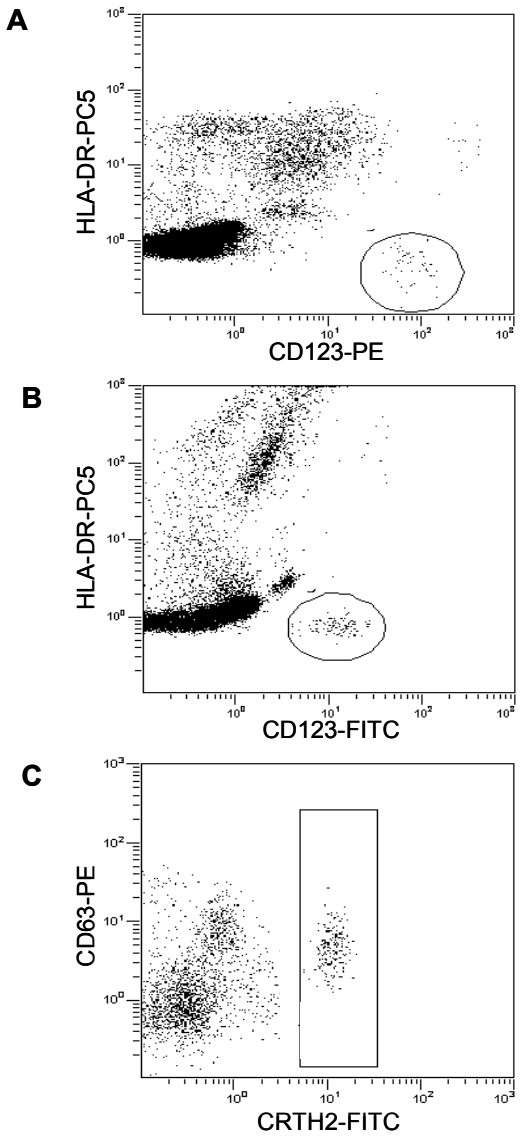

Figure 4. Gating strategies of basophils in flow cytometry
(A) Strategy 1: Basophils gated as HLA-DR-PC5^{neg} and CD123-PE^{pos} cells. (B) Strategy 2: Basophils gated as HLA-DR-PC5 ^{neg} and CD123-FITC^{pos}. (C) Strategy 3: Basophils gated as CD3-PC7^{neg}, CRTH2-FITC^{pos} cells (CD3-PC7-negative gate not shown).

When the three gating strategies were compared with respect to basophil reactivity, strategy 1 yielded the highest basophil responses while strategy 3 significantly attenuated basophil reactivity to stimulation with anti-IgE antibody (Figure 5).

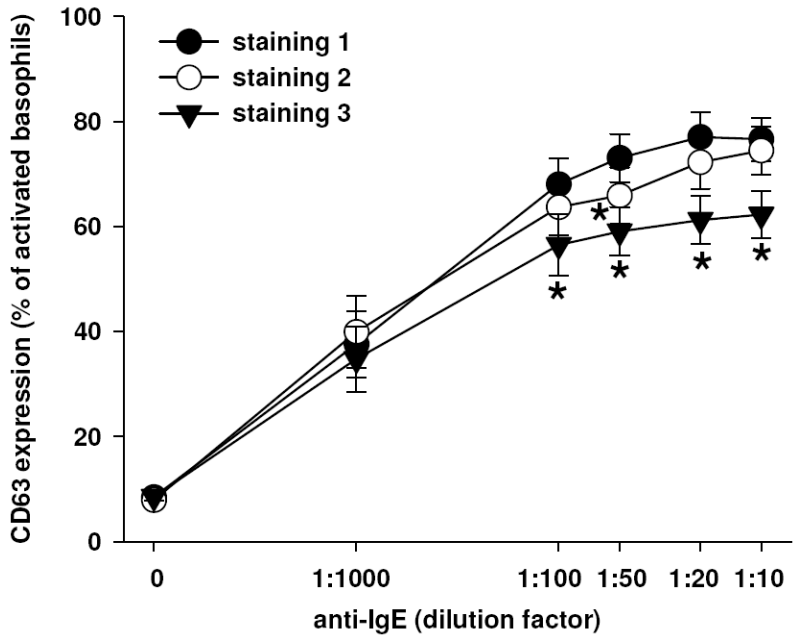

Figure 5. Effect of different staining strategies on basophil activation
Staining 1: HLA-DR-PC5^{neg}, CD123-PE^{pos}, CD63-FITC^{pos}; staining 2: HLA-DR-PC5^{neg}, CD123-FITC^{pos}, CD63-PE^{pos}, staining 3: CD3-PC7^{neg}, CRTH2-FITC^{pos}, CD63-PE^{pos}. Staining 1 shows consistently higher CD63 expression compared to staining 3. The generally weaker CD63 expression of staining 2 is only significant at the 1:50 dilution of anti-IgE. Data are expressed as means ± SEM, $n = 16$, * $p < 0.05$ *versus* staining 1.

Flow cytometer systems

In a next step we determined whether testing on two different cytometers might yield different data with respect to CD63 expression and BAT. For this purpose, the Beckman Coulter FC 500 and the BD FACSCalibur, were compared. As shown in Figure 6, a difference of 6.8 to 10.3% in activated basophils was found in favor of the FC500 cytometer, depending on the dilution of anti-IgE

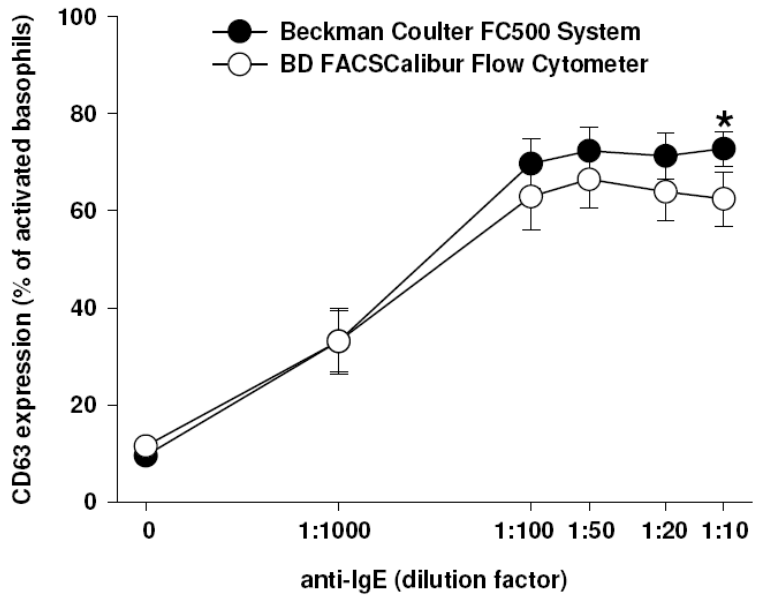

Figure 6. Comparison of FC 500 and FACSCalibur
Detection of CD63 expression levels yielded slightly different results at dilutions of 1:10, 1:20, 1:50 and 1:100 of anti-IgE. However, the difference was only significant at 1:10. Data are expressed as means ± SEM, $n = 15$, * $p < 0.05$. Experiments performed by Ines Freistätter

Influence of antihistamines and glucocorticoids on basophil activation

Finally, we investigated the influence of anti-allergic drugs on basophil activation. In vitro pretreatment of the samples with dimetindene and prednisolone had no effect on BAT data at concentrations that corresponded to therapeutic plasma levels of the drugs (Figure 7). Basophil activation, however, was significantly attenuated at concentrations fifty-fold higher than the therapeutic level. As the intake of antihistamines is a frequent problem in the diagnosis of allergies, BAT was additionally performed in 10 subjects before, and three hours after, taking 10 mg of desloratadine. As shown in Figure 8, no significant effect on test results was observed.

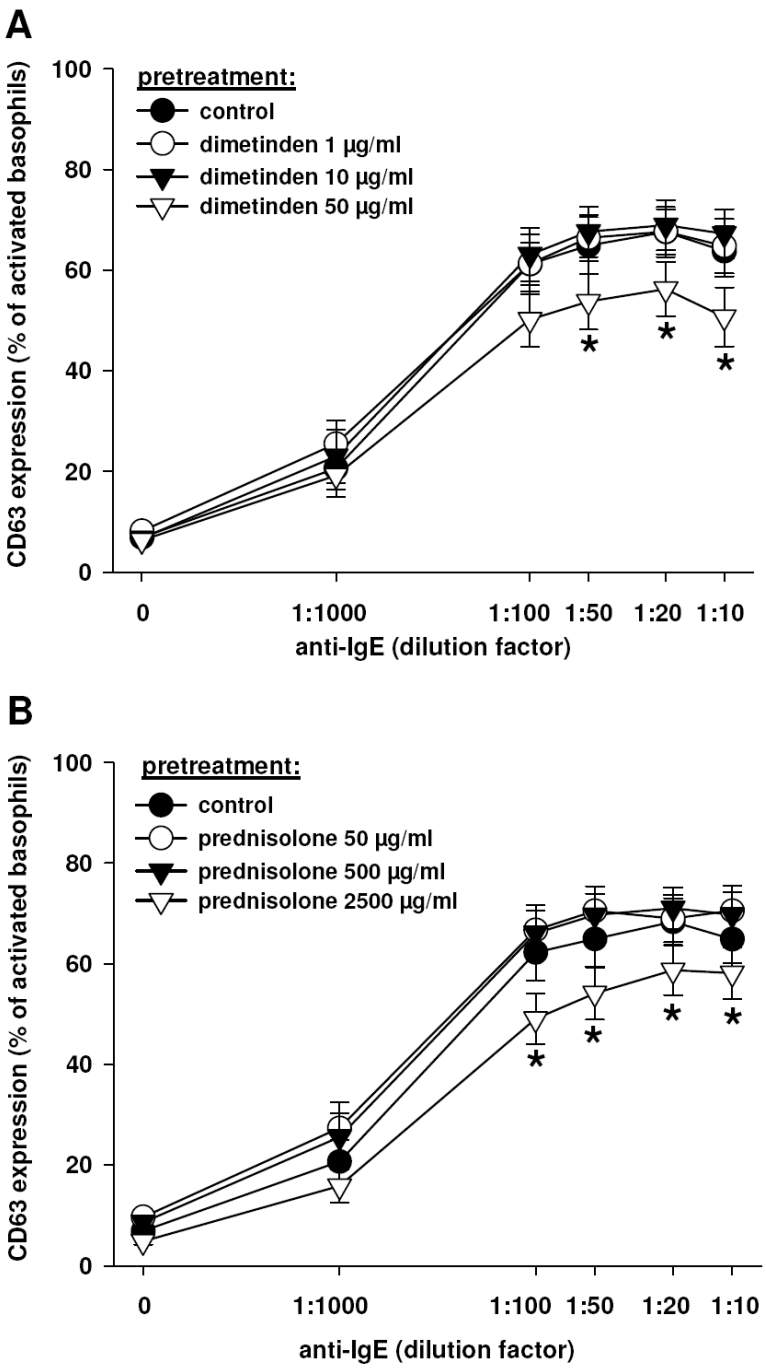

Figure 7. Influence of in vitro pre-treatment of basophils with antihistamines and glucocorticoids on the results of the basophil activation test
At concentrations up to ten-fold higher than the therapeutic range, no significant reduction of basophil reactivity as determined by stimulation with anti-IgE antibody was observed after pre-treatment of cells with the H_1 histamine receptor antagonist dimetindene or the glucocorticoid prednisolone for 30 min. Basophil activation, however, was significantly attenuated at concentrations fifty-fold higher than the therapeutic level. Data are expressed as means ± SEM, $n = 17$, * $p < 0.05$ *versus* control.

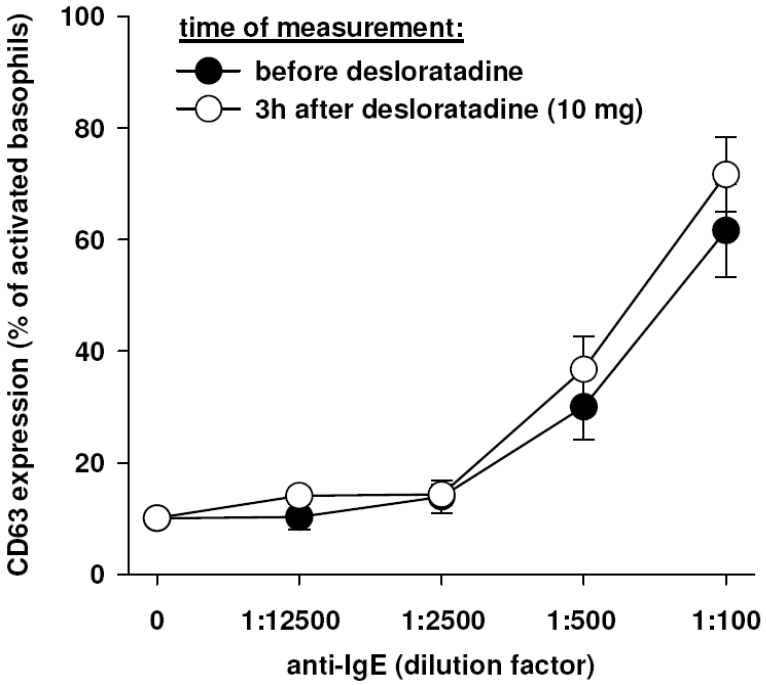

Figure 8. Influence of antihistamine intake on the results of the basophil activation test
Blood samples were taken before, and three hours after the intake of 10 mg desloratadine. Basophil reactivity to anti-IgE stimulation was not altered when responses before and after treatment were compared. Data are expressed as means ± SEM, $n = 10$.

Discussion

Several studies have confirmed the usefulness of BAT as a routine diagnostic tool [31, 33, 38, 67, 71] and as a valuable test to identify the culprit insect in ambiguous cases (e.g., negative skin tests) [72, 73]. However, the debate on its reliability and validity is still ongoing and an agreement on a standardized protocol is poor. In the present study we addressed technical aspects to be considered when performing this test, and evaluated factors that might influence the test results.

We were able to confirm the stimulatory effect of IL-3 described in the published literature [74, 75]. However, cytochalasin B and pre-warming of samples and reagents exerted no such effect. The positive effect of pre-warming was recently discussed at the first European Workshop on Flow cytometry in Allergy held in Pamplona, Spain. Possibly, pre-warming of cells has no effect when fresh blood is used: in our experiments we processed all samples within 30 to 60 minutes after taking blood.

We observed a reduction of basophil activation by anti-IgE antibody by up to 58.5% after a storage time of 48 hours. More importantly, even after 4 hours basophil activation decreased. Nevertheless, the test results remained positive after 48 hours in the anti-IgE dilution series (threshold of 25% activated basophils). This could be explained by the strong response to anti-IgE; up to 81.7% activated basophils were seen at the time point of 0 h. Conversely, stimulation of basophils with bee and wasp venom after 18 hours resulted in several false-negative results. In contrast to stimulation with anti-IgE, the maximum response to Hymenoptera venoms was considerably lower at 63.0% even immediately after blood sampling. While loss of basophil reactivity is comparable with that of stimulation with anti-IgE, the results become readily negative due to the overall lower reactivity to allergens. Therefore, BAT should preferably be performed using fresh blood. It is not advisable to store blood samples or mail them to specialized laboratories.

In further experiments, we evaluated various staining protocols. Best results were obtained with the HLA-DR-PC5/CD123-PE/CD63-FITC staining. The same staining strategy, but with switched fluorochromes (HLA-DR-PC5/CD123-FITC/CD63-PE), resulted in reduced mean activation. One explanation might be that FITC is less bright compared to PE. Therefore, under sub-optimal conditions, the basophil population is insufficiently separated from other cells using FITC-conjugated antibodies. For this reason the percentage of activated basophils might have been influenced by the presence of other cell types. This assumption is confirmed by a completely different staining strategy, namely the CD3-PC7/CRTH2-FITC/CD63-PE staining, which again relies on FITC conjugates (CRTH2) and also shows reduced mean activation.

In a next step we compared two different flow cytometer systems with various softwares. In fact, there was a difference of up to 10.3% in mean activation of basophils between the two flow cytometers.

Taken together, quantitative results may vary due to the above mentioned, as well as other yet untested, factors. Differing quantitative outcomes of BAT could be explained by varying storage times, stainings and flow cytometers, among other factors. Thus, it is virtually impossible to compare qualitative results unless different research groups use identical settings. Qualitative results such as simply “positive” or “negative” may be comparable, but further studies are needed to confirm this assumption. Due to variations in quantitative results it is questionable whether thresholds of 5% for BAT with drugs, as proposed by some research groups, are meaningful. The diagnostic sensitivity and specificity values of the BAT are usually high when basophils show a strong response to given allergens, as is the case for Hymenoptera venoms [31, 38, 67] or inhalant allergens such as grass pollen [76], house dust mite extracts [77] or tree pollen [78]. In drug allergy, basophil responsiveness to the allergens or haptens is usually poor. Initial data concerning the usefulness of BAT in betalactam allergy [79, 80] and anti-inflammatory drug hypersensitivity [81] are discussed controversially [34, 82].

Finally, we were able to show that especially antihistamines do not interfere with basophil reactivity. As antihistamines are H1-receptor antagonists, they only block the effect of histamine but not mediator release from basophils or mast cells. Antihistamines therefore do not reduce CD63 expression; this has been already mentioned by Erdmann in a short comment [83]. Glucocorticoids reduced basophil reactivity only at unusually high concentrations *in vitro* with a short incubation time. However, we did not investigate 24 hour incubation with glucocorticoids as whole blood cannot be stimulated over several hours. As they mainly rely on nuclear action, and this mechanism requires possibly several hours, no conclusion can be drawn for patients taking glucocorticoids for several days.

In conclusion, BAT has been repeatedly shown to be a reliable diagnostic tool in hymenoptera allergy. In the current study we determined some factors that might influence the results of BAT. However, larger studies are needed to address yet unresolved issues, which would include a comprehensive comparison of the available staining strategies as well as microscopic investigation of the purity of gating strategies, further evaluation of different storage temperatures and media, and a comparison of whole blood and isolated leukocyte protocols. As a number of potentially confounding factors have to be taken into account, the use of BAT should currently be limited to experienced laboratories.

2. CD203c-based basophil activation test in allergy diagnosis: Characteristics and differences to CD63 upregulation

Introduction

Hypersensitivity to hymenoptera venom is a common and potentially life-threatening condition[84-87]. Large local reactions occur in up to 26.4%[88] of the general population after hymenoptera stings and up to 7.5% are reported to have experienced systemic anaphylactic reactions[2]. Current standard diagnostic procedures, based on personal history, skin testing and the determination of allergen-specific IgE serum antibodies, allow an accurate diagnosis in the majority of cases, but there is still concern about their sensitivity and specificity[7, 12, 16, 17]. Hence, in the last years a considerable progress in the development of novel functional *in vitro* tests took place and the flowcytometric basophil activation test (BAT) became generally accepted as an additional and reliable diagnostic assay. The first CD63-based protocol for allergy diagnosis was developed by Sainte-Laudy and Sabbah in 1996[66]. In 2000, an excellent sensitivity and specificity of BAT in the diagnosis of hymenoptera allergy was described[67] and subsequently confirmed in several studies[31-33, 37, 38]. Data of the first CD203c-based protocol for hymenoptera allergy was published in 2001[63] and was recently validated by others[33]. Currently, BAT with CD63 is considered as the best clinically validated test[49] while BAT based on CD203c still requires further investigation.

The ectoenzyme CD203c (E-NPP3; pyrophosphatase/phosphodiesterase 3) was found to be expressed on blood basophils, tissue mast cells and their CD34+ progenitor cells, but not on other blood leukocytes[60, 61]. In contrast to CD63, resting basophils show some degree of constitutive CD203c expression on their plasma membrane, whereas CD63 expression is closely related to basophil degranulation[43]. Similarly to CD63, basophil CD203c expression rapidly increases after allergen challenge in sensitized individuals[36, 62, 63]. On the one hand, some authors claimed the advantages of CD63 over CD203c[39-41], but on the other hand

several studies addressed the pivotal role of CD203c as the most promising new activation marker for flow cytometry-based allergy diagnosis. Already in 2003 Boumiza *et al.* pointed out that due to superior gating of basophils and a higher range of activation in response to allergen, the basophil activation test is markedly improved by use of CD203c instead of CD63[42]. Recently, a comparative study emphasized the reliability of BAT using either CD63 or CD203c as an *in vitro* diagnostic tool for Hymenoptera venom allergy with a higher sensitivity for the CD203c protocol[33]. Additionally, a study concerning the use of basophil activation markers in the diagnosis of cat allergy demonstrated that the measurement of basophil CD203c up-regulation is as reliable as CD63-based BAT[35]. Moreover, Abuaf *et al*. demonstrated that CD203 seems to be a more sensitive basophil activation marker than CD63 for the diagnosis of amoxicillin allergy[34].

In spite of the widespread application, BAT protocols using either CD63 induction or CD203c up-regulation are generally not standardized and their potentials and pitfalls are still a point at issue. However, pitfalls are rarely reported in the published literature; most reports are based on personal communication and small numbers of patients[70]. We recently addressed important technical issues regarding BAT based on CD63[89], but there is urgent need for further investigation addressing the potential factors that might influence CD203c-based BAT. The goal of the present study was to establish an optimized BAT protocol based on CD203c up-regulation for the diagnosis of hymenoptera allergy and to point out potential influencing factors including anti-allergic drugs.

Methods

Reagents and Antibodies

All laboratory reagents were obtained from Merck (Whitehouse Station, NJ, USA) or Sigma-Aldrich (St. Louis, CA, USA) unless otherwise specified. Dulbecco's modified phosphate-buffered saline (PBS; with or without Ca^{2+} and Mg^{2+}) was purchased from PAA (Pasching, Austria). CellFix and antibodies to CD123 (PE-conjugated) were supplied by Becton Dickinson (Franklin Lakes, New Jersey, USA). Dimetindene, desloratadine and prednisolone were purchased from Novartis (Basel, Switzerland), Schering-Plough (Kenilworth, NJ, USA) and Merck (Whitehouse Station, NJ, USA), respectively. Mouse IgG1 isotype controls (FITC and PE-conjugated), antibodies to HLA-DR (PC5-conjugated), CD63 (FITC-conjugated) and monoclonal antibodies to IgE (clone E124.2.8) were purchased from Beckman Coulter (Fullerton, CA, USA). Antibodies to CD123 (FITC-conjugated) and CD203c (pure and PE-conjugated) were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany) and antibodies to CD63 (pure) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse IgG (Alexa Fluor 488-conjugated) antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Mouse IgG1 isotype controls, cytochalasin B and interleukin (IL)-3 were supplied by Sigma-Aldrich. Latrunculin B was purchased from Calbiochem, Merck Chemicals (Nottingham, UK). Ultra V Block blocking solution was purchased from Lab Vision (Fremont, CA, USA) and antibody diluent was purchased from Dako (Golstrup, Denmark). Easy Sep Basophil Enrichment Kit and the “Big Easy” Silver Easy Sep Magnet were purchased from StemCell Technologies (Vancouver, Canada). Vectashield Mounting Medium was purchased from Vector Laboratories (Burlingame, CA, USA). Aqueous bee and wasp venom (ALK aqueous SQ, corresponding to raw venom after microfiltration) were purchased from ALK-Abelló (Hørsholm, Denmark).

Wash buffer: Dulbecco's modified phosphate-buffered saline containing 0.2 g/L KCL, 0.2 g/L KH_2PO_4, 8 g/L NaCl, and 1.15 g/L anhydrous Na_2HPO_4.

Stimulation buffer: Dulbecco's modified phosphate-buffered saline containing 0.2 g/L KCL, 0.2 g/L KH_2PO_4, 8 g/L NaCl, 1.15 g/L anhydrous Na_2HPO_4, 0.132 g/L $CaCl_2 \cdot 2H_2O$ and 0.1 g/L $MgCl_2 \cdot 2H_2O$.

Lysis solution: The composition of the lysis solution used for erythrocyte elimination from whole blood samples was 155 mM NH_4Cl, 10 mM $KHCO_3$ and 100 µM Na_2-EDTA.

Fixative solution: BD CellFIX (10X; 10% paraformaldehyde, 1% sodium azide) was diluted 1/80 in PBS (without Ca^{2+} and Mg^{2+}).

Selection of study subjects

Blood was collected from healthy (not bee- or wasp venom allergic) volunteers as well as from bee- or wasp venom-allergic patients according to a protocol approved by the Ethics Committee of the Medical University of Graz. Eighty-two age (42.0 ± 13.2) and sex-matched (45 women, 37 men) control subjects and 79 allergic patients (39 women, 40 men; mean age 39.0 ± 13.0) were included in this study. In hymenoptera venom allergic patients, the personal history of a systemic sting reaction was confirmed by intradermal testing and IgE determination. Intradermal testing was performed with 0.02 mL of 0.01, 0.1 and 1 µg/mL of purified bee and wasp venom extracts (ALK aqueous SQ). The test was considered to be positive in the presence of a wheal ≥5 mm in diameter. Determination of total IgE and specific IgE to bee venom (i1) and wasp venom (i3) in the patients' serum were measured by the ImmunoCAP 1000 (Phadia, Uppsala, Sweden) according to the manufacturer's instructions.

Only patients with generalised symptoms were included; median total IgE level of the allergic patients was 85.1 (38.7;281.0) kU/L. None of the patients/control subjects were on systemic medication at the time of testing.

Experimental procedure

Immunofluorescence staining of isolated basophils

Leukocytes from citrated whole blood were separated by means of dextran sedimentation and centrifugation on Histopaque 1077 into a polymorphonuclear cell fraction (eosinophils and neutrophils) and a mononuclear cell fraction (basophils, monocytes, and lymphocytes)[90, 91]. The resulting PBMC layer was incubated with the StemSep Basophil Enrichment Cocktail and colloidal magnetic particles, and unlabeled basophils were separated from other cell types by passage through a magnetic field according to the manufacturer's instructions (StemCell Technologies). Cell purity was determined by staining with fluorochrome-labeled anti-CD123 and anti-HLA-DR antibodies and was found to be 95% basophils, with a cell yield of 7×10^4 to 5×10^5/donor (from 70 ml of citrated blood). Contaminating cells had forward scatter (FSC)/side scatter (SSC) characteristics of lymphocytes.

Isolated basophils were treated with or without (control) anti-IgE (1:100 dilution) for 20 min in a 37°C water bath. The samples were then transferred to an ice water bath for 5 min to terminate the reaction and washed in PBS (without Ca^{2+} and Mg^{2+}). Unspecific binding sites were blocked with Ultra V Block blocking solution for 30 min at 4°C and cells were washed in PBS (without Ca^{2+} and Mg^{2+}). For cell surface staining cells were incubated with CD63 or CD203c specific primary antibodies and stained with a FITC-conjugated secondary antibody for 30 min at 4°C, respectively. The cells were washed and resuspended in PBS (without Ca^{2+} and Mg^{2+}). Cytospin slide preparations were fixed with 3.7% formaldehyde for 10 min and mounted with Vectashield medium. Then the samples were analyzed on an Olympus IX70 fluorescence microscope and an Olympus UPlanApo - 60x/1.20 lens, using Hamamatsu ORCA-ER digital camera and Olympus Cell^P software.

Basophil activation test – Standard protocol

EDTA whole blood was stained with anti-CD123 FITC-conjugated antibody, anti-HLA-DR PC5-conjugated antibody and anti-CD203c PE-conjugated antibody for measuring CD203c expression, or with anti-CD123 PE-conjugated antibody, anti-HLA-DR PC5-conjugated antibody and anti-CD63 FITC-conjugated antibody in the CD63-based protocol. In all experiments samples were stained for 10 min at room temperature with an antibody-to-whole blood ratio of 1:50. Basophil reactivity was measured using serial dilutions of bee or wasp venom (1000, 100, 10, 1 ng/mL) or serial dilutions of anti-IgE antibody (1:10-1:3200 dilution). Hundred µL aliquots of whole blood were mixed with 100 µL of the respective stimulant or buffer (PBS with Ca^{2+} and Mg^{2+}) and incubated for 20 min in a 37°C water bath. Subsequently, the reaction was terminated by adding 100 µL of a 3.8% EDTA stop solution. In order to achieve erythrocyte lysis, 2 mL of lysis solution was added to each sample. After 15-min incubation at room temperature in the dark, the samples were centrifuged (5 min, 200 x g) and the supernatants discarded. The resulting cell pellets were washed in 3 mL PBS (without Ca^{2+} and Mg^{2+}) and resuspended in 400 µL ice-cold fixative solution.

Incubation time

The expression of CD203c and CD63 is described to be linked to two different mechanism of basophil activation which is indicated by different time kinetics of upregulation[44]. In order to define an ideal incubation time for BAT protocols, the maximum expression of CD203c and CD63, with or without IL-3 (300 pM) priming was determined in a time-course study. Basophil activation was induced by anti-IgE at a dilution of 1:100 and samples were incubated for up to 3 h. The data of all three protocols were analyzed for every point of time by adjusting the threshold in non-stimulated samples to contain 10% of CD203c or CD63 expressing basophils (baseline).

Storage time

Samples of EDTA anti-coagulated whole blood from control subjects were stored for 4, 24, 28 and 48 hours at 4°C while samples from allergic patients were stored for 18 hours at 4°C. BAT was performed immediately after blood-taking and at the indicated time-points. For stimulation, serial dilutions of anti-IgE, or bee or wasp venom were used as described above.

Basophil priming

IL-3, a potent basophil-priming cytokine, is used in several CD63-based BAT protocols to enhance basophil responsiveness. As IL-3 is also described to enhance the levels of CD203c without promoting the expression of CD63[92], the effect of IL-3 pretreatment on the up-regulation of CD203c and CD63 was examined. For this purpose, EDTA whole blood samples of healthy subjects were incubated with vehicle or IL-3 (300, 150 or 75 pM) and with anti-CD123 FITC-conjugated antibody, anti-HLA-DR PC5-conjugated antibody and anti-CD203c PE-conjugated antibody for measuring CD203c expression, or with anti-CD123 PE-conjugated antibody, anti-HLA-DR PC5-conjugated antibody and anti-CD63 FITC-conjugated antibody in the CD63-based protocol, for 10 min at room temperature.

Similarly, we investigated the effect of cytochalasin B (5µg/mL)[31, 89] and latrunculin B (5µg/mL and 0.1 µg/mL)[93, 94], both inhibitors of actin polymerization, on CD203c as well as CD63-based BAT results. In all cases, serial dilutions of anti-IgE were used for basophil stimulation as described above.

The data were analyzed by adjusting the threshold in non-stimulated samples to contain 10% of CD203c or CD63 expressing basophils (baseline) or by adjusting the threshold in non-stimulated, but IL-3 primed, or cytochalasin B or latrunculin B pretreated samples to contain 10% of CD203c or CD63 expressing basophils (baseline).

Influence of anti-allergic drugs

To investigate the effect of prednisolone and dimetindene on the up-regulation of basophil CD203c expression, EDTA whole blood samples derived from healthy subjects were pre-treated with vehicle, 50, 500 and 2500 µg/mL of prednisolone or vehicle, 1, 10 and 50 µg/mL of dimetindene for 30 min at 37°C. Subsequently, BAT was performed using serial dilutions of anti-IgE in accordance with the standard protocol. To examine the influence of desloratadine, the assay was performed before and three hours after the intake of the two-fold daily dose (10 mg) of desloratadine.

Cytometer and fluorochrome setting

Flow analysis was performed using the FC500 single laser flow cytometer from Beckman Coulter[89]. This instrument has a 3,300 events/sec capability, five color detection and a scatter and fluorescence carryover < 1%. The FC500 is equipped with FL-1 525/40 nm, FL-2 575/40 nm, FL-3 620/20 nm, FL-4 675/40 nm and FL-5 755/40 nm band pass filters. Analyses were performed with a high flow rate setting an excess limit of 100,000 events. The flow cytometer was calibrated daily for laser fluctuation and photomultiplier tube (PMT) voltage setting by using FlowCheck beads (Beckman Coulter). Daily fluctuation is logged and checked every week to monitor range in variation. To assess spectral overlap and to calculate the compensation for the assays a three-color compensation followed the cytometer manufacturer's instruction was performed. Therefore whole blood samples were single stained with anti-CD45 antibodies conjugated to FITC, PE or PC5, as CD45 is a highly expressed marker which produces a high intensity signal in all the fluorescent detectors.

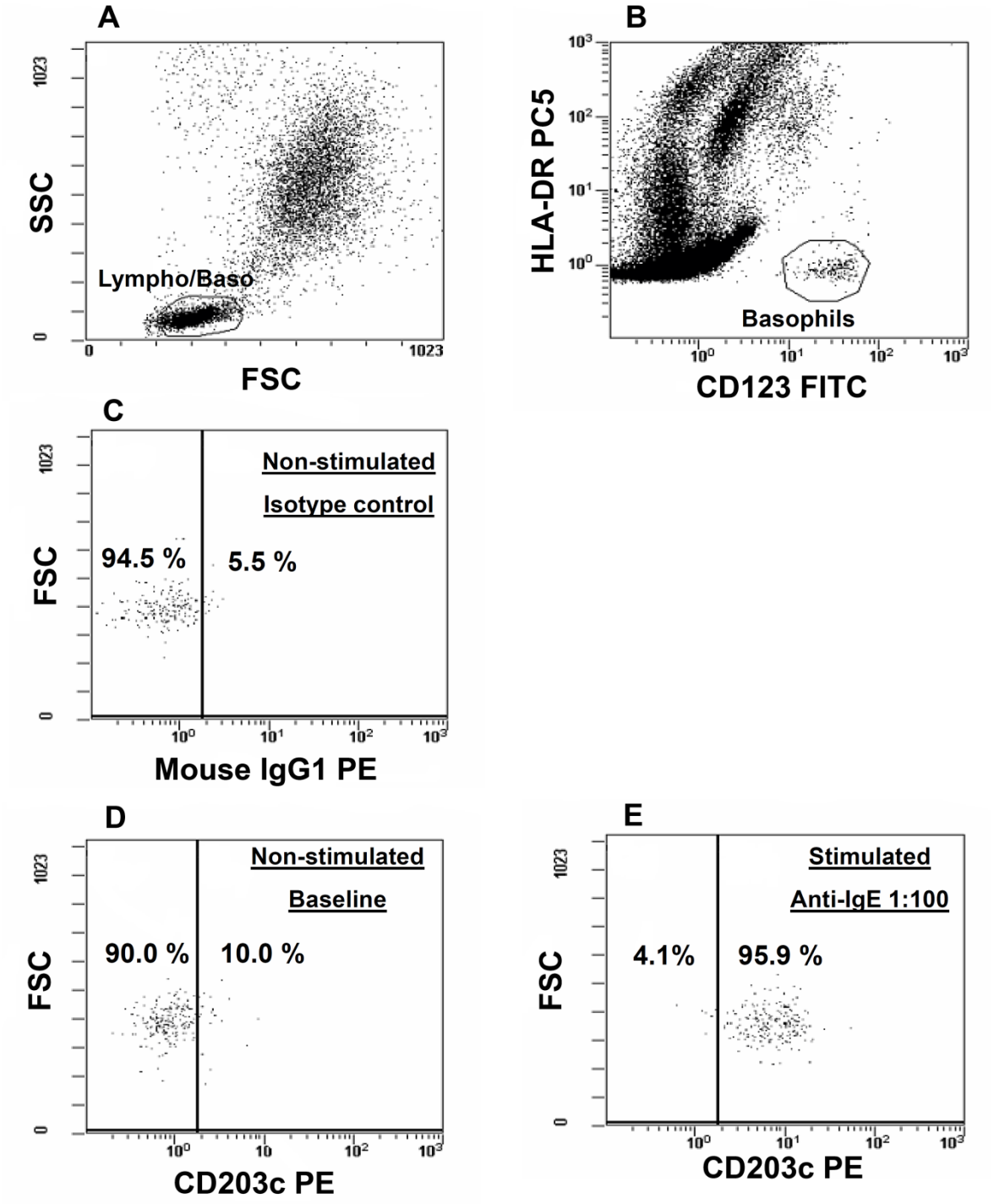

Figure 1. Gating procedure in CD203c-based BAT.

Basophils gated in the lymphocyte region of the SSC/FSC pattern were selected as a HLA-DR^{neg}/CD123^{pos} population (A; B). CD203c responses were quantified as percentages of CD203c expressing basophils in a higher FL-2 region which had been adjusted to contain 10% of activated basophils (D), in an unstimulated sample (i.e. negative control). A 2.5-fold increase in the number of activated basophils at any of the test concentrations of anti-IgE and bee or wasp venom was considered to be a positive response (E). To evaluate fluorochrome unspecific staining a mouse IgG1 isotype control conjugated to PE was used (C).

Gating procedure

Basophils were gated in the lymphocyte region of the side scatter (SSC)/ forward scatter (FSC) pattern (Fig. 1A). In the CD203c-based protocol basophils were identified as a single population of cells that stained positive for CD123 FITC (488 nm/520 nm; FL-1 channel) and negative for HLA-DR PC5 (488 nm/668 nm; FL-4 channel) (Fig. 1B). In the CD63-based protocol basophils were identified as a single population of cells that stained positive for CD123 PE (488 nm/578 nm; FL-2 channel) and negative for HLA-DR PC5 (488 nm/668 nm; FL-4 channel).

CD203c and CD63 surface expression

To evaluate fluorochrome unspecific staining, mouse IgG1 antibodies conjugated to FITC or PE (Fig. 1 C) were used as isotype controls in the preliminary procedure. Up-regulation of CD203c expression was indicated by an increase in fluorescence in the FL-2 channel (Fig. 1 D and E). Up-regulation of CD63 expression was indicated by an increase in fluorescence in the FL-1 channel. Acquisition was terminated after 300 basophil target events. Responses were quantified as percentages of CD203c expressing basophils in a higher FL-2 region or CD63 expressing basophils in a higher FL-1 region, which had been adjusted to contain 10% of basophils (i.e. activated basophils), in an unstimulated sample (i.e. negative control). A 2.5-fold increase in the number of activated basophils (>25%) as compared with the negative control (10%) at any of the test concentrations of anti-IgE and bee or wasp venom was considered to be a positive response.

Statistics

Data are shown as means ± SEM for *n* observations. Comparisons of groups of data were performed using repeated measures ANOVA. Probability values of $p < 0.05$ were considered

to be statistically significant. Data were analyzed using the SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

Immunofluorescence staining of CD63 and CD203c surface expression

In contrast to CD63, CD203c is described as a basophil specific activation marker with a basal low-level expression in resting basophils. To further confirm this observation and to emphasize the differences between CD63 and CD203c kinetics, the distribution of both markers on purified basophils was analyzed by immunofluorescence staining. In untreated cells a low CD203c basal expression was observed, while they lacked CD63 expression or only showed a discrete staining pattern (Fig. 2A and C). In anti-IgE (ratio 1:100) stimulated basophils there was a significant increase in surface expression, similar for both markers (Fig. 2B and C).

Comparison between CD203c and CD63 – based BAT results

A side-by-side comparison between CD63 and CD203c-based BAT was performed in 79 hymenoptera venom allergic patients (Table 1). Therefore, whole blood samples were stimulated with anti-IgE (ratio 1:3200 - 1:100) or with bee and wasp venom (1 – 1000 ng/mL). In the CD63 protocol whole blood samples were incubated with IL-3 (300 pM) for 10 min at room temperature prior to allergen or anti-IgE stimulation.

Results of CD63 and CD203c were comparable to a large extend, although CD203c showed a slight, but not statistically significant, higher sensitivity compared to the CD63 (85.0% vs. 79.7%, p=0.405) with regard to patients' history.

	CD63		CD203c	
	n	**%**	**n**	**%**
bee venom positive	11	13.9	11	13.9
wasp venom positive	34	43.0	39	49.4
bee venom/wasp venom positive	18	22.8	17	21.5
negative	16	20.3	12	15.2
anti-IgE positive	**79**	**100.0**	**79**	**100.0**

Table 1. Comparison between CD63 and CD203c expression in hymenoptera venom allergic patients. CD63 and CD203c – based BAT was performed in 79 allergic patients. Whole blood samples were stimulated with anti-IgE (ratio 1:3200 - 1:100) and with bee and wasp venom (1 – 1000 ng/mL).

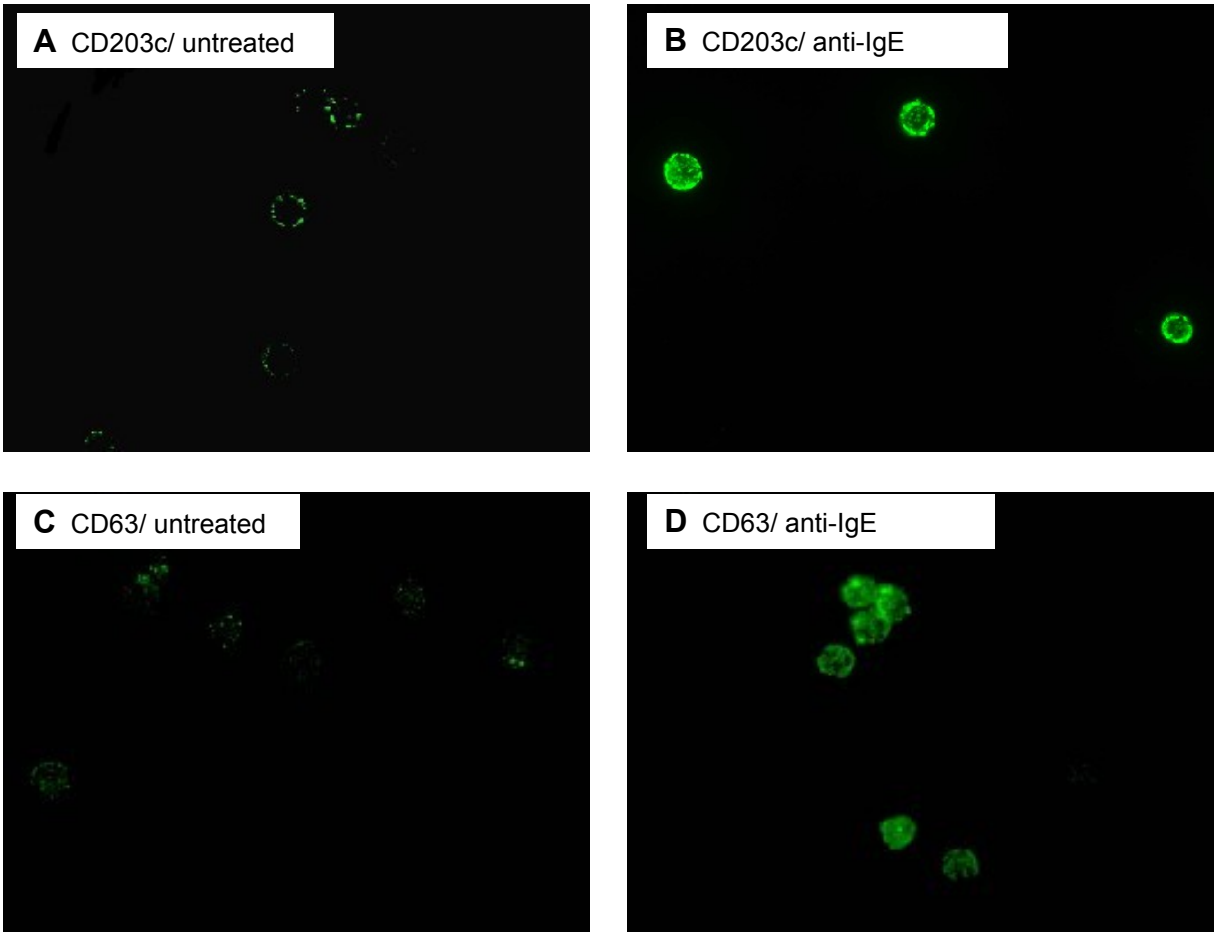

Figure 2. Immunofluorescence staining of CD63 and CD203c in isolated basophils. Purified basophils were stimulated with anti-IgE (ratio 1:100) or buffer for 20 min at 37°C. Samples were blocked and stained with CD63 and CD203c specific primary antibodies and a goat anti-mouse IgG (H+L) secondary antibody conjugated with Alexa Fluor 488. Cytospin slide preparations were fixed with 3.7% formaldehyde and mounted with Vectashield medium. Samples are analyzed on an Olympus IX70 fluorescence microscope and an Olympus UPlanApo - 60x/1.20 lens, using Hamamatsu ORCA-ER digital camera and Olympus Cell^P software. CD203c showed a higher expression than CD63 either under basal (A and C) and under stimulated conditions (B and D). Experiments performed by Eva Sturm

Time-course of CD203c up-regulation

As CD63 and CD203c up-regulation is suggested to exhibit different time kinetics we determined the optimum incubation time for our CD203c and CD63-based protocol using anti-IgE stimulation (ratio 1:100). The time kinetics of CD203c and CD63 were performed in 10 controls (3A). The time kinetics of CD203c compared to IL-3 (300 pM) primed CD63 expression were performed in 21 controls (3B). In the absence of IL-3, CD203c expression was more rapidly upregulated than CD63 (Fig. 3A). After 10 min only 62% of activated

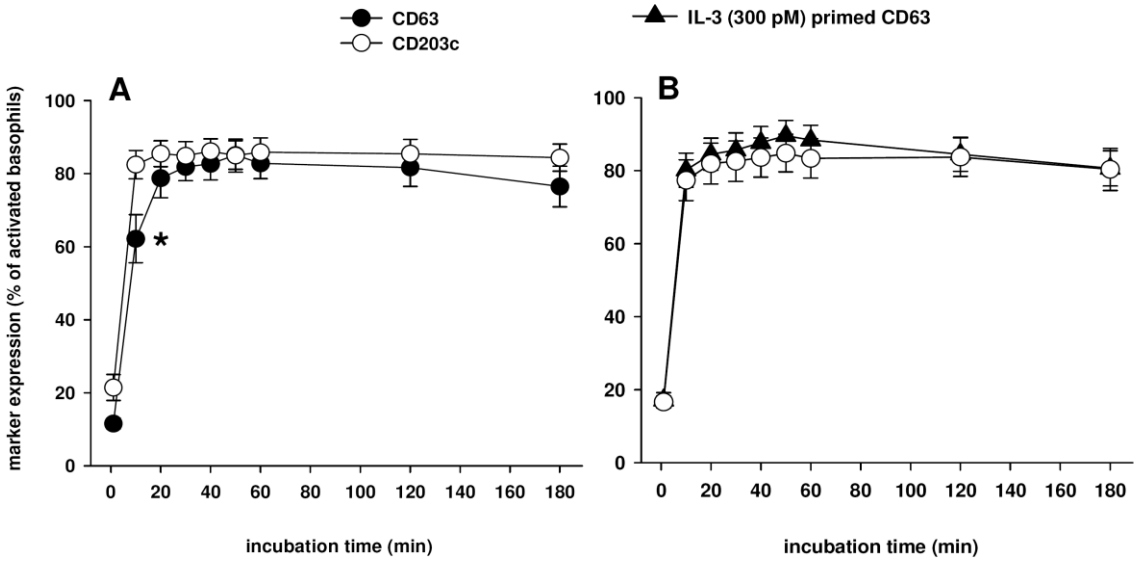

Figure 3. Time kinetics of CD203c and CD63 expression.

Samples were stimulated with anti-IgE (ratio 1:100) at 37°C and activation was stopped immediately or after the indicated incubation periods. The time kinetics of CD203c and CD63 were performed in 10 controls (A). The time kinetics of CD203c compared to IL-3 (300 pM) primed CD63 expression were performed in 21 controls (B). Data are expressed as mean ± SEM, n = 10-21; * $P < 0.05$ CD63 *vs* IL-3 primed CD63.

basophils that express CD63 were detected while CD203c was present on 82% of activated basophils. Priming of cells with IL-3 (300 pM) in the CD63 BAT corrected the difference between CD63 and CD203c kinetics (Fig. 3B). In all cases maximal expression of CD63 or CD203c was observed after 20 to 30 min reaching a plateau of 80-90% of activated basophils up to 180 min. Therefore, all further experiments were performed with an incubation time of 20 min.

Storage time and basophil activation

Next we examined the influence of different storage times of blood samples on basophil CD203c expression. Therefore, 18 control samples were stimulated by different dilutions of anti-IgE after storage of 0, 4, 24, 28 and 48 hours at 4°C, while samples from allergic patients were stimulated after storage of 0 and 18 hours at 4°C. As shown in Figure 4, basophil responsiveness decreased in a time-dependent manner: CD203c expression assayed after a storage time of 24 hours resulted in a loss of basophil reactivity, depending on the anti-IgE dilution, by 35.1 to 54.4%. After 48 hours the basophil responses were reduced by 48.1% to 56.1%. After 4 h of storage, basophil responses to higher concentrations of anti-IgE were unchanged as compared to samples assayed immediately after blood taking, while the response to low anti-IgE stimulation was attenuated by more than 50%.

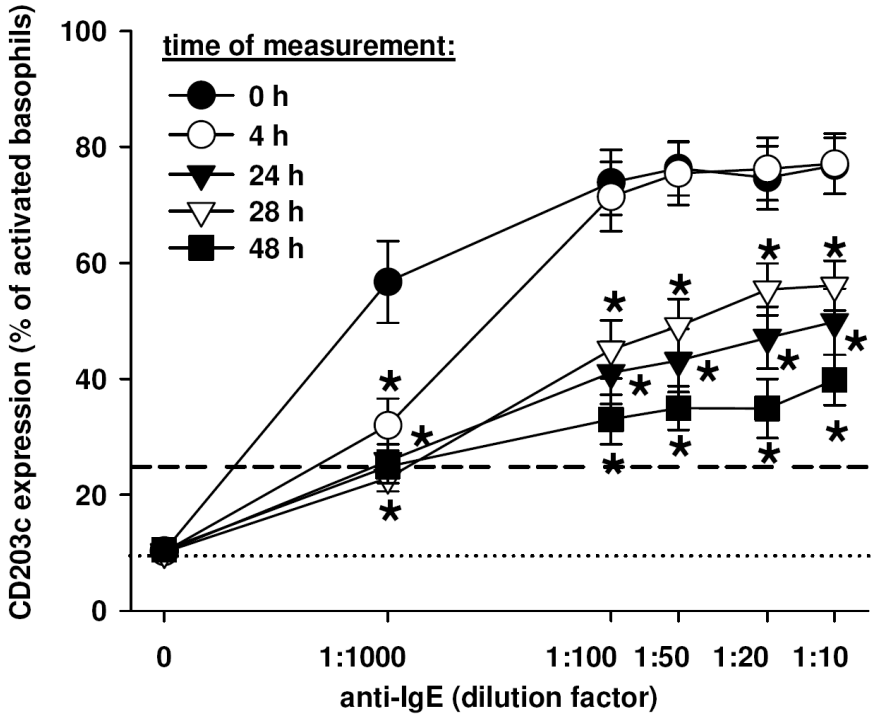

Figure 4. Storage time-dependent decrease in anti-IgE-induced basophil activation. Blood samples from controls were stored for up to 48 h at 4°C before BAT was performed (anti-IgE, 20 min, and 37°C). Best responses were obtained when blood samples were processed immediately. A storage time from 24 to 48 hours resulted in significantly reduced basophil activation. Data are expressed as means ± SEM, n = 18; * $P < 0.05$ *vs* 0 h. ……baseline response (10%); − − − threshold of 25% activated basophils.

Similar results were obtained with regard to bee and wasp venom: of initially 13 bee-venom-allergic and BAT-positive patients, only 6 were positive after 18 hours; of 18 wasp-venom-allergic patients, only 4 remained positive after 18 hours. A loss of reactivity by up to 70.9 % was found after 18 hours (Figure 5).

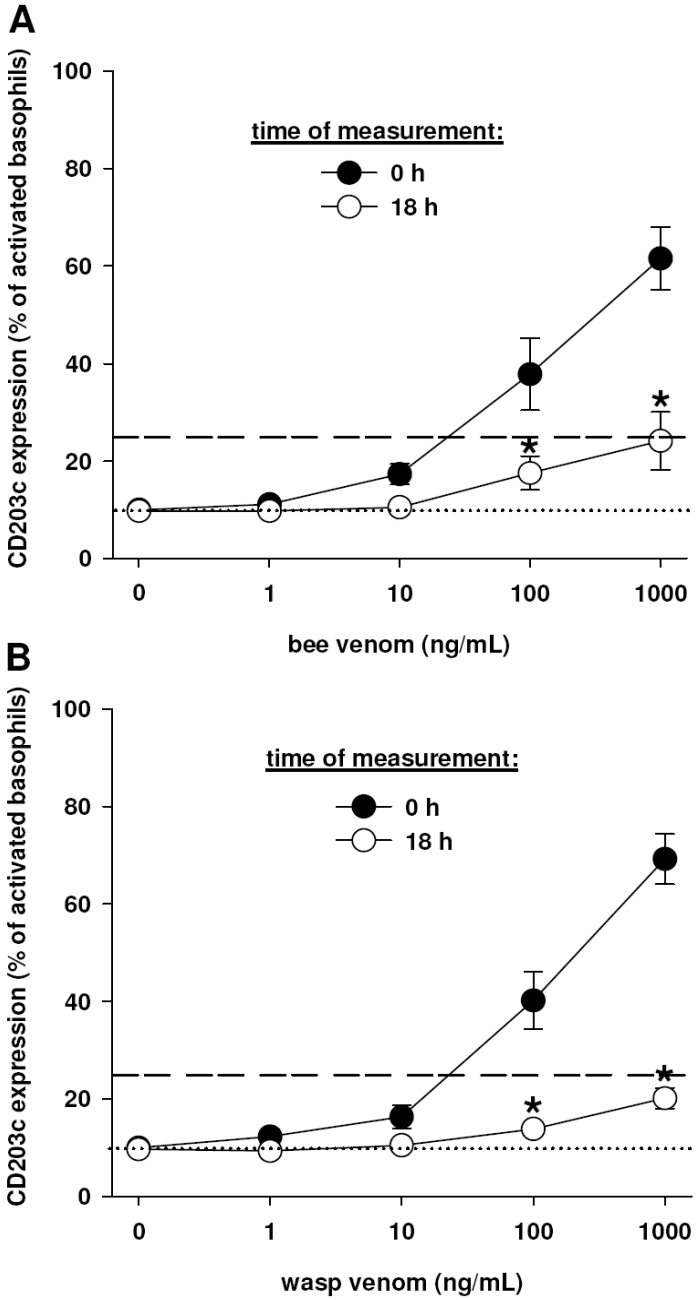

Figure 5. Storage time-dependent decrease in basophil activation to bee and wasp venom. BAT was performed immediately after blood taking or after storage of 18 h at 4°C with (A) bee (*n =13*) or (B) wasp venom (*n = 18*) at the indicated concentrations. Data are expressed as means ± SEM, *n* = 13-18; * $P < 0.05$ *vs* 0 h. ……baseline response (10%); − − − threshold of 25% activated basophils.

Experiments performed by Karin Laipold

Priming factors and basophil activation

We tested whether anti-IgE-induced up-regulation of CD203c expression is altered by pretreatment with the basophil-priming cytokine IL-3 or the degranulation-promoting substances cytochalasin B and latrunculin B. Therefore, IL-3, cytochalasin B (Figure 6) or latrunculin B (Figure 7) was added to EDTA whole blood samples before stimulation. As we reported previously[89], the concentrations of IL-3 (75 pM, 150 pM, 300 pM) significantly enhance basophil CD63 responses. Cytochalasin B (5µg/mL)[31] and latrunculin B[93, 94] (0.1 and 5 µg/mL) were applied at effective concentrations. As shown in Fig. 6, IL-3 priming significantly enhanced the basal expression levels of CD203c and CD63 in the non-stimulated samples as well as basophil responsiveness to FcεRI cross-linking. After priming with IL-3 (300 pM), CD203c and CD63 expression at baseline rose from 10% to 53% and 35% of basophils, respectively (Fig. 6A and C). To scrutinize the effect of IL-3 priming on BAT results, we reanalyzed the data by adjusting the threshold in non-stimulated but IL-3 primed samples to contain 10% of CD203c expressing basophils (baseline). In this manner, our recent data from the CD63-based BAT confirmed that IL-3 significantly enhances the test results. By contrast, when reanalyzing the CD203c data IL-3 priming diminished the CD203c-based test results by reducing the difference between baseline (unstimulated) and anti-IgE stimulated responses (Fig. 6B).

In the same way we reanalyzed the data by adjusting the threshold in non-stimulated but cytochalasin B or latrunculin B pretreated samples to contain 10% of CD203c or CD63 expressing basophils (baseline). We found that preincubation with cytochalasin B or latrunculin B was without effect on anti-IgE-induced CD203c results (Fig. 6A/B and 7A), but enhanced the basal CD63 expression levels and thus impaired the CD63-based test results by reducing the difference between baseline (unstimulated) and anti-IgE stimulated responses (Fig. 6C and 7B).

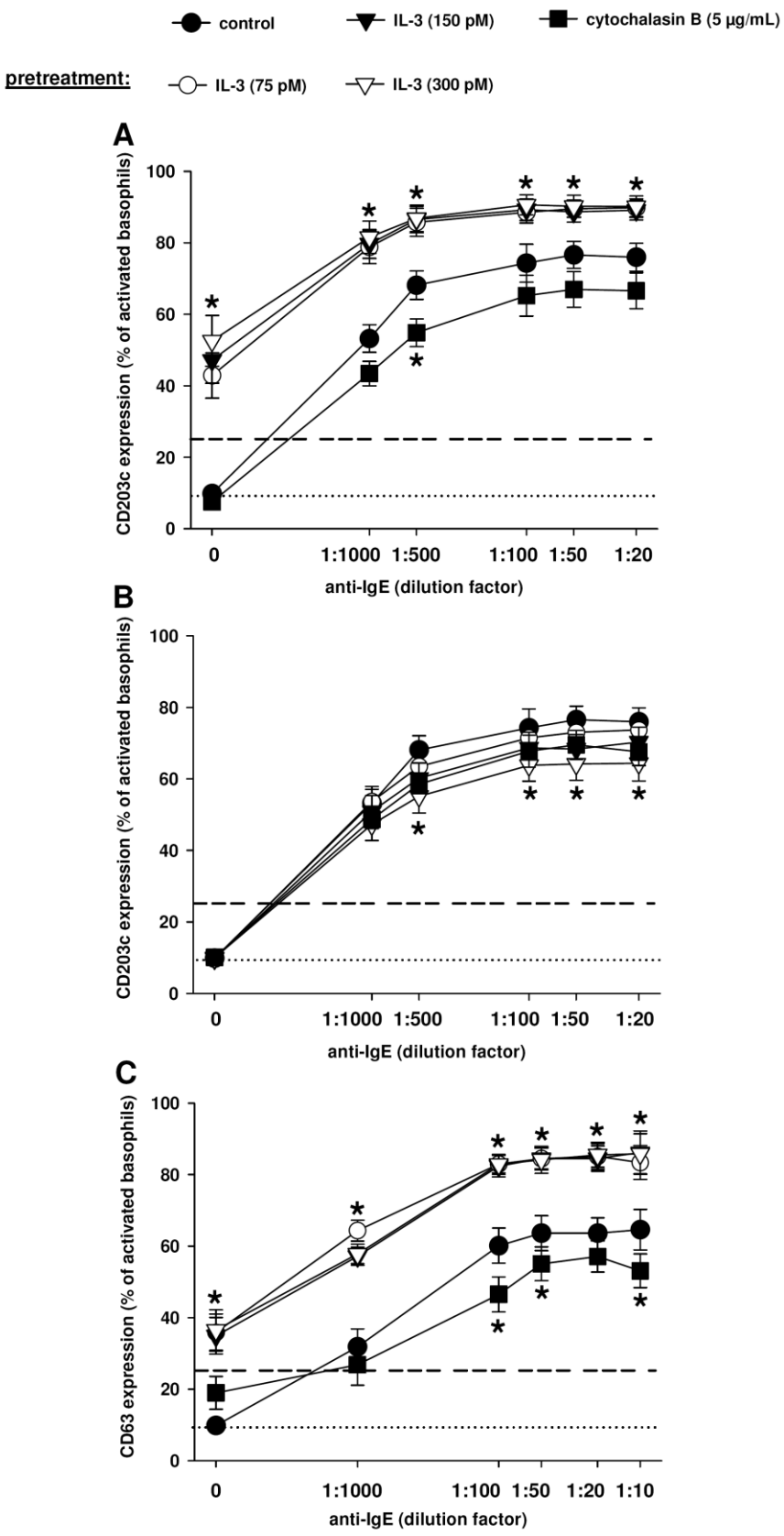

Figure 6. Influence of IL-3 priming and cytochalasin B on CD203c responses. Samples were pretreated with IL-3 (75 pM, 150 pM, 300 pM) or cytochalasin B (5 µg/mL) for 10 min at room temperature before BAT with anti-IgE stimulation for 20 min was performed. IL-3 enhanced CD203c (A) as well as CD63 (C) baseline levels, but significantly decreased CD203c-based BAT results by reducing the difference between baseline and stimulated samples (B). Cytochalasin B slightly affected CD203c levels (A) but was without effect on CD203c-based BAT results when the data were reanalyzed by adjusting the threshold in non-stimulated but cytochalasin B treated samples to contain 10% of CD203c expressing basophils (baseline) (B). Data are expressed as mean ± SEM, $n = 16$; * $P < 0.05$ *vs* control ..... baseline response (10%); – – – threshold of 25% activated basophils.

Experiments performed by Karin Laipold

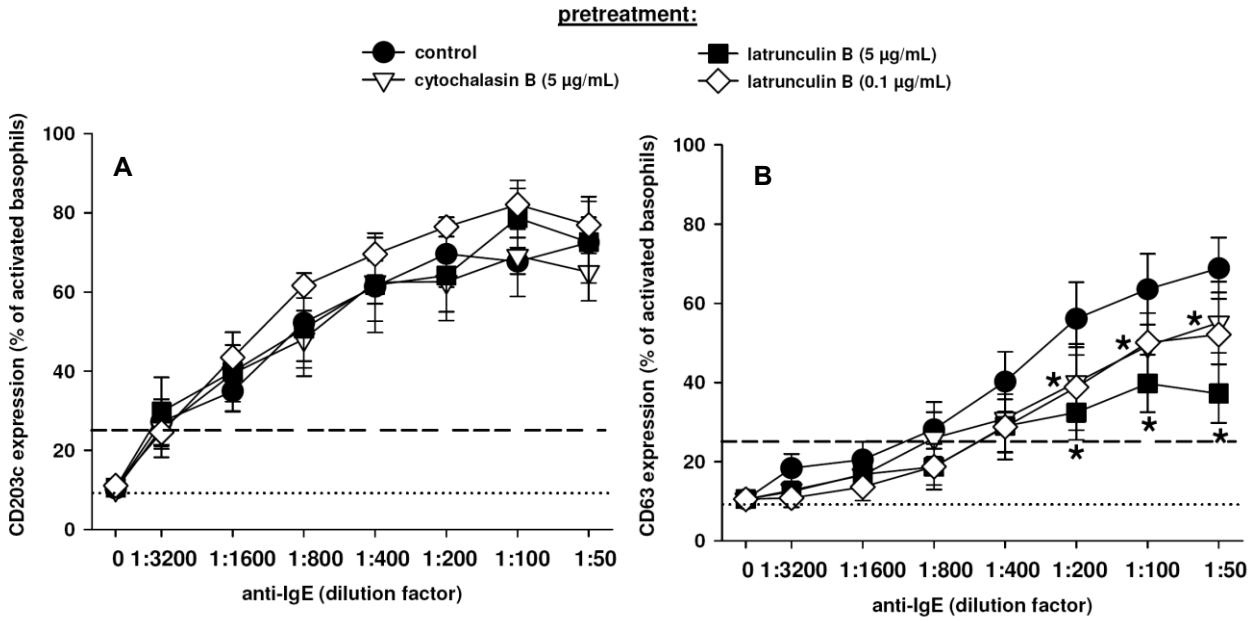

Figure 7. Influence of latrunculin B on CD203c and CD63 responses. Similar to cytochalasin B, pretreatment for 10 min at room temperature with latrunculin B (0.1 or 5 µg/mL) did not affect CD203c-based BAT results, but enhanced CD63 baseline levels and thus significantly diminished CD63-based BAT results by reducing the difference between baseline and stimulated samples. Data are expressed as mean ± SEM, $n = 5$; * $P < 0.05$ *vs* control. ……baseline response (10%); − − − threshold of 25% activated basophils. Experiments performed by Karin Laipold

Influence of antihistamines and corticosteroids on basophil activation

It may be assumed that some allergic patients are already under systemic anti-allergic medication when BAT is performed. Thus, we investigated whether anti-allergic drugs such as corticosteroids and antihistamines might influence CD203c-based BAT results. The *in vitro* pretreatment of blood samples with dimetindene and prednisolone had no effect on the up-regulation of CD203c at concentrations that corresponded to therapeutic plasma levels of the drugs (Figure 8). Only at concentrations fifty-fold higher than the therapeutic level, dimetindene significantly attenuated basophil activation. Since the intake of antihistamines is a frequent problem in the diagnosis of allergies particularly with skin testing, BAT was additionally performed in 10 subjects before, and three hours after, taking 10 mg of desloratadine. As shown in Figure 9, no effect on test results was observed.

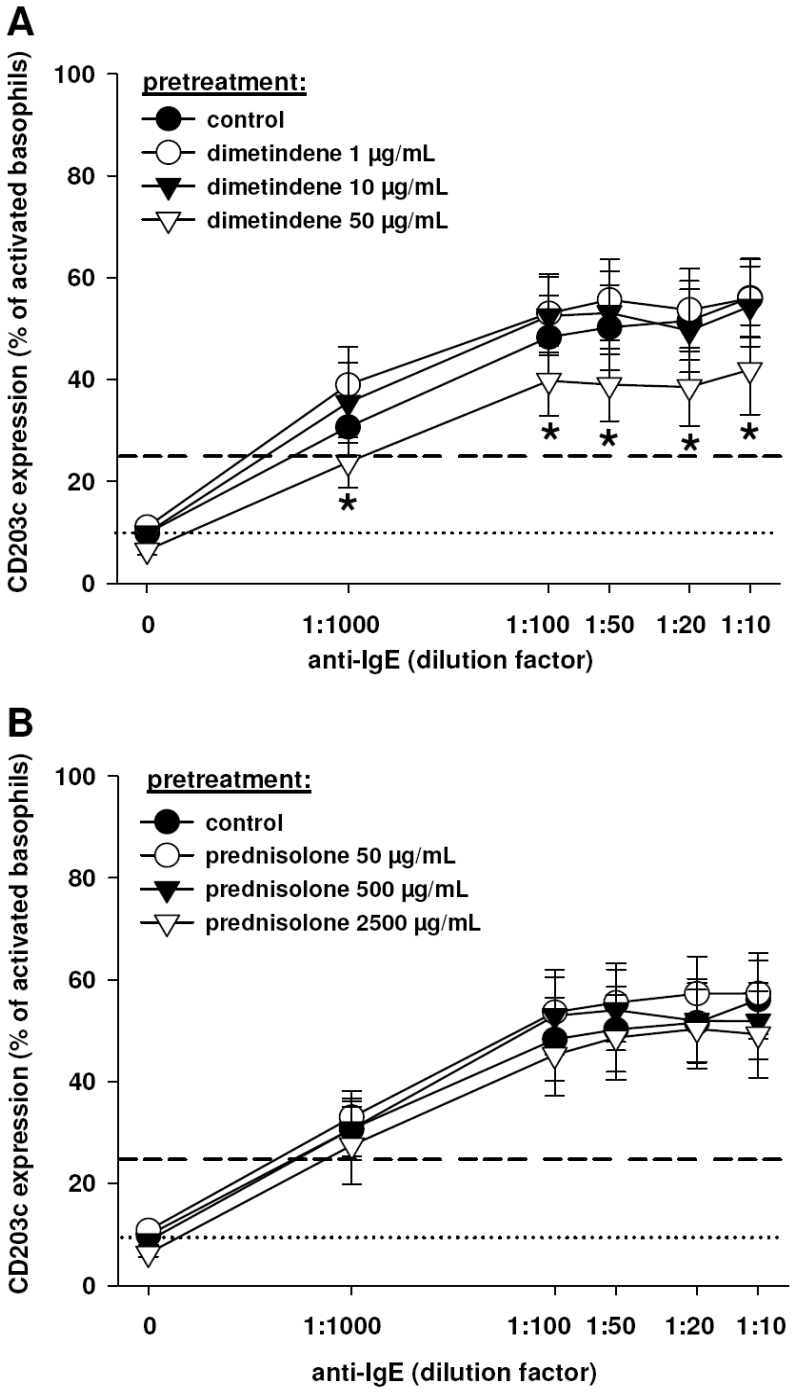

Figure 8. Influence of *in vitro* pretreatment of basophils with antihistamines and corticosteroids on CD203c up-regulation. Samples were preincubated with various concentrations of the H_1 histamine receptor antagonist dimetindene (A) or the corticosteroid prednisolone for 30 min (B) and CD203c expression was stimulated with anti-IgE antibodies. At concentrations up to ten-fold higher than the therapeutic range, no significant reduction of basophil reactivity was observed after dimetindene or prednisolone. Only at fifty-fold higher concentrations than the therapeutic level basophil activation was significantly attenuated by dimetindene. Data are expressed as means ± SEM, $n = 12$; * $P < 0.05$ *vs* control.
……baseline response (10%); − − − threshold of 25% activated basophils.

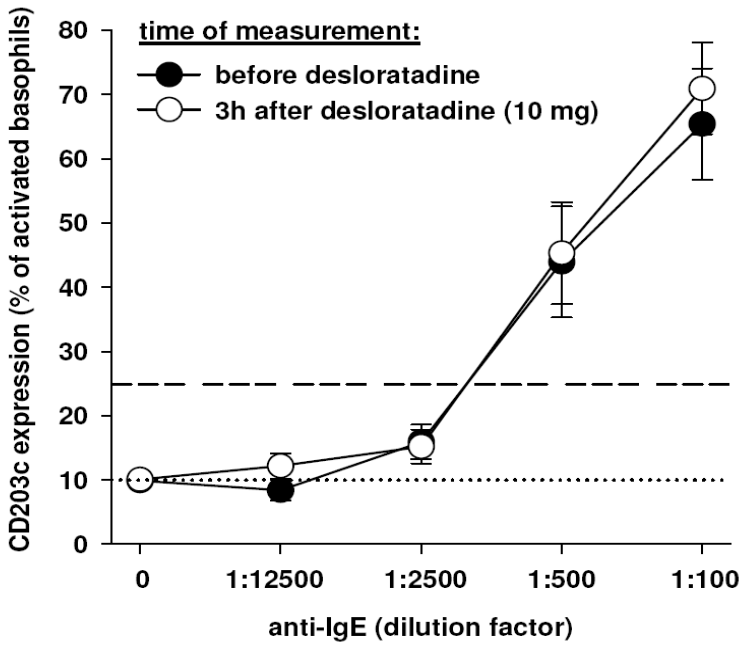

Figure 9. Influence of antihistamine intake on CD203c up-regulation. Blood samples were taken before and three hours after the intake of 10 mg desloratadine. Basophil reactivity to anti-IgE stimulation was not altered when responses before and after treatment were compared. Data are expressed as means ± SEM, $n = 10$.
……baseline response (10%); − − − threshold of 25% activated basophils.

Discussion

The basophil activation test based either on CD63 or CD203c up-regulation has been proposed as a promising alternative diagnostic tool for hymenoptera venom allergy. Nevertheless, BAT protocols are generally not standardized and their 'pros and cons' are still a point at issue. Therefore, the objective of the present study was to define optimized conditions for a CD203c based BAT protocol and to point out potential influencing factors, and we found that longer periods of sample storage and the use of priming factors might confound BAT results based on CD203c determination.

The expression of CD203c and CD63 is described to be linked to different pathways of basophil activation as indicated by different time kinetics of upregulation[44]. Using the present BAT protocol we found that both markers are rapidly upregulated to the cell surface. Already after 10 min, 73% of the maximum CD63 response and 91% of the maximum CD203c response were reached. When primed with IL-3 even 89% of the maximum CD63 response was achieved. In all three cases the optimum incubation time was found to be 50 min with 85% of activated basophils that express CD63 and 90% of activated basophils that express CD63 under IL-3 priming conditions and 85% of activated basophils that express CD203c. Given that already after 10 to 20 min both markers nearly reached their maximum expression levels, an incubation time of 20 min appears sufficient for meaningful test results.

In further experiments we explored the impact of different blood storage times on BAT results. We observed a decline of anti-IgE-induced basophil activation up to 56.1% after a storage time of 48 hours, and even after 4 hours basophil responses were slightly decreased. More substantially, when basophils were stimulated with bee or wasp venom after storage for 18 hours, BAT resulted mostly in false-negative results. Compared to anti-IgE stimulation, the maximum response to Hymenoptera venoms was lower at 69.3%, even immediately after blood sampling. In addition, a higher loss of basophil reactivity by up to 70.9% was observed and results became readily negative due to the overall lower reactivity in response to

allergens. Therefore, CD203c-based BAT should best be performed immediately after blood taking. Until now, there is no general consensus about the usage of priming and degranulation-stimulating factors in BAT. Concerning CD63-based protocols it has been claimed, that short IL-3 pretreatment does not induce CD63 upregulation by itself but acts as a basophil-priming cytokine as it enhances the responsiveness to several basophil activating substances[35, 48]. In contrast, IL-3 was found to enhance CD203c expression by resting, non-activated basophils but to decrease the sensitivity of BAT[35]. Our data confirm that IL-3 treatment for 10 min not only enhances CD203c expression on unstimulated basophils, but we also found that IL-3 up-regulates CD63 expression at baseline. Further on, we were able to confirm that IL-3 priming is not beneficial for this CD203c protocol, as it significantly reduced the difference between baseline (unstimulated) and stimulated responses.

Similarly, in a previous study we found that cytochalasin B significantly inhibits CD63-based BAT results in whole blood[89], as it enhances CD63 baseline expression and also reduces the difference between baseline and stimulated responses. Our current data showed that cytochalasin B and latrunculin B treatment is without effect on CD203c responses and therefore emphasize that the use of degranulation-enhancing substances is generally not recommended in BAT.

Consequently, the extent of basophil responses may vary due to the above factors, i.e. storage time and priming factors. Thus, it is impractical to compare quantitative results unless different working groups use the same standardized protocols. Qualitative results such as "positive" or "negative" may be comparable, but further studies are needed to corroborate this notion.

Concerning CD63-based BAT Erdmann *et al*. already mentioned that antihistamines do not affect basophil responses[83], this was recently confirmed[89], but until now no data have been available regarding CD203c. As antihistamines are selective H1 receptor antagonists, they only block the histamine/receptor interaction but do not interfere with mediator release from

basophils or mast cells. Antihistamines therefore do not reduce CD63 expression. Similarly, we could demonstrate that anti-allergic drugs do not affect basophil responsiveness, as measured by CD203c up-regulation, which occurs independently from degranulation and mediator release. Beside that, *in vitro* pre-treatment of samples with corticosteroids at high concentrations, which may have been expected to exert a membrane-stabilizing effect and hence attenuating degranulation, did not impair basophil reactivity. However, we did not investigate 24-h incubation with glucocorticoids as whole blood cannot be stimulated over several hours. As they mainly rely on nuclear action and this mechanism requires possibly several hours, no conclusion can be drawn for patients taking glucocorticoids for several days. Therefore, how *in vivo* treatment of allergic patients with corticosteroids alters basophil reactivity also awaits further investigation. Other issues that need to be addressed in future studies include different staining and gating strategies, evaluation of different storage temperatures and media, and a comparison of BAT performed in either whole blood or isolated cells.

In conclusion, in the current study we characterized several factors that could impair CD203c-based BAT results and we optimized a fast protocol applicable for CD203c as well as CD63 determination.

3. A critical evaluation of the BAT in cases of double sensitization in hymenoptera venom allergy

Introduction

Personal history, skin testing, and detection of sIgE, are the mainstays of the diagnostic procedure in cases of hymenoptera venom allergy. Although sensitization to both, honeybee and vespid venom, is observed in up to 59% of patients[65], clinically relevant DS is rare and patients usually react either to bee or to wasp stings. Therefore, in clinical routine it can be sophisticated to find the relevant venom for specific immunotherapy with common diagnostic tests.

There are several reasons for (irrelevant) DS: Generally, a true DS with antibodies to different bee and vespid venom allergens should be considered. DS can also be a result of an around 50% sequence identity of the hyaluronidases in bee and vespid venom. However, a recent study revealed that the wasp hyaluronidase is only a minor allergen, and cross-reactivity between vespid and honeybee venom is not due to protein cross-reactivity, but is mainly caused by CCDs[95]. Generally, CCDs are a frequent cause for double positivity as CCD-sIgE mimics DS in vitro. Asparagine linked carbohydrate moieties of plant and insect glycoproteins are the structural basis of CCDs. In hymenoptera venom, these moieties are found in honeybee venom phospholipase A2 (Api m 1) and hyaluronidase (Api m 2), in vespid venom only in hyaluronidase (e.g. Ves v 2). CCD-sIgE is believed to be clinically irrelevant, although the underlying mechanisms are not completely understood[96, 97]. In cases of double positivity, also characteristics of different methods of serum IgE determination should be regarded: Depending on the method, frequencies of double-positive test results vary and range from 10 to 59%[65, 98]. In this context, affinity may play an important role. Affinity is largely determined by the stability of the allergen/IgE complex; therefore low affinity is usually correlated with a rapid dissociation of the complex. To

efficiently activate mast cells or basophils, high affinity antibodies are required. Most of the current systems of IgE determination use high doses of allergen for IgE detection due to the binding competition with specific IgG. As a consequence low affinity IgE antibodies[99], which are thought to be less relevant for eliciting an allergic reaction[100], are bound as well. Nevertheless, low affinity IgE is not completely irrelevant: in the presence of high affinity IgE it may also activate basophils[101].

The intradermal test is considered to be not influenced by CCDs, as low affinity antibodies itself are not able to cause positive reactions. However, clinically irrelevant positive test results at 1,0 µg/ml are frequently observed[102] and side effects cannot be ruled out[103].

Several studies confirmed the usefulness of the CD63 based BAT as a routine diagnostic tool[31, 38, 67] and as a valuable test in patients with inconclusive tests and history (negative skin tests, undetectable sIgE or unknown stinging insect)[73, 104]. Compared with the IgE determination in the serum, BAT has the advantage of demonstrating functional responses: Positive test results will only occur after successful cross-linking of two identical FcεRI-bound IgE antibodies and not by monovalent binding like in IgE assays.

Recently, the component resolved diagnosis (CRD) has been described as useful tool to facilitate the diagnosis of bee and vespid venom allergy[65, 105]. Nevertheless, in these studies only rApi m 1 and rVes v 5 were employed to discriminate between true and CCD-based DS. But it is crucial to additionally determine Ves v 1, otherwise 10-13% of vespid venom allergic patients will not be diagnosed due to a mono-sensitization to Ves v 1[65, 95]. Treatment of double positive patients with both venoms is a pragmatic way, but frequently not justified because of asymptomatic sensitization or cross-reactions caused by CCDs. Therefore there is still need for a test which is able to discriminate between clinically relevant or irrelevant sensitization in order to reduce the burden of treatment and to keep therapy as cost-efficient as possible.

In clinical routine, we observed a high frequency of double positivity in the IgE determination by the CAP system and a markedly lower frequency of double positive results obtained by the BAT. Giving this background, we initiated a prospective study to evaluate the usefulness of new diagnostic approaches for the routine diagnosis of hymenoptera venom allergy. For this purpose, we aimed to compare the outcomes of the BAT with the IDT as well as with three different methods of IgE determination (CAP, ADVIA, Immulite) regarding the frequency of double positive results. To study IgE binding patterns, western blot (WB) inhibition as well as a CRD with native and recombinant Api m1, Ves v 1 and Ves v 5 were performed in all patients with DS.

Methods

Patients

One hundred and seventeen consecutive patients, who had been admitted to our outpatient clinic because of systemic allergic reactions with at least generalized skin symptoms after a hymenoptera sting, were screened. Their personal history was taken and the current standard diagnostic procedures (intradermal tests, IgE determination by CAP) were performed. As wasp and European hornet belong to the family of Vespidae and their venoms contain the same major antigens, we did not differentiate between these genera. Additionally, sIgE was determined by ADVIA, and the Immulite; basophil responsiveness was analyzed by a CD63 based BAT. In 72 patients showing specific IgE to honeybee and vespid venom in the CAP system, IgE patterns were determined by WB inhibition and CRD. This study has been approved by the ethics committee of the Medical University of Graz.

Personal history

According to the modified classification of Ring and Messmer, generalized skin symptoms such as flush, urticaria and angioedema were classified as grade I reaction. Mild to moderate pulmonary, cardiovascular or gastrointestinal symptoms were rated as grade II reaction. Bronchoconstriction, emesis, anaphylactic shock, and loss of consciousness were classified as grade III reaction.

Reagents

All laboratory reagents were obtained from Merck (Whitehouse Station, NJ, USA) or Sigma-Aldrich (St Louis, CA, USA) unless otherwise specified. Dulbecco's modified phosphate-buffered saline (PBS; with or without Ca^{2+} and Mg^{2+}) was purchased from Gibco-Invitrogen (Carlsbad, CA, USA). CellFix and anti-CD123 (PE-conjugated) were supplied by Becton

Dickinson (Franklin Lakes, NJ, USA). Antibodies to HLA-DR (PC5-conjugated), CD63 (FITC-conjugated), and monoclonal antibodies to IgE were purchased from Beckman Coulter (Fullerton, CA, USA). Honeybee and vespid venom for the skin tests and BAT were purchased from ALK-Abelló (Hørsholm, Denmark). Honeybee venom and vespid venom sac extracts (mixture of *Vespula vulgaris* and *germanica*) were kindly provided by Vespa Laboratories, PA, USA.

Skin tests

The nature of sensitization was confirmed by standardized end-point titration IDTs (0.02 mL of 0.001, 0.01, 0.1 and 1 μg/mL solution) using purified honeybee and vespid venom extracts. IDTs were considered to be positive in the presence of a wheal ≥5 mm in diameter and erythema.

Determination of sIgE and tIgE

Specific and total IgE antibody levels in the patients' serum were measured using ImmunoCAP 1000 (Phadia, Uppsala, Sweden), ADVIA Centaur, and Immulite 2000 (both: Siemens, Tarrytown, NY, USA) according to the manufacturer's instructions. The CRD with native and recombinant nApi m 1 and rApi m 1 was done on the ImmunoCAP 1000. Diagnosis with the major wasp allergens nVes v 1 and nVes v 5 as well as with nApi m 1 was done on the ADVIA Centaur platform by the Department of I+D, ALK-Abelló, Madrid, Spain.

Basophil activation test (BAT)

BAT was performed as previously described[89]. In brief, EDTA whole blood was stained with anti-CD123 PE-conjugated antibody (1:50), anti-HLA-DR PC5-conjugated antibody (1:50) and anti-CD63 FITC-conjugated antibody. Basophil reactivity was measured using serial dilutions of honeybee or vespid venom (1000, 100, 10, 1 ng/mL) or serial dilutions of anti-IgE antibody (1:10-1:1000 dilution).

Finally, cell samples were analyzed by three-color flow cytometry (FC 500, Beckman Coulter). Basophils were identified as a single population of cells that stained positive for CD123 (FL-2) and negative for HLA-DR (FL-4). Up-regulation of CD63 expression was indicated by an increase in fluorescence in the FL-1 channel. Acquisition was terminated after 500 basophil target events. An approximately 2.5-fold increase in the number of activated basophils (>25%) as compared with the negative control (10%) at any of the test concentrations of the allergen was considered to be a positive response. This threshold was determined by ROC analysis as described earlier[31].

Western blots and western blot inhibition

Honeybee venom and vespid venom were separated by SDS-PAGE using 13.5% resolving and 5.7 % stacking gels under reducing conditions using dithiothreitol and heat. Electrophoretically separated proteins were blotted onto nitrocellulose membranes and single strips (6 µg venom/strip) blocked with PBS buffer (50 mM sodium phosphate, pH 7.5, 0.5% Tween 20, and 0.05% NaN_3) containing 0.5% BSA at room temperature for 1 h. Subsequently, strips were incubated overnight with 1 mL of serum (diluted 1:5 - 1:10) at 4^{o}C under continuous shaking. After washing twice with PBS buffer for 30 min, bound IgE was detected by ^{125}I-labelled rabbit anti-human IgE (Phadia, Uppsala, Sweden). After overnight

incubation at room temperature, washed and dried strips were exposed to a high-performance autoradiography film (Hyperfilm MP, Amersham, England) at -70^{o}C for 5-10 days.

To discriminate between IgE specific for peptide or carbohydrate epitopes, antibody binding to CCDs was inhibited by preincubating sera with 5 μg/mL of MUXF-BSA as done in previous studies[106]. MUXF-BSA is a synthetic glycoprotein obtained by coupling purified N-glycans from pineapple stem bromelain to BSA[107], whereby MUXF (or more exactly MUXF^3) stands for the glycan structure Manα1-3(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAcβ1.

Data analysis

All data are expressed as medians (25%; 75% percentiles) on the raw scale, unless otherwise indicated. Data were tested for normality using the Kolmogorov-Smirnov test. Continuous variables were analysed by the Kruskal Wallis test; categorical variables were compared by the Chi-square test or Fisher's exact test. To check agreement between the tests, Cohen's kappa coefficient was calculated. The level of significance was set at $p<0.05$. The SPSS 17.0 software (SPSS Inc, USA) was used for statistical analysis.

Results

History and demographic data

One hundred and seventeen patients with a unequivocal history of a systemic sting reaction were included in the study. Fifty-eight (49.6%) were female, and 59 (50.4%) male. Median age was 42.0 (30.5; 53.0) years; the majority of patients (45.3%) were in the age group between 30 and 50 years.

Four patients (3.4 %) had a history of grade I reactions, 80 patients (68.4 %) had experienced grade II reactions and 33 patients (28.2 %) grade III reactions. Thirty-eight (32.5%) identified a honey bee as culprit insect, 55 (47.0%) a wasp, and 24 (20.5%) could not identify the insect. None of the patients reported systemic sting reaction after both, honeybee and wasp stings.

Double sensitization

Frequency of DS differed considerably among performed diagnostic tests and ranged from 63.7% with the Immulite to 17.1% with the BAT (Figure 1). Generally, agreement of tests was fair with 53.1% (kappa 0.318; p<0.0001)

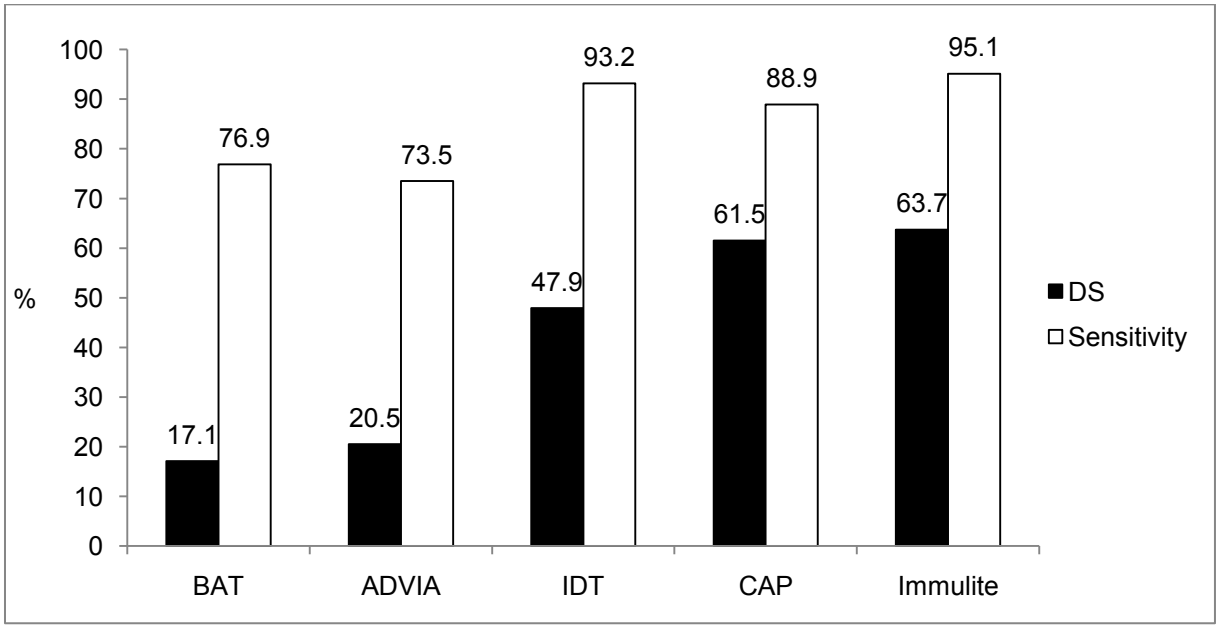

Figure 1. Frequency of double sensitization
The rate of DS in 117 consecutive patients differed significantly (p<0.0001) and ranged from 17.1% with the BAT to 63.7% with the Immulite.

Differences between mono and double sensitized as well as double negative patients

In all tests except in the BAT, tIgE levels were up to 2.3-fold higher in double sensitized patients compared to mono sensitized patients. Conversely, patients with double negative results had lower tIgE levels compared to mono or double sensitized patients (Table 1).

	Double sensitization	Mono sensitization	Double negative	p
BAT	54.3 (24.5; 217.3)	64.7 (36.9; 151.0)	58.9 (22.7; 112.0)	0.463
ADVIA	117.0 (50.9; 397.6)	51.7 (30.3; 123.4)	35.9 (10.4; 142.4)	0.008
IDT	88.7 (45.2; 252.0)	53.9 (29.4; 103.5)	35.3 (8.7; 69.0)	0.014
CAP	90.8 (48.9; 230.0)	43.3 (29.1; 64.2)	28.0 (10.6; 66.0)	0.000
Immulite	87.2 (42.6; 246.0)	51.5 (19.6; 87.5)	8.0 (2.7; 95.6)	0.006

Table 1. Correlation between tIgE (kU/L) and test results
In all tests except in the BAT, double sensitized patients showed higher levels of tIgE compared to mono sensitized patients. Conversely, double negative patients had lower tIgE levels compared to mono- and double sensitized patients.

The comparison of mean age between the three categories revealed no significant difference. Additionally, regression analysis to check the influence of the severity of sting reaction, sex, age and tIgE on DS was performed: The frequency of DS was influenced by tIgE levels in the CAP (e^b 1.005, p=0.035) and ADVIA (e^b1.003, p=0.048). Additionally, higher age of the patients was associated with a lower frequency of DS in the CAP (e^b 0.966, p=0.038). The rate of DS in the BAT, IDT, and Immulite was not influenced by the tested variables.

Subgroup analysis of double sensitized patients in the CAP

IgE determination by CAP yielded together with the Immulite the highest frequency of double positive results. As the CAP system is widely used, and this group of 72 patients comprised virtually all patients with double positive results in supplemental tests, further analysis regarding the individual IgE pattern was done in this subgroup.

First, the rate of DS of each commercially available and experimental test was determined to identify the most specific test to reduce the high frequency of clinically not relevant DS. As

expected, CRD analysis solely done with the native main allergen components nApi m 1, nVes v 1, and nVes v 5 led to a slightly reduced, but still high frequency of DS. The use of non-glycosylated rApi m 1, nVes v 1 and nVes v 5 reduced the frequency considerably by 49.0%. Similar lower rates of DS were observed with the WB inhibition, ADVIA and BAT, while the Immulite and the IDT revealed high frequencies of DS (Figure 2).

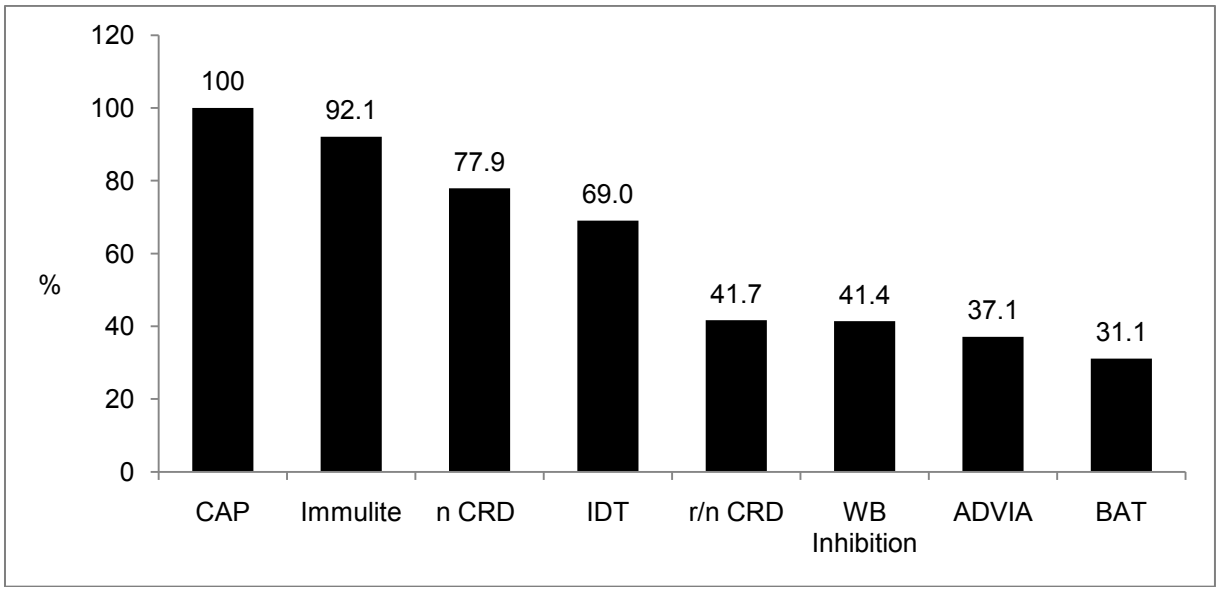

Figure 2. Frequency of double sensitization in supplemental tests in 72 CAP double positive patients
n CRD: native component resolved diagnosis with nApi m 1, nVes v 1, nVes v 5
r/n CRD: combined component resolved diagnosis with recombinant rApi m 1, and native nVes v 1, nVes v 5.
BAT (p=0.324) and ADVIA (p=0.874) showed a similar frequency of DS compared to WB inhibition and r/n CRD, although they were performed with native venom extracts.

IgE patterns of CAP double sensitized patients with WB inhibition

The WB was not interpretable in 11 of 72 patients. Among the remaining patients, true DS was diagnosed in 24 of 61 patients, putative cross-reactivity due to hyaluronidase in 6 patients, and double positive results caused by CCD alone in 31 patients (typical IgE patterns see Figure 3).

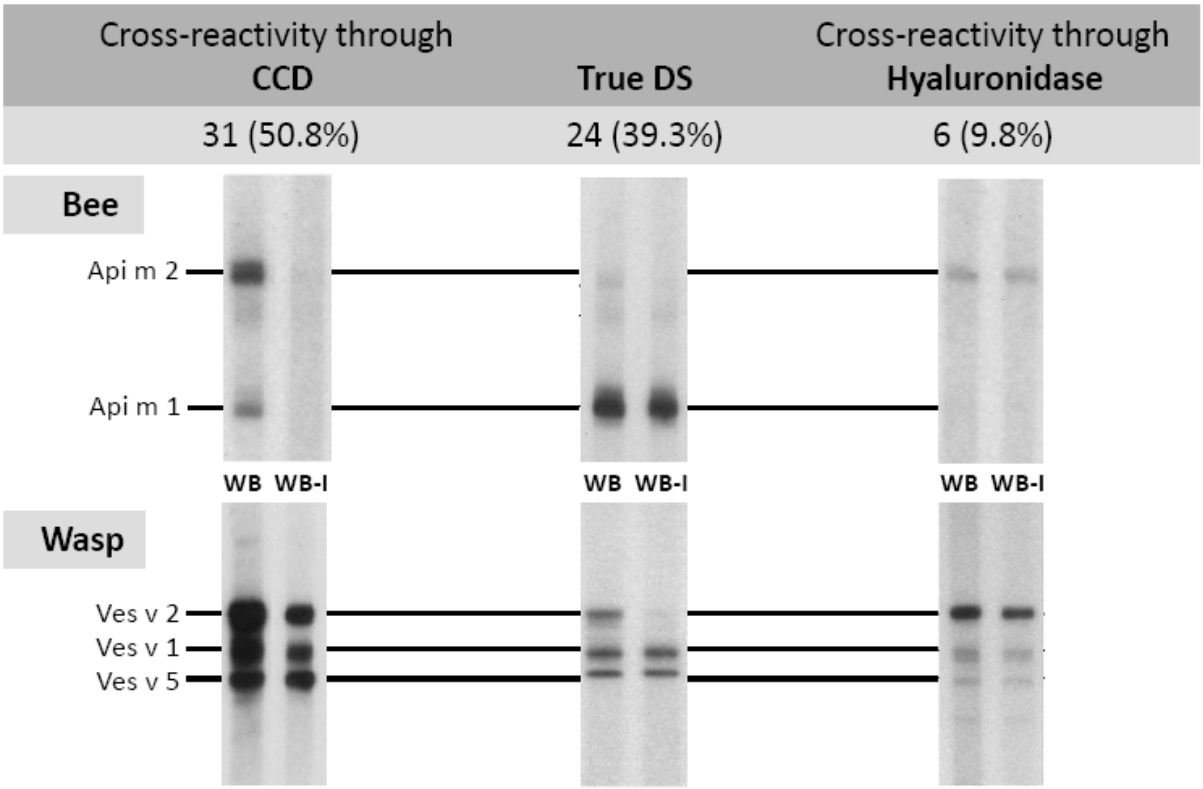

Figure 3. Frequency of typical IgE patterns obtained by western blot inhibition in CAP double sensitized patients.
CCD: cross-reactive carbohydrate determinants, True DS: true double sensitization, WB: western blot, WB-I (western blot inhibition): To discriminate between IgE specific for peptide or carbohydrate epitopes, antibody binding to CCDs was inhibited by preincubating sera with MUXF-BSA.
Among these patients the majority of DS was CCD-dependent. DS due to protein components of hyaluronidases played a minor role. n=61. Experiments performed by Chunsheng Jin

CRD in CAP double sensitized patients

As at the time when the study was performed rApi m 1 was not available for the ADVIA, and vice versa nVes v 1 and nVes v 5 not for the CAP, rApi m1 was determined with the CAP and nVes v 1 and nVes v 5 with the ADVIA. To check compatibility, nApi m 1 was determined on the CAP as well as ADVIA. In contrast to the IgE determination with bee and vespid extracts, the test results with native components were coinciding with 92.3%, assuming an almost perfect agreement.

Finally, CRD with recombinant and native allergens was performed in 64 of 72 CAP double positive sera; four patients were negative for the tested bee and vespid venom allergens

(Figure 4 A+B). There was a substantial agreement between the WB and the CRD for rApi m 1 with 88.5% (kappa 0.770, $p<0.0001$), and nVes v 5 with 87.7% (kappa 0.744, $p<0.0001$). The agreement for nVes v 1 was only fair with 71.9% (kappa 0.377, $p=0.005$).

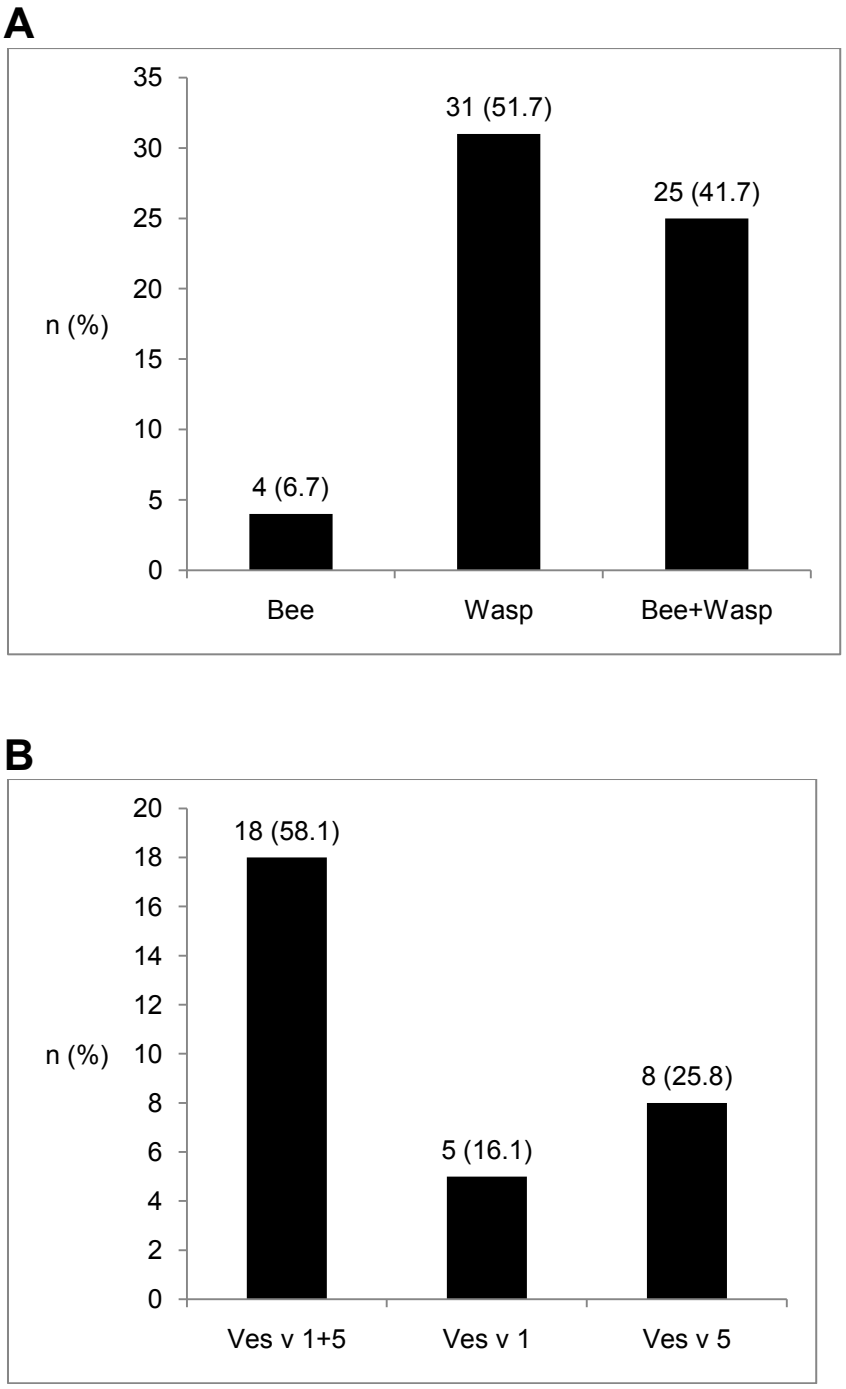

Figure 4. Component resolved diagnosis in CAP double sensitized patients

(A) Sensitization to bee and/or vespid venom in the component resolved diagnosis
Positive for bee venom: rApi m 1^{pos} / nVes v 1^{neg} and nVes v 5^{neg};
Positive for wasp venom: rApi m 1^{neg} / nVes v 1 and/or nVes v 5^{pos};
DS: rApi m 1^{pos} / nVes v 1 and/or nVes v 5^{pos}.
n=60

(B) Sensitization pattern in vespid venom allergic patients
The majority of patients were sensitized to both vespid major allergens (nVes v 1 and nVes v 5). Nevertheless, a considerable proportion had a mono sensitization to nVes v 1 or nVes v 5.
n=31

BAT compared to CRD and WB inhibition in CAP double sensitized patients

Beside the component-specific tests (CRD, WB) only the BAT and ADVIA showed a comparable low frequency of DS despite the use of conventional allergen extracts. As ADVIA is no longer available, further analysis was only done with the BAT in 72 patients; 11 were negative for both venoms (Figure 5).

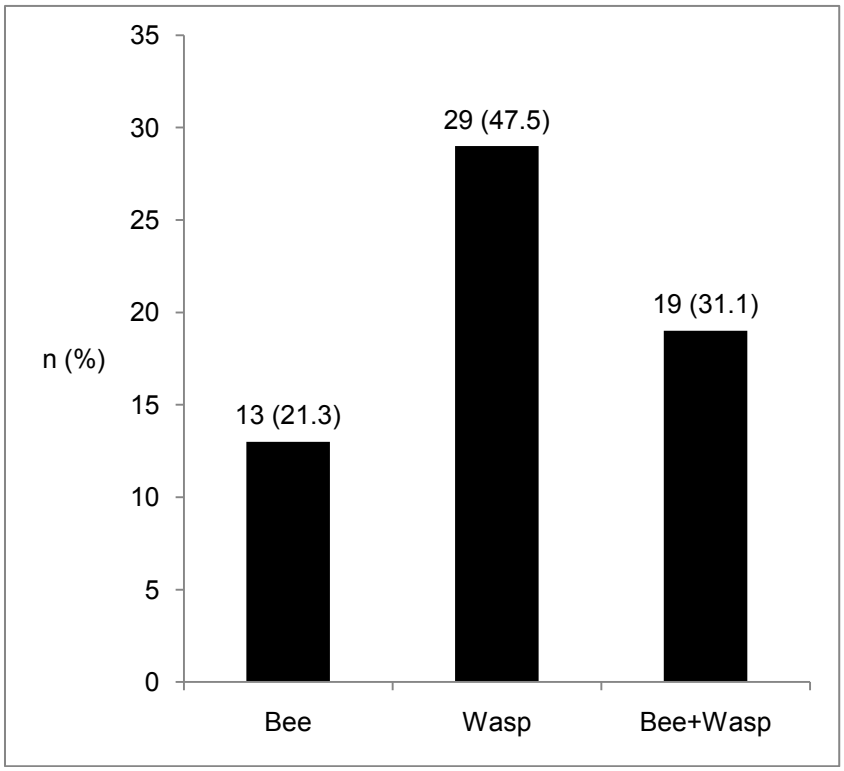

Figure 5. Frequency of sensitization to bee and/or vespid venom in the basophil activation test
n=61

Noteworthy, in 11 patients with DS in the CRD, basophils were only activated by one venom in the BAT. Conversely, 7 BAT double positive patients showed only a mono sensitization in the CRD. There was a similar picture with the WB inhibition: 13 double positive patients in the WB inhibition were only positive to one venom in the BAT and 7 BAT double positive patients showed only a mono sensitization in the WB inhibition. Generally, results of the BAT were in fair agreement with those of the CRD (Figure 6).

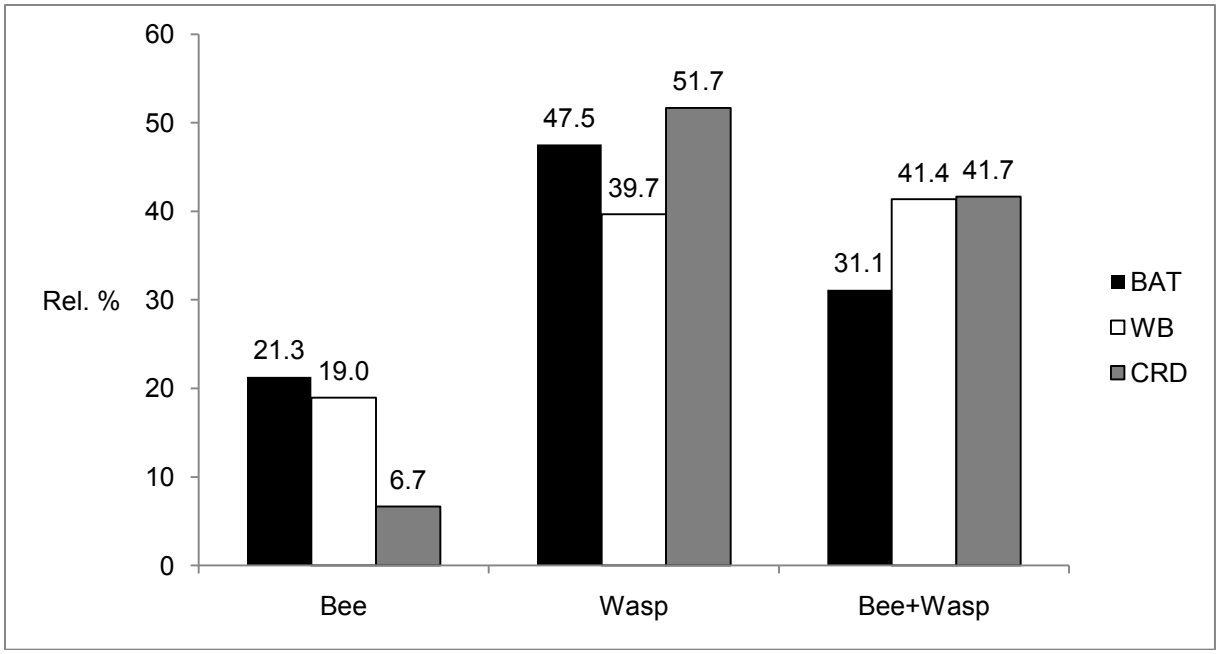

Figure 6. BAT results in relation to western blot inhibition and component resolved diagnosis
Although BAT was performed with native venom extracts, frequency of mono- and double sensitization was comparable with component based methods. Results of the BAT were in fair agreement with those of the CRD (60.0%, kappa 0.373, p<0001) and WB (59.6%, kappa 0.377, p<0001). Interestingly, the frequency of honey bee sensitization obtained with the CRD was markedly lower compared to BAT and WB, which could indicate a lower sensitivity of rApi m 1.

sIgE to MUXF (CCD)

Determination of sIgE to MUXF in the CAP (CCD-IgE) was not appropriate to distinguish between true DS and CCD based DS. 16 of 30 patients with true DS in the WB (sensitization to major allergens or hyaluronidase) had detectable sIgE to MUXF and conversely, only 16 of 31 patients with a verified CCD-based DS by the WB inhibition had sIgE to MUXF (Figure 7). Additionally, also 15 of 25 (60.0%) patients with true DS verified by CRD had sIgE to MUXF.

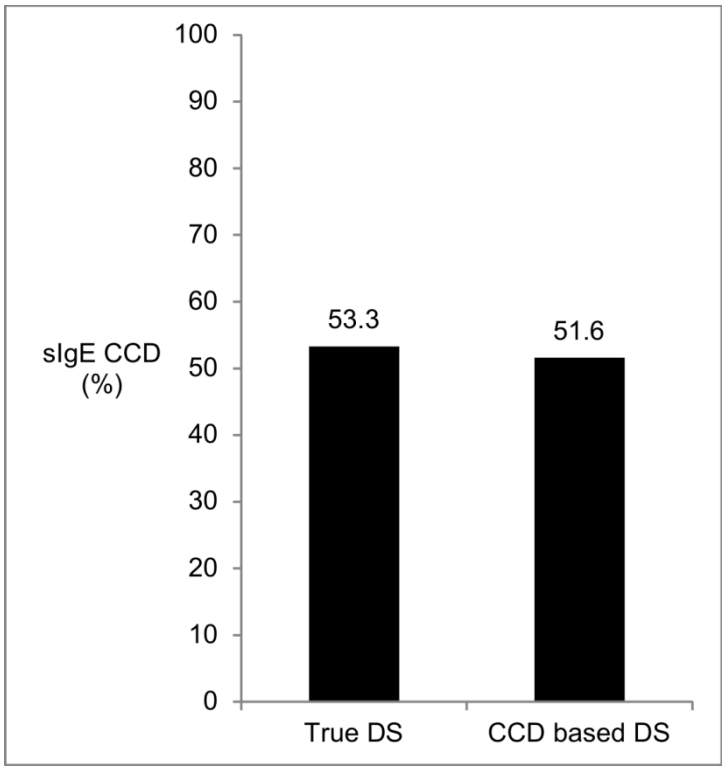

Figure 7. Determination of sIgE to MUXF (CCD) was not appropriate to distinguish between true and CCD-based double sensitization
Patients with true DS in the WB (sensitization to major allergens or hyaluronidase) had detectable sIgE to MUXF and conversely, patients with a verified CCD-based DS by the WB inhibition had no detectable sIgE to MUXF.
As the coincidence of true DS and detectable sIgE to MUXF was high, results could be misinterpreted and true DS could be easily overlooked.

Double positive results in CCD-dependent DS

CCD dependent DS was verified by WB inhibition in 31 patients. Depending on the test, frequency of DS ranged from 12.0% to 89.3% (Figure 8).

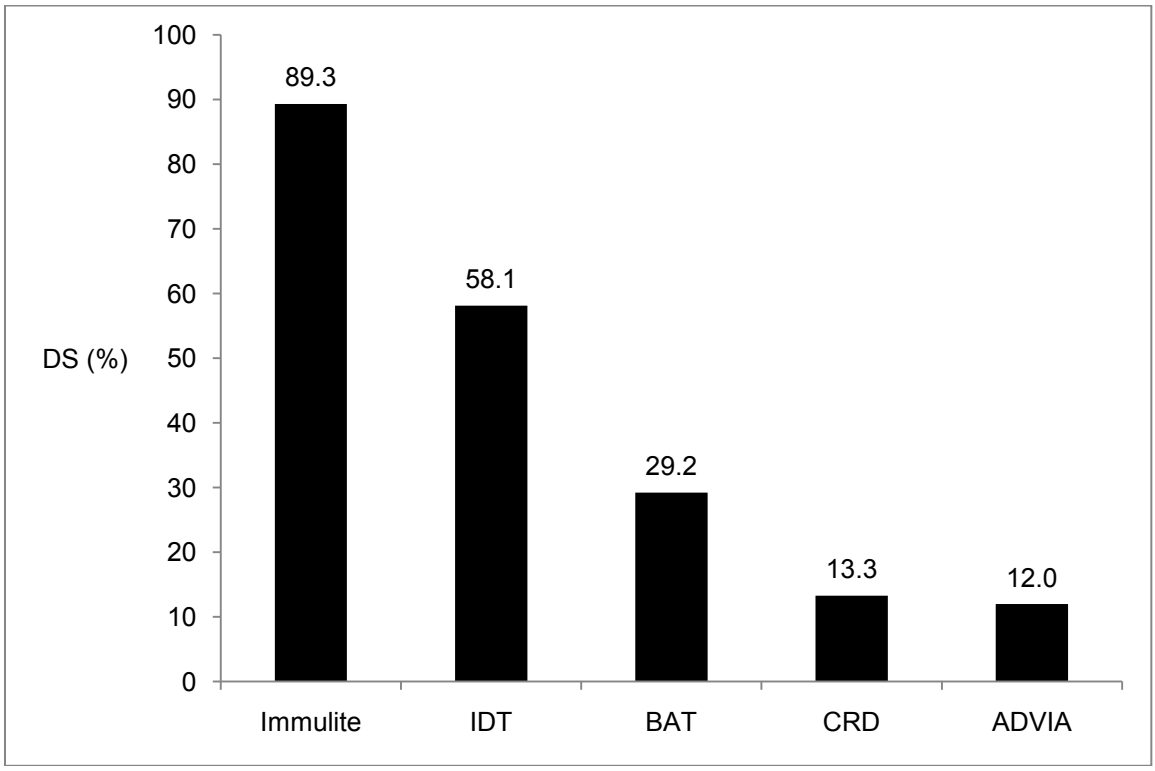

Figure 8. Double positive results in CCD-dependent double sensitization
CCD-dependent DS was verified with WB inhibition in 31 patients. The Immulite and IDT revealed the highest rates of DS in these patients ($p<0.001$; n=24-31)

Discussion

Positive test results to bee and vespid venom are frequently observed in the routine diagnosis of hymenoptera venom allergy and raise problems to determine the causative insect for a correct treatment. Treatment with two venoms is generally accepted in patients with severe sting reactions and inconclusive test results. Nevertheless, there is a high risk of overtreatment, and even for a novel sensitization, if positive results are unspecific and caused by weakness of diagnostic methods or by CCDs.

In the current study, we performed an extensive evaluation of various conventional, recently established, and experimental test methods. We could demonstrate that the BAT had the lowest frequency of DS and thus correlated best with the patients' history. Nevertheless, the BAT showed double positive results in nearly one third of patients with CCD-based DS, and vice versa was sometimes only positive for one venom in patients with DS in the WB inhibition and CRD. CCDs can lead *in vitro* to a stimulation of basophils[108, 109] and the question of clinical relevance of these positive results remains still unanswered. Conversely, even a true (double-) sensitization must not be clinically relevant[64, 110]. In these case, the BAT as functional test may be helpful to find the culprit venom. IgE determination by the ADVIA also resulted in a low frequency of DS, even though it was slightly higher compared to the BAT. However, the ADVIA platform is no longer available for routine diagnosis as it has been taken off the market despite of its revolutionary concept of IgE determination and its excellent performance. Additionally, we could show that the intradermal test was not beneficial in the discrimination between mono- and double sensitization because it revealed DS in as much as 69% of patients. This may either reflect false-positive reactions due to histamine liberating substances or toxic effects of the venom, as well as some mast cell activation by CCDs at very high venom concentrations (1 µg/ml). As expected, the CRD with recombinant and native CCD-free allergens discriminated well between CCD based and true

DS, and hence represents a clear step forward in the diagnosis of hymenoptera venom allergy. Importantly, the sensitization patterns of the CRD correlated well with those of the western blot. Nevertheless, the CRD revealed a markedly lower frequency of honey bee sensitization compared to BAT and WB which could indicate an insufficient sensitivity of rApi m 1 and the need for additional honeybee venom allergens.

Clinically relevant DS is rarely observed: in a large European (EAACI) multicenter study regarding side-effects during immunotherapy only 58 of 840 (6.9%) were treated with two venoms[111]. At the same time, asymptomatic sensitization is observed in 27.1 to 66.7% in the general population depending on the test method of IgE determination and tIgE levels [64, 98]. Depending on the methods and venoms used, the specificity of serum IgE determination ranges between 60% and 94%[31]. Leading manufacturers of automated lab systems generally postulate high sensitivities and specificities for their IgE determination. However, the studies leading to these results must be viewed critically: control subjects with high tIgE levels, positive skin tests and an atopic disposition are generally ruled out in order to obtain optimum specificities[112, 113].

Generally, methods of serum IgE determination differ considerably and therefore results are difficult to compare. In CAP, the allergen is bound to a solid cellulose sponge matrix. After incubation with the patient's serum sIgE and also specific IgG is bound to the covalently coupled allergen. To quantify sIgE levels, sIgE is detected by enzyme-labeled anti-IgE. To minimize competition between the low quantity of IgE and the substantial quantity of IgG a very high amount of allergen is bound to the immunosorbent. Therefore also low-affinity cross-reacting sIgE like those to CCDs with questionable clinical relevance are detected. The same might be valid for the Immulite, although it depends on another principle: In brief, ligand-labeled liquid allergens first bind to anti-ligand-coated polystyrene beads; after adding the patient's serum, sIgE is bound to the allergen. Again, sIgE is detected by anti-IgE. High

doses of allergen to avoid displacement of sIgE antibodies in both tests would explain the similar frequency of DS with 61.5% and 63.7%, respectively.

The concept of the ADVIA is completely different to exclude interference with non-IgE antibodies like IgG. Anti-IgE is coupled to paramagnetic particles that catch all IgE in the serum. Then biotin-labeled allergen is added and bound sIgE reacts with the allergen in suspension. Finally sIgE is detected indirectly with acridinium ester labeled streptavidin[114]. The main advantage of this approach is that much less allergen is needed and therefore the affinity of sIgE is better considered. This explains the good performance of the ADVIA despite of the native venom extracts used.

The IDT and the BAT have the advantage of demonstrating functional responses as positive results usually only occur after cross-linking of two identical cell-bound IgE antibodies. Nevertheless, we observed a considerable difference in the occurrence of DS: The IDT was positive for bee and vespid venom in 47.9% of patients compared to 17.1% double positive results obtained by the BAT. The high frequency in the IDT might be explained by the irritant effect of the venom at higher doses and, as mentioned earlier, by the activation of some mast cells by CCDs at very high venom concentrations. On the other hand, the low rate of DS in the BAT with native venom extracts supports the hypothesis, that the BAT is able to demonstrate a functional response without possible irritant reactions as seen in the IDT and without considerable influence of CCDs on test results as obtained with the CAP or Immulite. Recently, up to 67% double positive results were reported with the CD203c based BAT[115], this is contrary to our findings. This extraordinary high rate of DS might not depend on the different activation marker CD203c, but on an internationally uncommon protocol and unusual interpretation of results. Nevertheless, there still remain a few open questions: In our study, the BAT showed in 29% of patients with a verified CCD-based DS double positive results and vice versa the BAT was sometimes only positive for one venom despite that the CRD and WB inhibition revealed double positive results, respectively. The role of CCDs for

eliciting clinical symptoms is still unclear. There are several hypotheses why sIgE to CCDs are not relevant, one of them is that patients are constantly exposed to these carbohydrate structures and therefore produce blocking IgG_4 antibodies, comparable with the effect of immunotherapy[97]. This might explain that basophils can be activated in the BAT, but not in vivo.

The application of recombinant or native CCD-free allergens will be a considerable progress in the diagnosis of hymenoptera venom allergy. nApi m 1 showed clearly more positive results compared to rApi m 1, again indicating the crucial role of CCDs in DS. Thus makes it inevitable to use components which are CCD-free by nature or to produce recombinant allergens without CCDs. Importantly, the generally accepted use of sIgE to CCD as marker for CCD-based cross-reactivity has to be viewed critically and must be considered obsolete. As shown in the WB, the presence of IgE to CCDs does not exclude true DS, therefore true DS can be easily overlooked, which may result in fatal reactions.

To summarize, BAT and CRD showed the lowest rates of DS, but inconsistent results were common. Although each test alone seems to help finding the clinically relevant venom, it is still unclear which test represents the most accurate. Therefore, studies with sting challenges to check the accurate negative predictive value of the BAT and CRD in otherwise double sensitized patients would be preferable. At present, no routinely employed test can be regarded as gold standard to distinguish between clinically relevant bee and wasp venom sensitization.

4. Correlation of the BAT and routine diagnostic tools with the outcome of sting challenges in asymptomatically sensitized subjects

Introduction

Depending on the climate, the incidence of hymenoptera stings ranges from 56.6% to 94.5% in the general adult population[116]. 0.3 to 7.5 % and 2.4 to 26.4% of the general population are reported to have experienced systemic sting reactions (SSR) or large local reactions (LLR) to hymenoptera stings, respectively[2]. Recently, we carried out the first epidemiological survey on hymenoptera venom allergy in Austria and found 3.3% who have already experienced SSR and 4.6% who reported LLR, respectively[117]. However, asymptomatic sensitization to bee and wasp venom occurs frequently in *in vitro* tests and 27.1 to 40.7% of the general population have detectable sIgE to hymenoptera venom[64, 110]. Furthermore, asymptomatic sensitization (AS) is related to tIgE levels and in the case of high tIgE in up to 66.7% of healthy subjects sIgE is demonstrable[64]. Therefore, current criteria to diagnose hymenoptera venom allergy cannot accurately predict the occurrence or severity of anaphylactic symptoms after a sting. The main cause of AS in double sensitized subjects to bee and wasp venom is the presence of sIgE to cross-reactive carbohydrate determinants (CCDs) in the serum. Many of bee venom allergens and some of wasp venom allergens bear CCDs; therefore sensitization to CCDs can mimic double sensitization to hymenoptera venoms. However, sIgE to CCDs as a cause for AS in mono sensitized subjects are typically not observed[64]. Also methodological characteristics of different systems of serum IgE determination should be regarded. Most of the current systems of IgE determination use high doses of allergen for IgE detection due to the binding competition with specific IgG. As a consequence low affinity IgE antibodies[99], which are thought to be less relevant for eliciting an allergic reaction[100], are bound as well. Affinity is largely determined by the stability of the allergen/IgE complex; therefore low affinity is usually correlated with a rapid dissociation of the complex. But to efficiently

activate mast cells or basophils, high affinity antibodies are required. Beyond this point, methods of serum IgE determination differ considerably and therefore results are difficult to compare[118]. Nevertheless, a large part of subjects are sensitized to non-glycosylated venom allergens and tolerate hymenoptera stings well. Given that the prevalence of SSR in Austria is 3.3%, and that 40.7% of the general population are sensitized to at least one venom, it can be assumed that the majority of sensitized subjects do not experience SSR. But subjects could have recently been clinically relevant sensitized and could therefore potentially react to the next sting. The relevance of these sensitizations has not been elucidated yet. Therefore we conducted a prospective study to clarify the impact of detectable sIgE to hymenoptera venoms for the next sting. For this purpose, sting challenges with living bees and wasps were performed. Then we correlated available diagnostic tools like intradermal test, component resolved IgE diagnosis, and the basophil activation test with the outcome of the sting challenge. A mere of 5.3% showed SSR after the sting challenge, but the majority of subjects showed an increase of sIgE to the respective venom after sting challenge.

Methods

Subjects

Subjects, who tolerated previous hymenoptera stings well, were initially screened for sIgE to hymenoptera venom. Subjects with detectable sIgE, who met all other inclusion and exclusion criteria (Table 1), were asked to participate in the study. Finally, 110 subjects were enrolled.

Inclusion criteria
Legally competent male and female patients aged from 18 to 65 years
Detectable IgE antibodies (>0.35 kU/L) to bee or wasp venom
No history of a systemic anaphylactic reaction after a Hymenoptera sting
For female patients: effective contraception
Exclusion criteria
Clear history of a systemic anaphylactic reaction after a Hymenoptera sting
Individuals who have received immunotherapy with bee or wasp venom
Individuals with severe chronic illness
Severe asthma (FEV1) < 80% of predicted, FEV1/FVC ratio < 70%
Severe disorders of the lungs, liver, kidneys or nervous system
Clear chronic or acute cardiovascular failure
Hypertension and/or severe chronic ischaemic heart disease
Patients on ACE-inhibitor or beta-blocker treatment
Severe psychological disorders
For females: pregnancy and breast-feeding

Table 1. Inclusion and exclusion criteria

After a complete health check including physical examination, laboratory tests, spirometry, and ECG, diagnostic tests for hymenoptera venom allergy were carried out. To check clinical

relevance of sIgE to bee and wasp venom, a total of 131 sting challenges were performed: 41 with a bee, 37 with a bee and a wasp, and 16 with a wasp.

Classification of systemic sting reactions

According to the modified classification of Ring and Messmer, generalized skin symptoms such as flush, urticaria and angioedema were classified as grade I reaction. Mild to moderate pulmonary, cardiovascular or gastrointestinal symptoms were rated as grade II reaction. Bronchoconstriction, emesis, anaphylactic shock, and loss of consciousness were classified as grade III reaction.

Skin tests

The nature of sensitization was confirmed by means of standardized end-point titration skin prick tests (10, 100, 300µg/mL) and intradermal tests (0.02 mL of 0.01, 0.1 and 1 µg/mL solution) using purified honeybee and vespid venom extracts. Prick tests and intradermal tests were considered to be positive in the presence of a wheal ≥3 mm and ≥5mm in diameter and erythema, respectively.

Determination of sIgE and tIgE

Specific and tIgE antibody levels in the patients' serum were measured using ImmunoCAP 1000 (Phadia, Uppsala, Sweden), and Immulite 2000 (Siemens, Tarrytown, NY, USA) according to the manufacturer's instructions.

Component resolved diagnosis with rApi m 1 and rVes v 5 was done on the ImmunoCAP 1000. Diagnosis with nVes v 1 and nVes v 5 as well as with nApi m 1 was done on the ADVIA Centaur platform at the Department of I+D, ALK-Abelló, Madrid, Spain.

Basophil activation test (BAT)

BAT was performed as previously described[31, 89]. In brief, EDTA whole blood was stained with anti-CD123 PE-conjugated antibody (1:50), anti-HLA-DR PC5-conjugated antibody (1:50) and anti-CD63 FITC-conjugated antibody. Basophil reactivity was measured using serial dilutions of honeybee or vespid venom (1000, 100, 10, 1 ng/mL) or serial dilutions of anti-IgE antibody (1:10-1:1000 dilution).

Cell samples were analyzed by three-color flow cytometry (FC 500, Beckman Coulter). Basophils were identified as a single population of cells that stained positive for CD123 (FL-2) and negative for HLA-DR (FL-4). Up-regulation of CD63 expression was indicated by an increase in fluorescence in the FL-1 channel. Acquisition was terminated after 500 basophil target events. An approximately 2.5-fold increase in the number of activated basophils (>25%) as compared with the negative control (10%) at any of the test concentrations of the allergen was considered to be a positive response.

Sting challenge test

The insect sting challenge test was carried out using a definitely identified living bee (Apis melifera) and/or wasp (V. germanica, V. vulgaris) supplied by the Institute of Zoology, Graz. The challenge was performed on the upper forearm under partial inpatient condition with intensive medical stand-by and a continuous infusion; it was considered valid if a wheal ≥5 mm in diameter and erythema after 15 minutes at the site of the sting occurred.

Determination of prevalence rates for SSR and LLR

Prevalence rates for SSR and LLR were determined in a representative sample in Austria. Therefore, a telephone survey was conducted among 1,401 subjects to determine the prevalence of SSR with an accuracy of ±1%. Subjects were randomly and equally selected

from all political districts of Styria (a state in southern Austria) using an online telephone directory. The interviews were performed by instructed medical students on the basis of a structured questionnaire.

Data analysis

All data are expressed as medians (25%; 75% percentiles) on the raw scale, unless otherwise indicated. Data were tested for normality using the Kolmogorov-Smirnov test. Continuous variables were analysed by the Kruskal Wallis test; categorical variables were compared by the Chi-square test or Fisher's exact test. The level of significance was set at $p<0.05$. The PASW Statistics 18.0 software (IBM Inc, Somers, NY, USA) was used for statistical analysis.

Results

Epidemiology

Initially, we determined the prevalence of SSR and LLR in Styria. 1,401 persons (51.8% female, 48.2% male; median age 49 years (25%, 75% percentiles: 36, 65 years) were interviewed. Of these, 46/1,401 (3.3%, CI 2.4%- 4.4%) reported SSR and 64/1,401 (4.6%, CI 3.5%-5.8%) LLR.

Outcome of sting challenges

In 94 subjects 131 sting challenges with bees and wasps were performed. Surprisingly, only 5 of 94 (5.3%) subjects showed SSR after the sting; they usually showed generalized skin symptoms. Only one subject developed abdominal pain and emesis; no moderate or severe affection of the cardiovascular system in any subject was

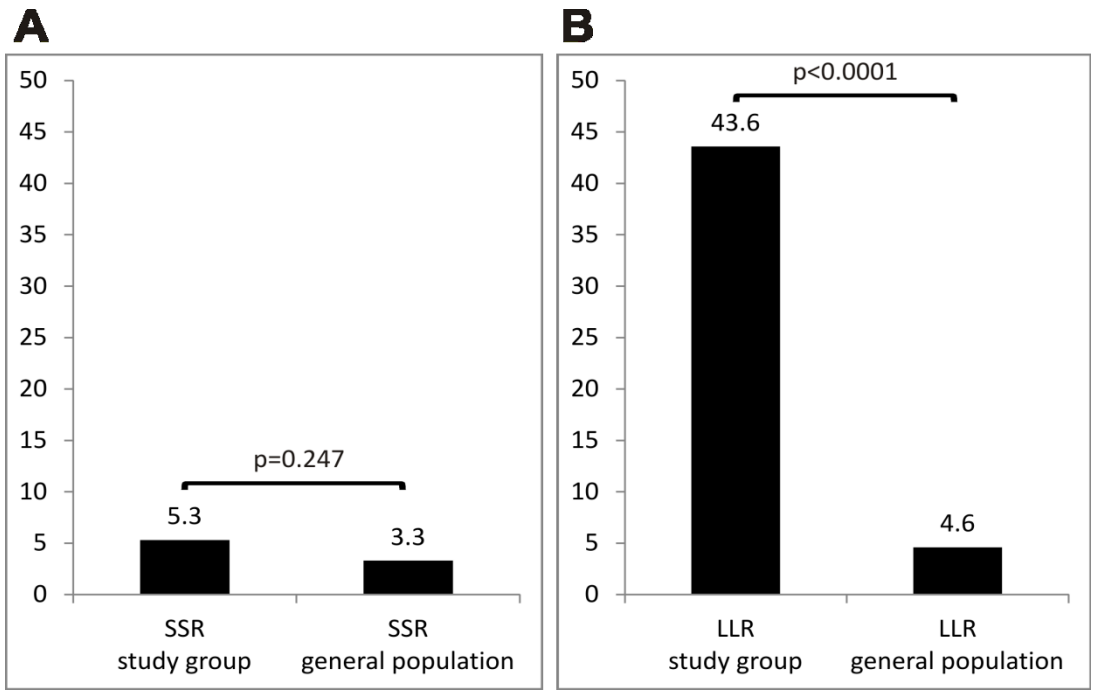

Figure 1. Frequency of SSR and LLR in the study group and general population

n study group=94, n general population=1,401

Data partly collected by Bettina Kranzelbinder & Danijela Bokanovic

observed. Large local reactions occurred in 41 of 94 (43.6%) subjects. Compared to the general population, these subjects had a comparable risk of SSR (p=0.247), whereas the frequency of LLR was 9.5 times higher (p<000.1; Figure 1).

Correlation between diagnostic tests and the outcome of sting challenges

78 subjects were stung by a bee and 75 of them tolerated the bee sting without any SSR. Nonetheless, of those who tolerated the bee sting, all had detectable sIgE to bee venom, 11 had sIgE to rApi m 1, 30 were positive to bee venom in the intradermal test, and 18 had a positive BAT result (Figure 2).

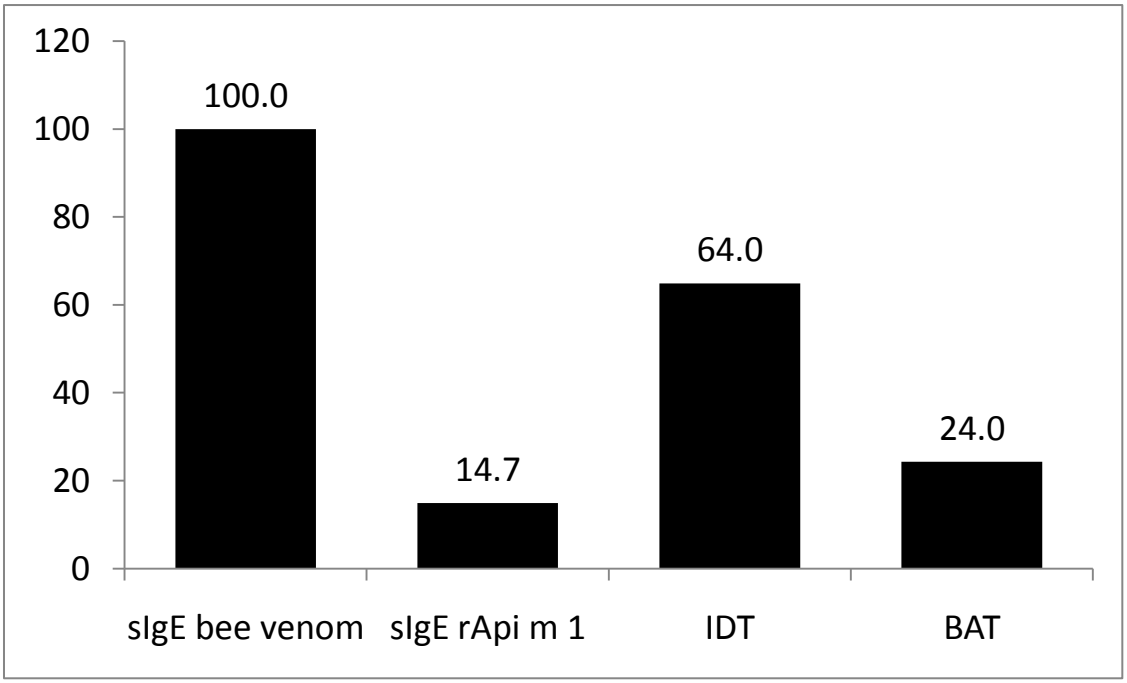

Figure 2. Positive tests (rel.%) to bee venom and negative bee sting challenge

53 subjects were stung by a wasp; 50 subjects tolerated the sting without any SSR. Of those who tolerated the sting, all had detectable sIgE to wasp venom, 15 had sIgE to rVes v 5, 28 were positive to wasp venom in the intradermal test, and 13 had a positive BAT result (Figure 3).

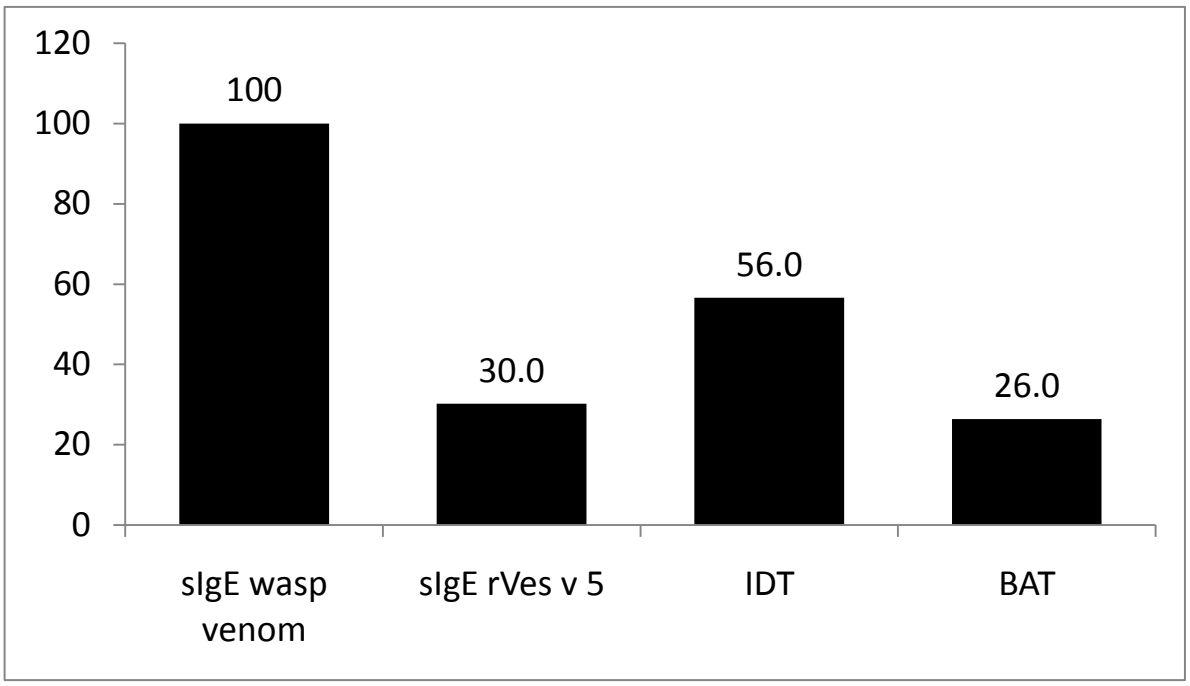

Figure 3. Positive tests (rel.%) to wasp venom and negative wasp sting challenge

Five subjects showed SSR, one of them developed urticaria after a bee sting as well as after a wasp sting. Three subjects reacted to a bee sting; all of them were identified by the IDT, but only 1 of them by the sIgE determination of rApi m 1 and by the BAT, respectively (Table 2).

Bee venom	positive	negative
rApi m1	1	2
BAT	1	2
IDT	3	0

Table 2.Comparison of test results in bee venom allergic subjects

Another three subjects showed SSR to a wasp sting; again all allergic subjects were identified by the IDT. 2 of 3 were identified by sIgE determination of rVes v 5 and by the BAT, respectively (Figure 3).

Wasp venom	positive	negative
rVes v 5	2	1
BAT	2	1
IDT	3	0

Table 3. Comparison of test results in wasp venom allergic subjects

Discussion

This study impressively illustrated that the majority of sensitized subjects without a sting reaction in the past tolerated the respective insect. Only 5 of 94 (5.3%) subjects showed SSR after the sting. We could previously show that asymptomatic sensitization to hymenoptera venoms is common in the general population[64], now we could confirm that these sensitizations are mostly not relevant for the persons concerned. However, asymptomatic sensitization still occurs despite the further development of diagnostic tests. Although the BAT seems to be closer to the in vivo allergy mechanism as component resolved serum IgE determination, it is still not possible to predict the occurrence and severity of allergic symptoms.

Conclusion

In the first step, we aimed to identify factors that may influence results of the CD63-based BAT. We found that it should be performed as early as possible after taking the blood sample, preferably within four hours. In contrast to the skin test, the CD63-based BAT could be performed in patients undergoing treatment with antihistamines. But due to multiple influencing factors, we recommend that the BAT should only be carried out at validated laboratories.

The next aim of the present study was to identify potent influencing factors of the CD203c-based BAT and to emphasize differences between CD63 and CD203c detection. We found that CD203c and CD63 expression was rapidly upregulated reaching a maximum after 20 to 30 min. Basophil CD203c up-regulation assayed after storage times up to 48 h declined already after 4h. IL-3 treatment increased CD203c and CD63 baseline levels and decreased basophil CD203c responses in a dose-dependent manner. Similar to CD63, CD203c-based BAT should be performed preferentially within 4 h after taking the blood samples. Priming and degranulation-enhancing factors were not required for CD203c-based BAT. In contrast to skin testing, CD203c-based BAT could also be performed in patients undergoing anti-allergic treatment.

In the next step, we aimed to compare currently available routine diagnostic tests as well as experimental tests including the BAT to identify the most accurate diagnostic tool. BAT, CRD, and ADVIA showed a low rate of DS. However, the rate of DS is higher than expected by personal history, indicating that the matter of clinical relevance is still not solved even by novel tests. Furthermore, the lack of agreement between these tests makes it difficult to distinguish between bee and wasp venom allergy. At present, no routinely employed test, not even the BAT, can be regarded as gold standard to find the clinically relevant sensitization.

In the final step, we focused on clinically irrelevant sensitization. Therefore, we initiated the next study to prove if sensitized subjects without a history of systemic sting reactions tolerate sting challenges with the respective insect. BAT and routine diagnostic tools were correlated with the outcome of sting challenges. Importantly, we could show that sensitizations in subjects not having shown SSR in the past do not constitute a high risk for SSR. However, even the BAT and CRD revealed positive results in subjects who tolerated stings well. Therefore, a test which is able to predict the occurrence and severity of future sting reactions is still needed.

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Abbreviations

AS	Asymptomatic sensitization
ADVIA	ADVIA Centaur®, Siemens, Tarrytown, NY, USA
BAT	basophil activation test
CAP	ImmunoCAP®, Phadia, Uppsala, Sweden
CCD	cross-reactive carbohydrate determinant
CD	cluster of differentiation
CCR3	C-C chemokine receptor-3
CR3	complement receptor 3
CRD	component resolved diagnosis
CRTH2	chemoattractant receptor homologous molecules on Th2 cells
DS	double sensitization
ECG	electrocardiography
E-NPP3	ectonucleotide pyrophosphatase/phosphodiesterase 3
FcεRI	high affinity IgE receptor
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
gp	glycoprotein
HLA	human leukocyte antigen
ICAM	intercellular adhesiomolecule
IDT	Intradermal test
Ig	immunoglobulin
IgE	immunoglobulin E
IL	interleukin
Immulite	Immulite 2000®, Siemens, Tarrytown, NY, USA
LAMP	lysosome-associated membrane protein
LIMP	lysosomal integral membrane protein
LT	leukotriene
Mac-1	macrophage adhesion molecule-1
MadCAM-1	mucosal addressin cell adhesion molecule-1
MLA	natural killer cells
MUXF	N-glycan: Manα1-3(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAcβ1
RAST	radioallergosorbant test
sIgE	specific IgE

SSR	systemic sting reaction
Th2	T helper cells 2
tIgE	total IgE
WB	western blot

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