

CD203c-Based Basophil Activation Test in Allergy Diagnosis: Characteristics and Differences to CD63 Upregulation

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Background: The basophil activation test (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 with that of CD63-based tests, as the mechanisms of CD203c versus CD63 induction differ considerably. The 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.

Methods: CD203c-based BAT was determined 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 upregulation. Furthermore, the influence of different storage conditions and incubation times was evaluated and the impact of antiallergic drugs on the test results was assessed.

Results: CD203c and CD63 expression was rapidly upregulated reaching a maximum after 20–30 min. Basophil CD203c upregulation assayed after storage times up to 48 h declined already after 4 h. IL-3 treatment increased CD203c and CD63 baseline levels and decreased basophil CD203c responses in a dose-dependent manner. In contrast, cytochalasin B and latrunculin B did not affect CD203c responses but decreased CD63-based BAT. Finally, therapeutic concentrations of dimetindene and desloratadine did not affect CD203c upregulation.

Conclusion: CD203c-based basophil activation test should be performed preferentially within 4 h after taking the blood samples. Priming and degranulation-enhancing factors are not required for CD203c-based BAT. In contrast to skin testing, CD203c-based BAT can be performed in patients undergoing anti-allergic treatment. © 2010 International Clinical Cytometry Society

Key terms: CD203c; CD63; BAT; allergy diagnosis; technical issues

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Hypersensitivity to Hymenoptera venom is a common and potentially life-threatening condition (1–4). Large local reactions occur in up to 26.4% (5) of the general population after hymenoptera stings and up to 7.5% are reported to have experienced systemic anaphylactic reactions (6). 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

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is still concern about their sensitivity and specificity (7–10). 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 (11). In 2000, an excellent sensitivity and specificity of BAT in the diagnosis of Hymenoptera allergy was described (12) and subsequently confirmed in several studies (13–17). Data of the first CD203c-based protocol for Hymenoptera allergy was published in 2001 (18) and was recently validated by others (16). Currently, BAT with CD63 is considered as the best clinically validated test (19) 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 (20,21). 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 (22). Similar to CD63, basophil CD203c expression rapidly increases after allergen challenge in sensitized individuals (18,23,24). On the one hand, some authors claimed the advantages of CD63 over CD203c (25–27), 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 (28). 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 (16). Additionally, a study concerning the use of basophil activation markers in the diagnosis of cat allergy demonstrated that the measurement of basophil CD203c upregulation is as reliable as CD63-based BAT (29). Moreover, Abuaf et al. demonstrated that CD203 seems to be a more sensitive basophil activation marker than CD63 for the diagnosis of amoxicillin allergy (30).

In spite of the widespread application, BAT protocols using either CD63 induction or CD203c upregulation 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 number of patients (31). We recently addressed important technical issues regarding BAT based on CD63 (32), 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 upregulation for the diagnosis of hymenoptera allergy and to point out potential influencing factors including antiallergic drugs.

METHODS

Reagents and Antibodies

All laboratory reagents were obtained from Merck (Whitehouse Station, NJ) or Sigma-Aldrich (St. Louis, CA) unless otherwise specified. Dulbecco's modified phosphate-buffered saline (PBS; with or without Ca^{2+} and Mg^{2+}) was purchased from PAA (Pasching, Austria). Cell-Fix and antibodies to CD123 (PE-conjugated) were supplied by Becton Dickinson (Franklin Lakes, NJ). Dimetindene, desloratadine, and prednisolone were purchased from Novartis (Basel, Switzerland), Schering-Plough (Kenilworth, NJ), and Merck, 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). 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). Goat anti-mouse IgG (Alexa Fluor 488-conjugated) antibodies were purchased from Invitrogen (Carlsbad, CA). 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) and antibody diluent was purchased from Dako (Gulstrup, 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). 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 PBS 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 was used as wash buffer.

Stimulation buffer. Dulbecco's modified PBS 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 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.1 g/L $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$ was used as stimulation buffer.

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_2EDTA .

Fixative solution. BD CellFIX (10 $\times$; 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 $39.0 \pm$

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

EXPERIMENTAL PROCEDURES

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) (33,34). 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-60 $\times$ /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 anti-

body, 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 to 1:3200 dilution). Hundred-microliter 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. To achieve erythrocyte lysis, 2 mL of lysis solution was added to each sample. After 15 min of incubation at room temperature in the dark, the samples were centrifuged (5 min, 200g) 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 up-regulation (35). 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 the three protocols were analyzed for every point of time by adjusting the threshold in nonstimulated samples to contain 10% of CD203c or CD63 expressing basophils (baseline).

Storage Time

Samples of EDTA anticoagulated whole blood from control subjects were stored for 4, 24, 28, and 48 h at 4°C while samples from allergic patients were stored for 18 h at 4°C. BAT was performed immediately after blood-taking and at the indicated time-points. For stimulation, serial dilutions of anti-IgE, 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 (36), 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 (5 µg/mL) (15,32) and latrunculin B (5 and 0.1 µg/mL) (37,38), both inhibitors of actin polymerization, on CD203c-based as well as CD63-based BAT results. In all the cases, serial dilutions of anti-IgE were used for basophil stimulation as described above.

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

Influence of Antiallergic Drugs

To investigate the effect of prednisolone and dimetindene on the upregulation of basophil CD203c expression, EDTA whole blood samples derived from healthy subjects were pretreated 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 h after the intake of the twofold daily dose (10 mg) of desloratadine.

Cytometer and Fluorochrome Setting

Flow analysis was performed using the FC500 single laser flow cytometer from Beckman Coulter (32). This instrument has a 3,300 events per s capability, five color detection, and a scatter and fluorescence carryover <1%. The FC500 is equipped with FL-1 525/40, FL-2 575/40, FL-3 620/20, FL-4 675/40, 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.

Gating Procedure

Basophils were gated in the lymphocyte region of the SSC or 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. 1C) were used as isotype controls in the preliminary procedure. Upregulation of CD203c expression was indicated by an increase in fluorescence in the FL-2 channel (Figs. 1D and 1E). Upregulation 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 $\pm$ 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, Chicago, Illinois).

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 (Figs. 2A and 2C). In anti-IgE (ratio 1:100) stimulated basophils, there was a significant increase in surface expression, similar for both the markers (Figs. 2B and 2C).

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 1:3,200 to 1:100) or with bee and wasp venom (1–1,000 ng/mL). In the CD63 protocol, whole blood samples

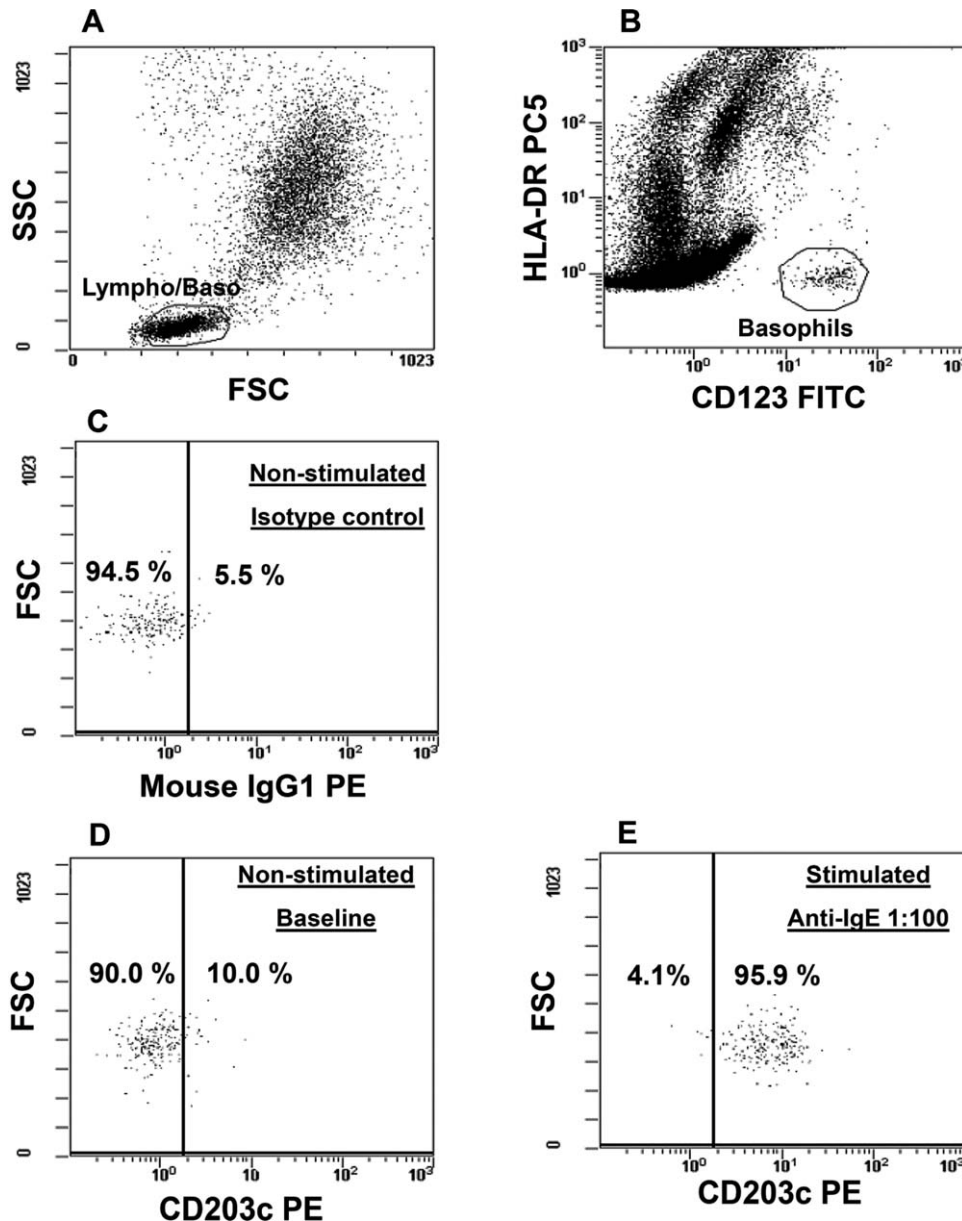


FIG. 1. Gating procedure in CD203c-based BAT. Basophils gated in the lymphocyte region of the SSC/FSC pattern were selected as a HLA-DR^{neg}/CD123^{pos} population (A, B). CD203c responses were quantified as percentages of CD203c expressing basophils in a higher FL-2 region, which had been adjusted 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 fluorescence unspecific staining, a mouse IgG1 isotype control conjugated to PE was used (C).

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 extent, 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.

Time-Course of CD203c Upregulation

As CD63 and CD203c upregulation is suggested to exhibit different time kinetics, we determined the opti-

mum 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 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

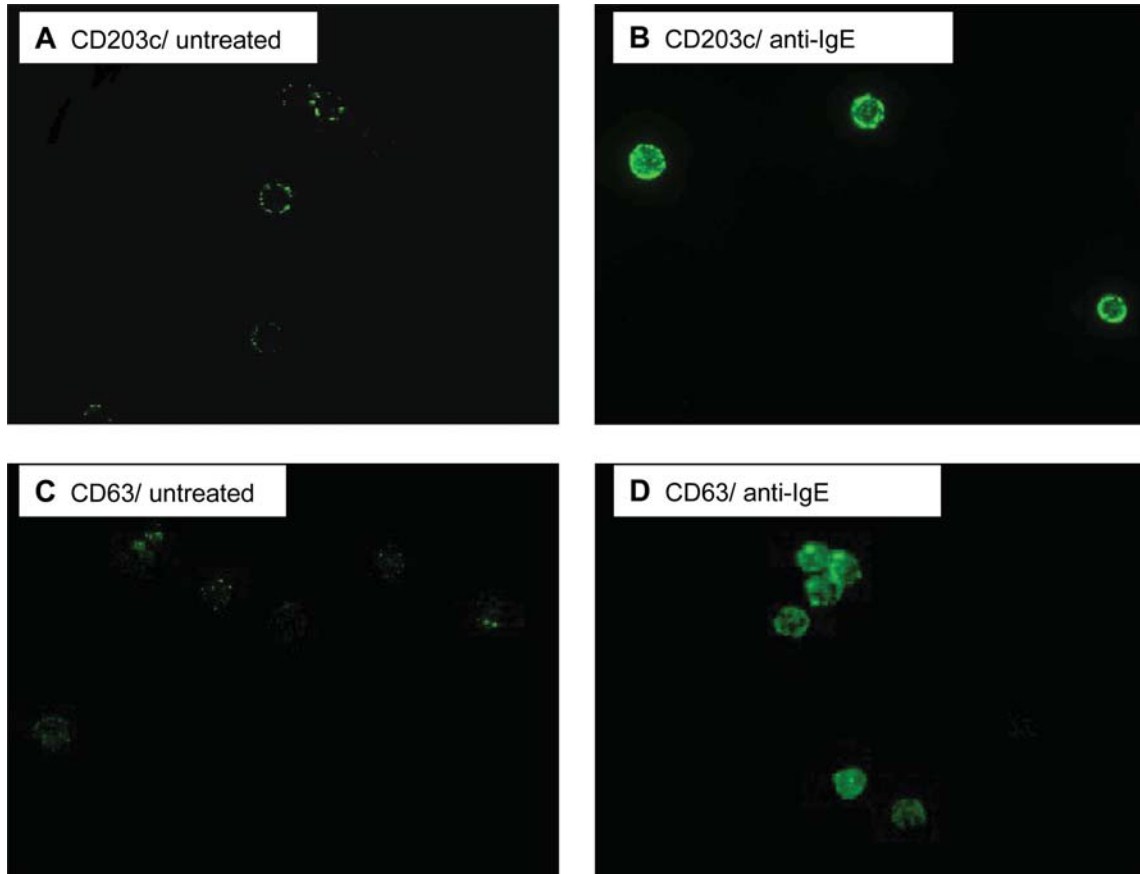


FIG. 2. Immunofluorescence staining of CD63 and CD203c in isolated basophils. Purified basophils were stimulated with anti-IgE (ratio 1:100) or buffer for 20 min at 37°C. Samples were blocked and stained with CD63 and CD203c specific primary antibodies and a goat anti-mouse IgG (H + L) secondary antibody conjugated with Alexa Fluor 488. Cytospin slide preparations were fixed with 3.7% formaldehyde and mounted with Vecta-shield medium. Samples are analyzed on an Olympus IX70 fluorescence microscope and an Olympus UPlanApo-60 $\times$ /1.20 lens, using Hamamatsu ORCA-ER digital camera and Olympus Cell^AP software. CD203c showed a higher expression than CD63 either under basal (A and C) or under stimulated conditions (B and D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 1
Comparison Between CD63 and CD203c Expression in
Hymenoptera Venom-Allergic Patients

	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

CD63- and CD203c-based BAT was performed in 79 allergic patients. Whole blood samples were stimulated with anti-IgE (ratio 1:3,200 to 1:100) and with bee and wasp venom (1–1,000 ng/mL).

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 h at 4°C, while samples from allergic patients were stimulated after storage of 0 and 18 h 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 h resulted in a loss of basophil reactivity, depending on the anti-IgE dilution, by 35.1–54.4%. After 48 h, the basophil responses were reduced by 48.1–56.1%. 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%.

Similar results were obtained with regard to bee and wasp venom: of initially 13 bee venom-allergic and BAT-positive patients, only six were positive after 18 h; of the 18 wasp venom-allergic patients, only four remained

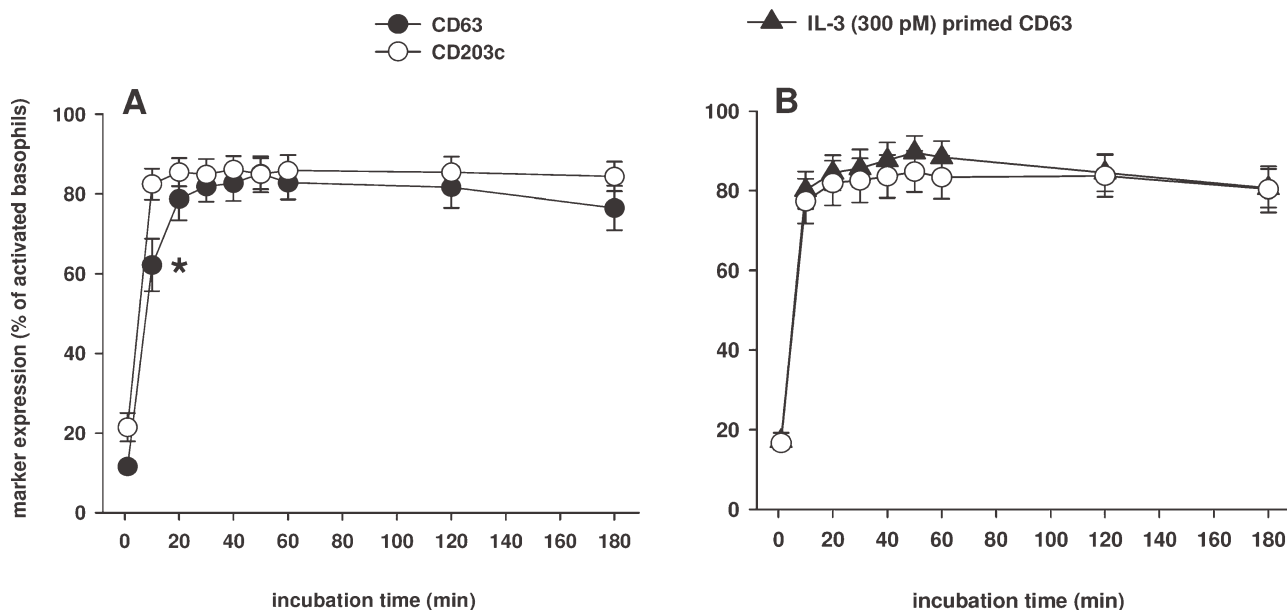


Fig. 3. Time kinetics of CD203c and CD63 expression. Samples were stimulated with anti-IgE (ratio 1:100) at 37°C and activation was stopped immediately or after the indicated incubation periods. The time kinetics of CD203c and CD63 were performed in 10 controls (A). The time kinetics of CD203c compared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

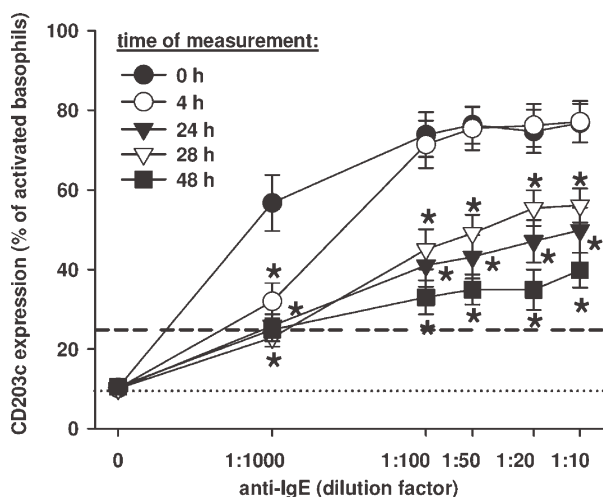


Fig. 4. Storage time-dependent decrease in anti-IgE-induced basophil activation. Blood samples from controls were stored for up to 48 h at 4°C before BAT was performed (anti-IgE, 20 min, and 37°C). Best responses were obtained when blood samples were processed immediately. A storage time from 24 to 48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

positive after 18 h. A loss of reactivity by up to 70.9 % was found after 18 h (Fig. 5).

Priming Factors and Basophil Activation

We tested whether anti-IgE-induced upregulation of CD203c expression is altered by pretreatment with the basophil-priming cytokine IL-3 or the degranulation-pro-

moting substances cytochalasin B and latrunculin B. Therefore, IL-3, cytochalasin B (Fig. 6) or latrunculin B (Fig. 7) was added to EDTA whole blood samples before stimulation. As we reported previously (32), the concentrations of IL-3 (75, 150, and 300 pM) significantly enhanced basophil CD63 responses. Cytochalasin B (5 μ g/mL) (15) and latrunculin B (37,38) (0.1 and 5 μ g/mL) were applied at effective concentrations. As shown in Figure 6, IL-3 priming significantly enhanced the basal expression levels of CD203c and CD63 in the nonstimulated samples as well as basophil responsiveness to Fc ϵ RI 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 (Figs. 6A and 6C). To scrutinize the effect of IL-3 priming on BAT results, we reanalyzed the data by adjusting the threshold in nonstimulated 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 nonstimulated 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 (Figs. 6A, 6B, and 7A) but enhanced the basal CD63 expression levels and, thus, impaired the CD63-based test results by reducing the difference between

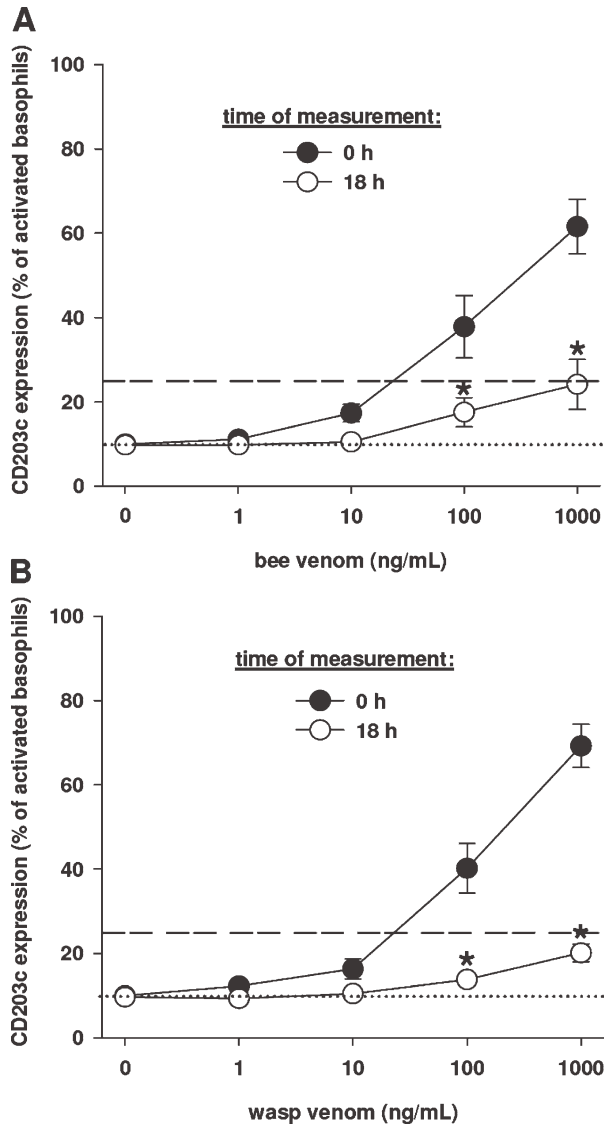


Fig. 5. Storage time-dependent decrease in basophil activation to bee and wasp venom. BAT was performed immediately after blood taking or after storage of 18 h at 4°C with (A) bee ($n = 13$) or (B) wasp venom ($n = 18$) at the indicated concentrations. Data are expressed as means $\pm$ SEM, $n = 13$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

baseline (unstimulated) and anti-IgE stimulated responses (Figs. 6C and 7B).

Influence of Antihistamines and Corticosteroids on Basophil Activation

It may be assumed that some allergic patients are already under systemic antiallergic medication when BAT is performed. Thus, we investigated whether antiallergic 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 upregulation of CD203c at concentrations that corresponded to therapeutic

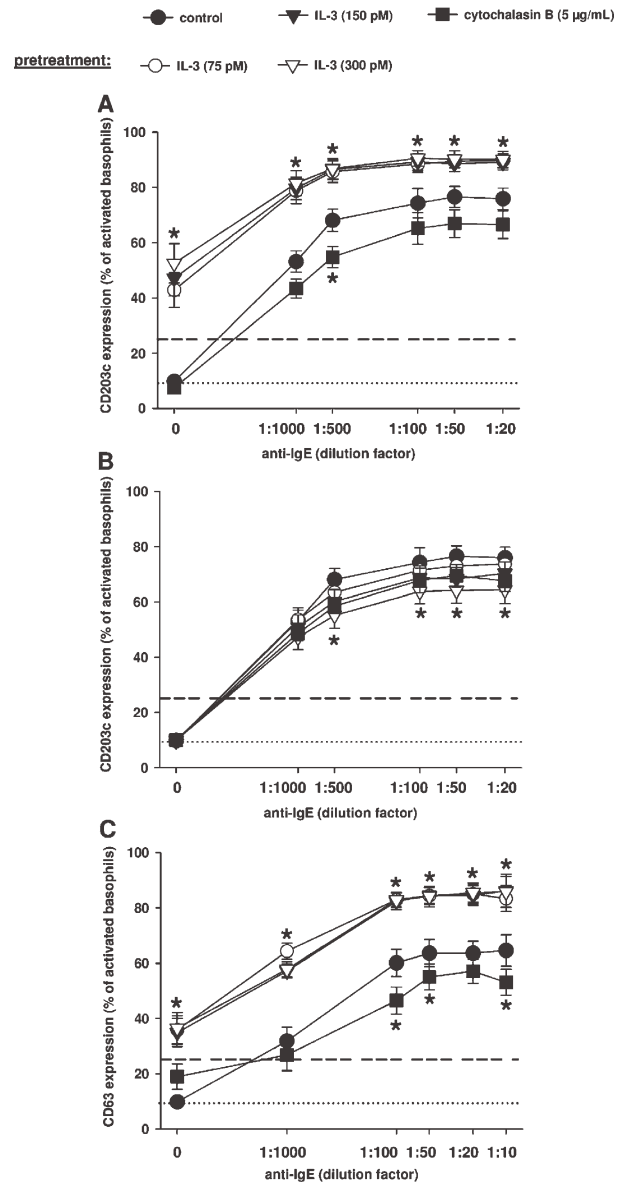


Fig. 6. Influence of IL-3 priming and cytochalasin B on CD203c responses. Samples were pretreated with IL-3 (75, 150, and 300 pM) or cytochalasin B (5 µg/mL) for 10 min at room temperature before BAT with anti-IgE stimulation for 20 min was performed. IL-3 enhanced CD203c (A) as well as CD63 (C) baseline 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 nonstimulated but cytochalasin B-treated samples to contain 10% of CD203c expressing basophils (baseline) (B). Data are expressed as mean $\pm$ SEM, $n = 16$; * $P < 0.05$ vs. control. baseline response (10%); - - - threshold of 25% activated basophils.

plasma levels of the drugs (Fig. 8). Only at concentrations 50-fold higher than the therapeutic level, dimetindene significantly attenuated basophil activation. As 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

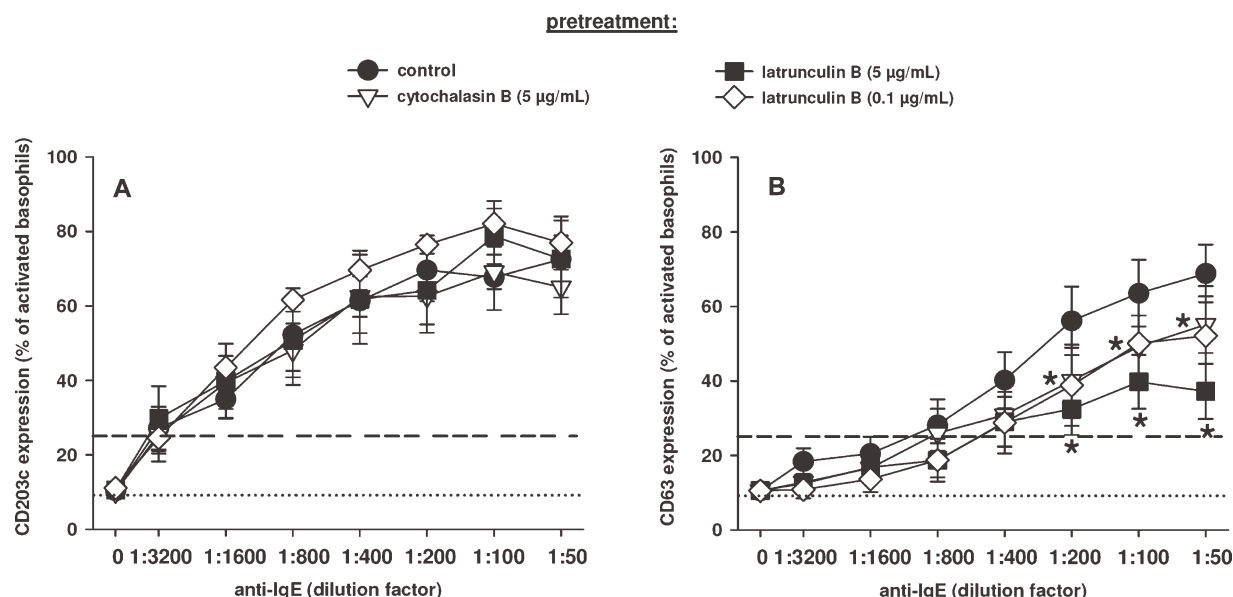


Fig. 7. Influence of latrunculin B on CD203c and CD63 responses. Similar to cytochalasin B, pretreatment for 10 min at room temperature with latrunculin B (0.1 or 5 µg/mL) did not affect CD203c-based BAT results (A), but enhanced CD63 baseline levels (B) and thus significantly diminished CD63-based BAT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

3 h after, taking 10 mg of desloratadine. As shown in Figure 9, no effect on test results was observed.

DISCUSSION

The basophil activation test based either on CD63 or CD203c upregulation 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 (35). 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 10–20 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 h, and even after 4 h, basophil responses were slightly decreased. More substantially, when basophils were stimulated with bee or wasp venom after storage for 18 h, BAT resulted mostly in false-negative results. Compared with 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 (29,39). In contrast, IL-3 was found to enhance CD203c expression by resting, nonactivated basophils but to decrease the sensitivity of BAT (29). 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 upregulates 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

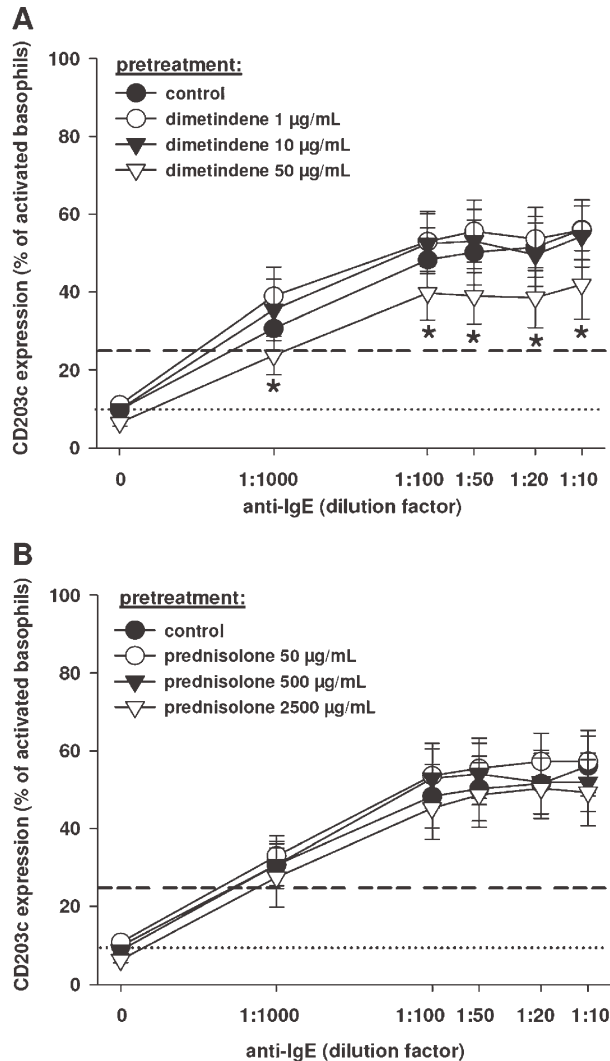


Fig. 8. Influence of in vitro pretreatment of basophils with antihistamines and corticosteroids on CD203c upregulation. Samples were preincubated with various concentrations of the H_1 histamine receptor antagonist dimetindene (A) or the corticosteroid prednisolone for 30 min (B) and CD203c expression 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 $\pm$ SEM, $n = 12$; * $P < 0.05$ vs. control. baseline response (10%); - - threshold of 25% activated basophils.

whole blood (32), 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

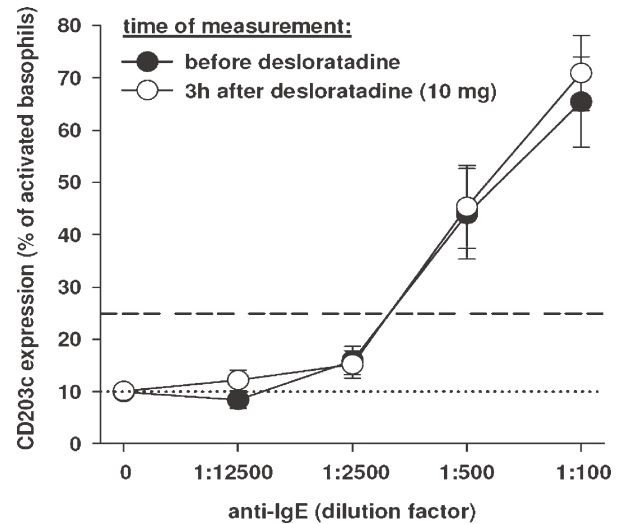


Fig. 9. Influence of antihistamine intake on CD203c upregulation. Blood samples were taken before and 3 h after the intake of 10 mg desloratadine. Basophil reactivity to anti-IgE stimulation was not altered when responses before and after treatment were compared. Data are expressed as means $\pm$ SEM, $n = 10$; baseline response (10%); - - threshold of 25% activated basophils.

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 (40), this was recently confirmed (32), but until now, no data have been available regarding CD203c. As antihistamines are selective H_1 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 antiallergic drugs do not affect basophil responsiveness, as measured by CD203c upregulation, which occurs independently from degranulation and mediator release. Beside that, in vitro pretreatment 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.

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