

Individual IL-3 priming is crucial for consistent *in vitro* activation of donor basophils in patients with chronic urticaria

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Background: The *in vivo* autologous serum skin test (ASST) is the diagnostic gold standard to detect autoantibodies against FcεRI or IgE itself, as well as other autoreactive serum components, in patients with chronic spontaneous urticaria (CU). Coincubation of patient sera with donor basophils and measuring their degranulation *in vitro* could be a safe alternative but has shown inconsistent results.

Objective: Optimization of the basophil activation test to detect autoreactive serum components in patients with CU.

Methods: The ability of patient sera to induce CD63 and CD203c in donor basophils (n = 15) was measured by means of flow cytometry. Sera of 20 patients with CU (10 with positive ASST results), 15 patients with cold urticaria, and 27 healthy control subjects were included to optimize test conditions with donor basophils and a basophil cell line (RBL703/21) followed by testing of 110 consecutive patients from clinical routine.

Results: We demonstrate that individual IL-3 priming normalized the initially inconsistent basophil reactivity and led to reproducible and comparable test results irrespective of the basophil donors used. CD203c as an activation marker and the use of a basophil cell line were less suitable for this purpose.

Conclusion: The basophil activation test with individualized IL-3 priming for each basophil donor is a reproducible and reliable alternative to the ASST. There are several advantages over the ASST: no risk of accidental infection, no influence of antihistamines on the test result, quantifiable results, and a potential in providing treatment monitoring. The exact nature of the degranulating factor or factors in patient sera remains an open question. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Autoantibodies, autologous serum skin test, basophil activation test, CD203c, CD63, chronic urticaria, flow cytometry, IgE receptor, IL-3

Abbreviations used

ASST: Autologous serum skin test
BAT: Basophil activation test
CU: Chronic spontaneous urticaria
fMLP: N-formyl-methionyl-leucyl-phenylalanine

Chronic spontaneous urticaria (CU) is defined as recurrent and transitory episodes of hives without an obvious cause present for more than 6 weeks. The quality of life of patients with CU is comparable with that of patients with 3-vessel coronary artery disease awaiting bypass grafting.¹ The cause of CU is heterogeneous, and in up to 80% of patients, a cause cannot be identified.² Therefore current guidelines for the diagnosis of CU³ still recommend the *in vivo* autologous serum skin test (ASST) to define a CU subgroup as having “autoreactive urticaria.” Several groups identified IgG autoantibodies against the α subunit of the high-affinity IgE receptor (FcεRIα) or IgE itself in a subpopulation of patients with CU.^{2,4-8} These autoantibodies might lead to mast cell or basophil degranulation, with release of histamine and other allergic mediators. However, their relevance in the pathogenesis of CU remains controversial.⁹ The ASST requires freshly prepared serum samples, and antihistamine treatment has to be stopped before testing. Interpretation is sometimes difficult, especially if a pronounced dermographism is present, with reports on the percentage of positive ASST results in the range of 4% to 76% of all patients with CU tested.¹¹ Furthermore, there are ethical concerns or even legal prohibitions to inject serum into patients because of the risk of accidental infection. Therefore several attempts have been made to develop sensitive and specific *in vitro* tests for the detection of anti-FcεRIα and anti-IgE antibodies in sera of patients with CU. ELISA-based tests did not discriminate between the low-affinity irrelevant antibodies occurring in most subjects and functionally relevant autoantibodies with high affinity.^{9,10,12} Therefore the basophil histamine release test or basophil activation test (BAT) with healthy donor basophils was investigated for their potential to detect the autoreactive serum components,¹³⁻¹⁸ and some of these tests are already commercially available (CU Index test, IBT Laboratories, Lenexa, Kan; Anti-IgE Receptor Ab, ADx Laboratories, Denver, Colo; HR-Urticaria Test,¹⁹ RefLab, Copenhagen, Denmark). Unfortunately, a high interlaboratory variability, most probably because of donor-to-donor variability, has been reported.²⁰

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TABLE I. Summary of basophil donor characteristics in regard to individual IL-3 concentration and expected values for positive controls serving as internal validation

Donor ID	Sex	Age (y)	Atopy status	CD63 upregulation (%)		Optimal IL-3 concentration (ng/mL)
				algE (100 ng/mL)	fMLP (1.5 ng/mL)	
1	M	26	No	82.2 ± 8.3	51.2 ± 2.6	10
2	M	35	Yes	47.3 ± 7.6	21.7 ± 3.5	—
3	M	27	Yes	69.4 ± 4.6	48.3 ± 3.3	1
5	F	58	No	38.6 ± 7.2	10.2 ± 1.5	10
6	F	26	No	49.0 ± 3.6	18.8 ± 2.6	10
7	F	39	No	69.5 ± 7.5	48.1 ± 2.8	1
9	F	35	Yes	33.1 ± 8.2	20.2 ± 1.3	0.1
10	M	36	No	25.1 ± 6.8	10.1 ± 3.2	0.1
13	M	25	No	57.3 ± 4.9	39.4 ± 3.7	1
14	M	34	No	18.8 ± 5.7	43.1 ± 6.5	10
15	F	53	No	47.8 ± 7.4	11.2 ± 3.7	10

Basophil donors 4, 8, 11 and 12 were nonresponders to the IgE-dependent positive control (algE). Data are presented as the percentage of CD63⁺ basophils (means ± SEMs). *algE*, Anti-IgE antibody; *F*, female; *M*, male.

Additionally, the fact that several basophil donors are needed to establish the test for daily routine diagnostics has not been investigated in detail. Basophil cell lines have rarely been used in this respect and did not show consistent results when tested in the setting of atopic disease.^{21,22} There is only 1 report describing the use of humanized RBL cells to measure β -hexosaminidase release from heat-treated sera of patients with CU.¹⁰ More recently, Nakamura et al²³ demonstrated the toxicity of higher human serum concentrations for RBL SX-38 cells, which was in part because of complement activation.

The main goal of this study focused on the BAT was to define the relevant parameters influencing test accuracy compared with that of the ASST as the current diagnostic gold standard. We systematically investigated different test conditions (cell separation and activation markers) and donor basophil characteristics (IL-3 sensitivity and IgE receptor density). Here we present, for the first time, an optimized test procedure of the BAT in patients with CU as an open-source protocol suitable to be implemented into routine laboratory diagnostics whereby human donor basophils could not be replaced by the basophil cell line used in this study.

METHODS

Serum samples of patients and healthy control subjects

Serum samples from 110 patients (67 women and 43 men; mean age, 42 years; age range, 12–77 years) with CU, which is defined as the spontaneous appearance of typical skin lesions at least 3 times a week for a minimum of 6 weeks without an identified cause, were recruited from our outpatient Department of Allergology in Bern, Switzerland. Patients with physical urticaria and urticarial vasculitis were excluded. Twenty-seven healthy subjects without urticaria (18 women and 9 men; mean age, 39 years; age range, 21–61 years) served as negative healthy control subjects. Additionally, we included 15 patients with cold urticaria (8 women and 7 men; mean age, 41 years; range, 28–59 years) as a further control group. All serum samples were stored at -20°C . All patients provided informed consent, and the study was approved by the local ethics committee.

ASST

ASST was performed according to international consensus.²⁴ Briefly, 0.2 to 0.3 mL of autologous serum, drawn and centrifuged immediately before testing, was injected intradermally (undiluted and 1:10) into the volar aspect of both forearms, avoiding skin areas affected within the last 24 hours.

Histamine and codeine (2 mg/mL; Allergopharma AG, Hamburg, Germany) and normal saline served as positive and negative controls, respectively. Results were evaluated 30 minutes after injection and were considered positive if the wheal diameter exceeded the injection wheal by 1.5 mm and was accompanied by a flare reaction.

Basophil donors

We recruited 15 healthy basophil donors (8 women and 7 men; mean age, 36 years; age range, 25–57 years; Table I). Five basophil donors were atopic based on history and skin test results to grass, weed, and birch pollen and house dust mites. Four were nonresponders lacking CD63 upregulation after stimulation with anti-IgE as a positive control; those donors were excluded from further analysis.

FcεRI density on the surface of donor basophils

To determine the surface density of FcεRI on donor basophils, we used QIFIKIT (Dako-Cytomation, Glostrup, Denmark) for quantitative flow cytometry, as described elsewhere.²⁵

BAT

All reagents were from Sigma-Aldrich (Buchs, Switzerland) unless stated otherwise. PBMCs were isolated from fresh heparinized peripheral blood by means of density gradient centrifugation and resuspended in assay medium (RPMI-1640, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, and 1% BSA) to a final concentration of 5×10^6 cells/mL. An individual concentration of IL-3 was added for each basophil donor. One hundred microliters of cells resuspended in the appropriate IL-3 medium was incubated with either 100 μL of patient serum or 100 μL of a healthy control serum for 30 minutes at 37°C . Anti-IgE (100 ng/mL; Beckman Coulter, Marseille, France) and assay medium served as positive and negative controls, respectively. In some experiments basophils were stimulated with N-formyl-methionyl-leucyl-phenylalanine (fMLP; 2.5 nmol/L) as an additional IgE-independent positive control. The cells were stained with anti-CCR3–allophycocyanin, anti-CD63–fluorescein isothiocyanate, and anti-CD203c–phycoerythrin, all of which were from BioLegend (San Diego, Calif), for 20 minutes at 4°C . Cells were washed in CellWASH and fixed with 1% paraformaldehyde. Basophils were acquired according to their granularity and CCR3 expression²⁶ on a FACSCanto. CD63 and CD203c served as activation markers and were expressed as the percentage of activated basophils.

Characterization of IL-3 sensitivity in donor basophils

To define the IL-3 sensitivity of a given basophil donor, we prepared different assay media with varying IL-3 concentrations (10–0.1 ng/mL

BioLegend) or without IL-3 for the subsequent BAT. The criteria to determine the optimal IL-3 concentration for a given basophil donor were that healthy control sera led to less than 5% upregulation of CD63 and optimal basophil reactivity with positive controls.

Basophil cell line

The human FcεRI-transfected RBL cell line RBL-703/21 was generated at Paul-Ehrlich-Institut (Langen, Germany)²⁷ and cultured in RPMI-1640 medium (Lonza, Switzerland) supplemented with 2 mmol/L L-glutamine (Gibco [now Life Technologies], Carlsbad, Calif), 5% neonatal heat-inactivated calf serum (Sigma-Aldrich), and 1 mg/mL geneticin sulfate (G-418; Sigma-Aldrich). The cells were cultured at 37°C in a 5% CO₂ atmosphere. RBL cell lines were harvested for use on reaching 85% to 95% confluence.

β-Hexosaminidase release assay

The β-hexosaminidase assay was performed as described by Vogel et al,²⁷ with some modifications. All reagents were from Sigma-Aldrich unless stated differently. RBL-703/21 cells were harvested by using 1× trypsin-EDTA solution, resuspended in the growth medium described above, seeded into Nunc 96-well cell-culture plates at 1.5×10^5 cells per well with or without sensitization with purified monoclonal human IgE (AbD Serotec, Oxford, United Kingdom), and grown overnight at 37°C in a 5% CO₂ atmosphere. The following day, the cells were washed twice with modified Tyrode buffer with 10 mmol/L CaCl₂ (Thermo Fisher Scientific, Loughborough, United Kingdom) and 1 g/L BSA adjusted to pH 7.45 with NaOH. Cells were stimulated with 100 μL of allergen challenge buffer, a mixture of 50% modified Tyrode buffer and 50% deuterium oxide (vol/vol) containing different dilutions of patient or control serum. Total β-hexosaminidase release was determined by comparison with cells treated with lysis buffer (0.5% Triton X-100). Cells treated with 1 μg/mL A23187 (Enzo Life Sciences, Farmingdale, NY) together with phorbol 12-myristate 13-acetate (10 μmol/L) or 1 μg/mL polyclonal goat anti-IgE (Vector Laboratories, Burlingame, Calif) in allergen challenge buffer were used as positive degranulation controls. Colorimetric quantification of β-hexosaminidase activity was performed as described by Vogel et al.²⁷

Detection of IL-13 in sera

Detection of IL-13 was done according to the manufacturer's protocol with the human IL-3 Flex Set (BD Cytometric Bead Array; BD, Franklin Lakes, NJ). Flow cytometric analysis was performed with a FACSCanto.

Statistical analysis

Data analysis was performed with FlowJo (version 9.2; Tree Star, Inc, Ashland, Ore) and Prism 5.0 (GraphPad Software, Inc, La Jolla, Calif) software. The 2-tailed Mann-Whitney test was applied, where appropriate. Correlations were analyzed by using the nonparametric Spearman test. Receiver operating characteristic curve analysis was performed to calculate the optimum cutoff value. *P* values of less than .05 were considered significant.

RESULTS

BAT with basophils of a single donor coincubated with different sera

As a proof of concept and according to the literature,¹³⁻¹⁸ basophils of 1 randomly selected donor were coincubated with patient sera (*n* = 67, 16 with positive ASST results) and healthy control sera (*n* = 27), and CD63 upregulation was measured. In these donor basophils sera from patients with CU with positive ASST results induced a median CD63 upregulation of $15.7\% \pm 7.4\%$ ($\pm$ SEM) compared with $3.7\% \pm 1.15\%$ in sera from patients with CU with negative ASST results and $1.1\% \pm 0.15\%$ in healthy

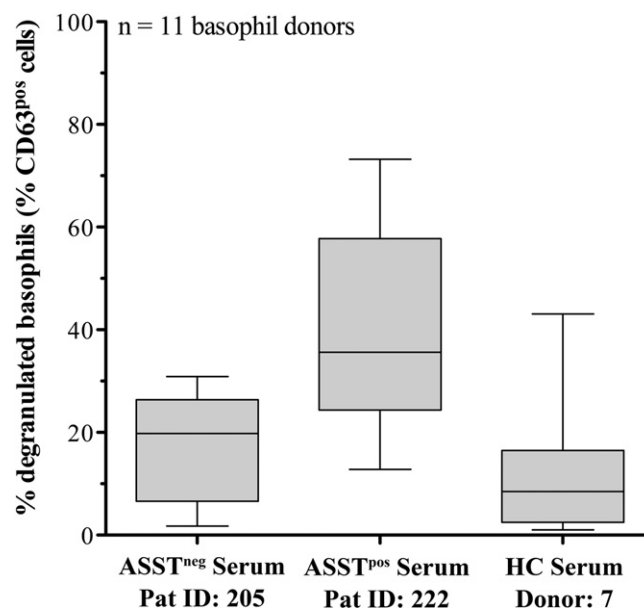


FIG 1. High variability of basophil activation under suboptimal IL-3 conditions. Basophils derived from 11 different healthy donors were coincubated with 2 patient sera (1 with a positive ASST result (ASST^{pos}) or serum from a healthy volunteer under standard IL-3 conditions (1 ng/mL). Data are presented as a percentage of CD63⁺ basophils for each basophil donor. ASST^{neg}, Negative ASST result; ASST^{pos}, Positive ASST result; HC, healthy control.

control sera (see Fig E1 in this article's Online Repository at www.jacionline.org).

Individual basophil response in different basophil donors

Several basophil donors are needed to establish the test for routine diagnostics. Sera of 2 representative patients and 1 healthy control subject were each coincubated with 11 different basophil donors to ensure test reproducibility (Fig 1). We observed a high interindividual variability of BAT results between the different basophil donors with limited correlation to the respective ASST result and, even more disturbing, false-positive results in certain basophil donors if coincubated with healthy control serum.

The mean IgE receptor (FcεRI) density of each basophil donor had no influence on the degree of basophil degranulation (see Fig E2 in this article's Online Repository at www.jacionline.org) and was therefore not responsible for the observed discrepancy.

It is well known that basophil reactivity is enhanced by IL-3²⁸; however, its concentration in the tested sera of patients and basophil donors is low (<5 pg/mL, see Fig E3 in this article's Online Repository at www.jacionline.org). In IL-3 titration experiments we could demonstrate an individual IL-3 sensitivity of each basophil donor. All tests were initially performed in the presence of the standard IL-3 concentration of 1 ng/mL. The standard IL-3 concentration of 1 ng/mL led to false-positive results with healthy control sera in certain basophil donors (Fig 2, A).

Individual basophil priming with IL-3 is crucial

To define an optimal IL-3 concentration for each basophil donor, avoiding false-positivity, we used 10 healthy control sera

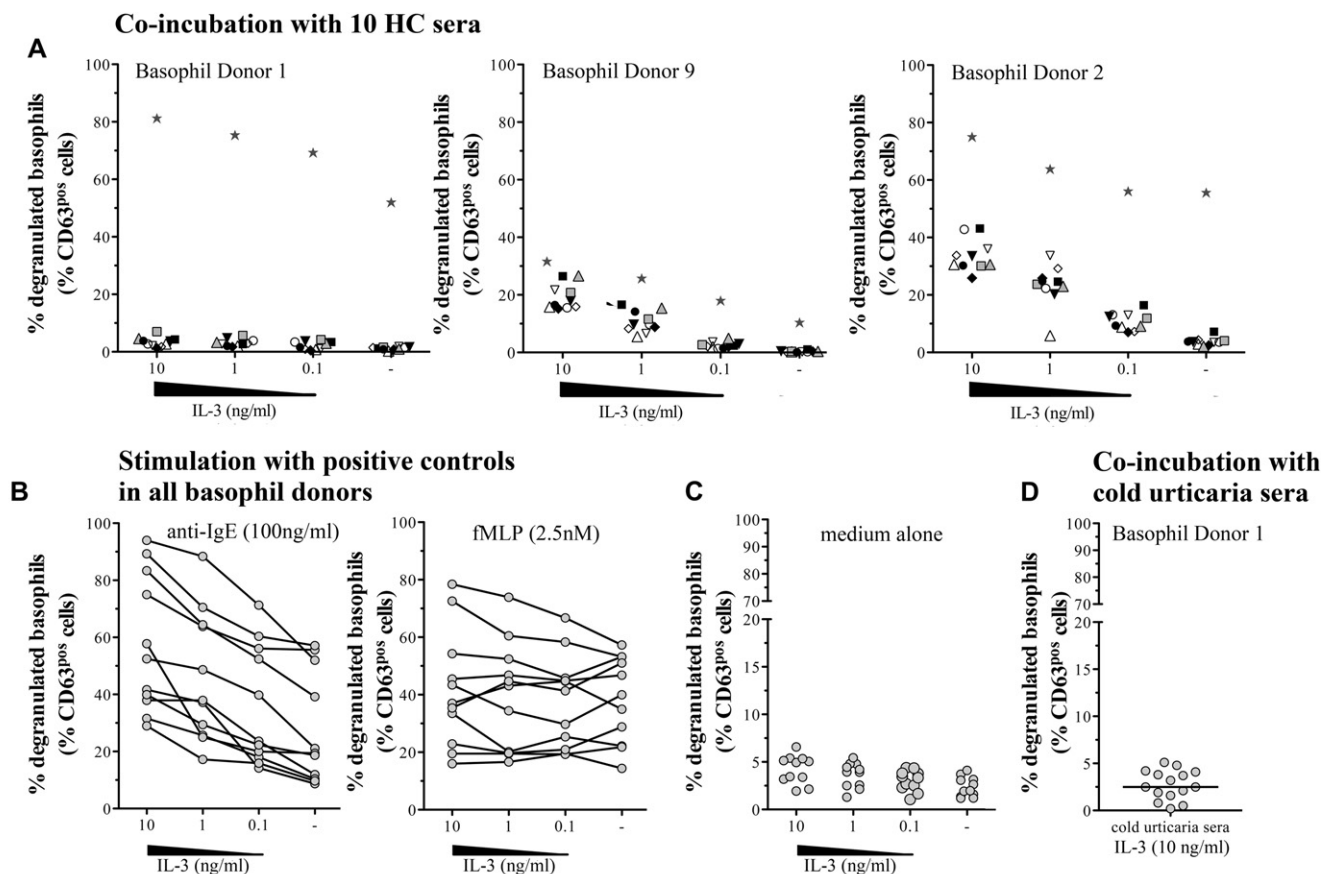


FIG 2. IL-3 priming is crucial for consistent *in vitro* basophil activation. **A**, Co-incubation of basophils with healthy control (HC) sera ($n = 10$) using different IL-3 concentrations (10, 1, and 0.1 ng/mL IL-3 or without IL-3) and assessment of CD63 upregulation. Positive controls (anti-IgE; 100 ng/mL) are indicated as asterisks. Data of 3 representative basophil donors are shown. **B** and **C**, CD63 upregulation under different IL-3 concentrations on stimulation with positive controls (anti-IgE and fMLP) or with medium alone. Data of 11 different basophil donors are shown. **D**, Co-incubation of basophils from donor 1 with sera from a patient with CU ($n = 15$) under optimal IL-3 priming conditions (10 ng/mL).

(Fig 2, A) and IgE-dependent (anti-IgE) and IgE-independent (fMLP) stimuli (Fig 2, B). As expected, anti-IgE elicited IL-3-sensitive basophil degranulation characteristics, whereas IgE-independent stimuli (fMLP) were independent of the IL-3 concentration used. Importantly, there was no unspecific upregulation of CD63 with IL-3 alone (Fig 2, C). Under optimal IL-3 concentrations, neither healthy control sera nor sera of patients with cold urticaria elicited basophil activation ($<5.1\%$; median, $2.5\% \pm 0.4\%$).

If the individual basophil priming with IL-3 was optimized for each basophil donor, testing with the defined patient sera mentioned above resulted in an improved interassay agreement irrespective of the basophil donor used. The results are shown for the patients with negative ASST results (Fig 3, A and B) and the patients with positive ASST results (Fig 3, C and D) separately.

Intraindividual variability of basophils over time

We further investigated the variability of IL-3 sensitivity and basophil degranulation characteristics over time within a given basophil donor. Therefore we stimulated basophils from several donors with sera from healthy control subjects, with sera from

patients with CU, and with unspecific stimuli in July (middle of the grass pollen season) and October (out of the pollen season). There were slight but insignificant fluctuations in basophil reactivity over time, with comparable results at the 2 time points for patient and healthy control sera (Fig 4, Table II, and see Fig E4 in this article's Online Repository at www.jacionline.org). Only on anti-IgE stimulation and restricted to the 2 atopic donors investigated could we detect a statistically significant difference of basophil reactivity over time ($P = .02$).

CD203c as an alternative basophil activation marker

We analyzed CD203c instead of CD63 as an alternative activation marker on donor basophils, as recommended by others.¹⁴ We co-incubated 2 sera of patients with positive ASST results and 4 healthy control sera with basophils from different donors (see Fig E5 in this article's Online Repository at www.jacionline.org). IL-3 could not be included in this approach because IL-3 alone leads to an enhanced expression of CD203c.²⁹ In contrast to the results obtained with CD63, the discrimination between healthy control sera and sera from patients with positive ASST results was no longer possible in certain basophil donors.

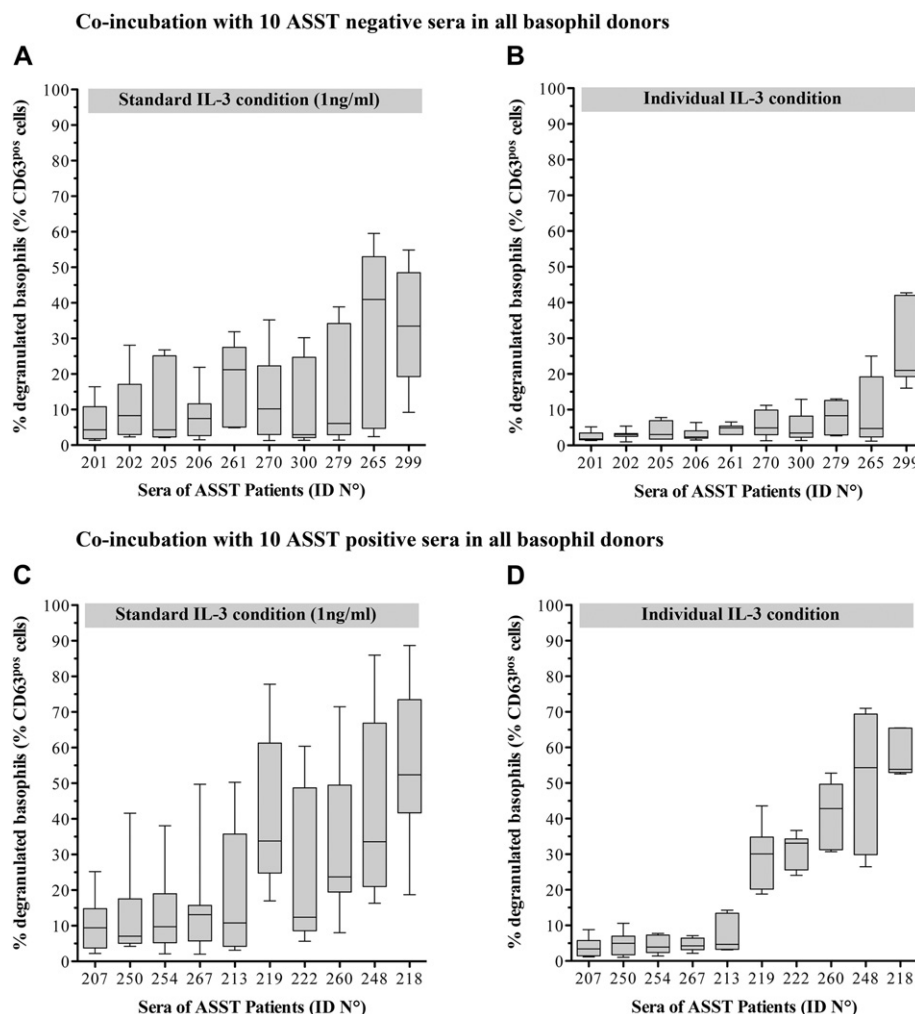


FIG 3. Individual IL-3 priming for consistent and reproducible test results. Coincubation of basophils from different donors ($n = 11$) with sera from patients with negative ASST results under standard IL-3 conditions (1 ng/mL; **A**) or with individual IL-3 priming for each basophil donor (**B**). **C** and **D**, Analogs to Fig 3, **A** and **B**, but with sera of patients with positive ASST results.

Putting the diagnostic system to the test

After the optimization of the BAT for CU diagnosis described above, we analyzed sera of patients with CU ($n = 110$) with ASST results available recruited from our outpatient department in Bern from January 2010 until January 2011. In this evaluation period basophils derived from all basophil donors described above were used according to the donors' availability. The optimal positivity threshold defined by receiver operating characteristic analysis was 7.7% activated basophils after incubation with patient sera. With this cutoff, the test result was positive in 53 of the 110 patients tested. We included a second method for cutoff determination by using the mean plus twice the SD, which resulted in a comparable cutoff point (6.7%). In these basophils from several donors, sera from patients with CU with positive ASST results induced a median upregulation of CD63 of $26.5\% \pm 5.3\%$ ($\pm$ SEM) compared with $8.36\% \pm 1.1\%$ in sera from patients with CU from negative ASST results ($n = 87$) and $3.7\% \pm 0.2\%$ in healthy control sera ($n = 27$). Thus the overall picture is comparable with the data obtained by using only 1 basophil donor. Fig 5 summarizes the BAT results of all cases compared with the respective ASST results.

Insufficient sensitivity of RBL703/21 cells as an alternative to human basophils

Sera were also tested by using the humanized basophil-like cell line RBL 703/21. One hour after stimulation with different dilutions (1:10, 1:100, and 1:1000) of patient or control sera, β -hexosaminidase activity released into the supernatant was determined and expressed as a percentage of total content determined by means of lysis (data not shown). We found no consistent differences between control and patient sera, and positive results were only obtained with the higher, potentially cytotoxic concentration of serum, as described by Nakamura et al.²³ Thus the use of the basophil-like cell line in its current form does not appear to be a viable alternative to the method presented in this article.

DISCUSSION

BAT with well-characterized donor basophils can be used in clinical routine for the detection of autoreactive serum components and might replace the ASST in a reliable and safe way. In contrast to ELISA, which cannot discriminate between autoantibodies of low and high affinity or detect the presence of an

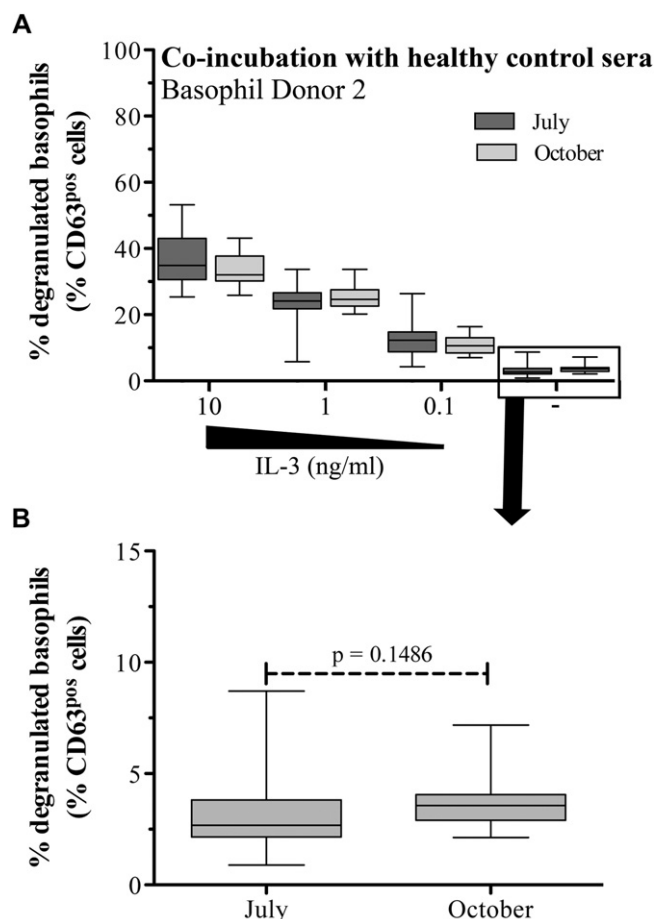


FIG 4. Intraindividual variability over time. **A**, Co-incubation of basophils from an atopic donor with healthy control sera ($n = 10$) under different IL-3 conditions analyzed at 2 different time points. **B**, Test results of this basophil donor at the 2 time points under optimal IL-3 concentrations (framed box) are shown in detail. P values are from 2-tailed Mann-Whitney tests.

autoreactive serum component, the ASST and BAT in particular might allow such a conclusion. However, we substantiated the observation by Najib et al²⁰ that different test conditions result in a low interlaboratory agreement. To reduce this variability by using only a single basophil donor is not feasible in laboratory routine for a disease as prevalent as CU. An alternative would be to use a suitable cell line. Unfortunately, in our hands the humanized RBL703/21 basophil-like cell line used in conjunction with β -hexosaminidase determination did not provide consistent results and did not reliably discriminate positive from control sera. Therefore recruiting and characterizing several basophil donors is currently the only solution for an application in routine diagnostics.

In contrast to other studies,^{13,14,17} we used density-separated and washed leukocytes instead of whole blood and thereby avoided possible interference of donor-derived plasma factors, such as interleukins. Second, we focused on CD63 upregulation. CD203c as an alternative activation marker led to variable activation patterns, rendering the discrimination between patient and healthy control sera impossible in certain basophil donors. Furthermore, those variations could not be normalized by individual IL-3 concentrations (see below) because IL-3 by itself leads to CD203c upregulation. Furthermore, in contrast to CD203c,

CD63 is directly linked to basophil degranulation.³⁰ Therefore CD203c is less suitable for this test purpose (see Fig E5).

The primary challenge was to eliminate the variability in basophil reactivity between the different donors. From our initial experiments, we could observe a high reactivity in certain basophil donors, even reacting with healthy control sera. Therefore we focused our investigation on the definition of basophil donor characteristics. The mean IgE receptor density of the individual basophil donor did not significantly influence the test result (see Fig E2). The mean IgE receptor density alone is not responsible for the magnitude of basophil degranulation. There are several other, mainly intracellular factors playing a role in the basophil activation process.³¹ Therefore we investigated the role of IL-3, which activates the Ras pathway through Janus kinase 2/signal transducer and activator of transcription 5³² and leads to an enhanced basophil degranulation.^{28,33} Some authors claim that IL-3 has to be avoided to improve test specificity.¹⁸ Indeed, this is the case in certain atopic basophil donors, in whom autocrine IL-3 priming might have already enhanced basophil reactivity,³⁴ and this phenomenon was already described for the histamine release test.³⁵ In our patients and basophil donors, IL-3 in sera was hardly detectable (see Fig E3), which is in concordance with earlier results.³⁶ Most cytokines are produced locally and have a predominantly local activity. Local tissue concentration of IL-3, as well as those of other cytokines, can only be estimated and might well be in the high nanogram range.³⁷ In contrast, nonatopic donors require IL-3 to achieve a comparable degranulation and histamine release of basophils.³⁸ To define the optimum test condition for each basophil donor, we adjusted the IL-3 concentrations with respect to avoidance of false-positive results ($<5\%$ CD63 upregulation if coincubated with healthy control sera) and conservation of activation characteristics (with anti-IgE as a positive control) at the same time. By individually priming the basophils with no, low, medium, or high concentrations of IL-3, we were able to normalize the basophil reactivity in all 11 basophil donors (Fig 3, B and D) and to achieve reproducible and comparable test results irrespective of the basophil donor used. Importantly, and as well reported by others,^{28,39} IL-3 alone never led to nonspecific basophil degranulation.

In a given basophil donor no variability of IL-3 sensitivity was observed over time (Fig 4 and see Fig E4), and the CD63 upregulation was reproducible when analyzed at different time points (Table II). Table I summarizes the characteristics of our basophil donors and is used as an internal test validation tool in our laboratory. For each test round, we controlled for the donor-specific activation characteristics. Again, within the same basophil donor, the results with our positive controls (anti-IgE and fMLP) were comparable (Table II). Of note, we observed a slightly higher CD63 upregulation during the pollen season only in the atopic basophil donors, but it reached statistical significance only with anti-IgE stimulation.

We and others⁴⁰ observed a discrepancy between ASST and BAT results in certain patients (Figs 3 and 5). Nevertheless, the BAT results for the single patient were consistent within all 11 basophil donors tested. Probably this observation is due to an inaccuracy of the ASST. In the group with negative ASST results, comedication can influence the skin test results, such as unreported antihistamine or antidepressant intake,⁴¹ as for example patient 299, who was taking trimipramine and paroxetine and consecutively only had positive BAT results. In the group with positive ASST results, mast cell hyperreactivity

TABLE II. Insignificant fluctuations in basophil reactivity: CD63 upregulation on different stimuli analyzed at 2 different time points

Stimuli	Nonatopic basophil donors					
	Donor 1			Donor 7		
	July	October	<i>P</i> value	July	October	<i>P</i> value
HC sera (n = 10)	2.9 ± 0.3	3.3 ± 0.2	.43	1.8 ± 0.2	1.5 ± 0.3	.97
ASST ^{pos} , patient ID 222	42.1 ± 1.8	46.3 ± 3.8	.34	36.6 ± 1.9	36.9 ± 1.3	.39
ASST ^{pos} , patient ID 248	61.3 ± 3.0	69.3 ± 2.9	.09	64.3 ± 6.4	74.3 ± 2.1	.14
ASST ^{neg} , patient ID 205	4.1 ± 0.6	3.2 ± 0.2	.67	2.1 ± 0.6	5.4 ± 1.3	.11
ASST ^{neg} , patient ID 261	6.3 ± 0.7	5.1 ± 0.5	.21	1.9 ± 0.4	2.8 ± 0.5	.11
alGE (100 ng/mL)	88.3 ± 2.1	83.3 ± 2.3	.12	68.3 ± 1.2	66.2 ± 2.4	.81

Stimuli	Atopic basophil donors					
	Donor 2			Donor 3		
	July	October	<i>P</i> value	July	October	<i>P</i> value
HC sera (n = 10)	2.6 ± 0.6	3.5 ± 0.4	.14	3.6 ± 0.5	2.0 ± 0.2	.13
ASST ^{pos} , patient ID 222	31.2 ± 1.4	28.3 ± 1.6	.12	24.2 ± 2.3	21.3 ± 1.7	.37
ASST ^{pos} , patient ID 248	36.3 ± 2.5	33.3 ± 1.6	.14	31.3 ± 5.2	32.2 ± 1.8	.81
ASST ^{neg} , patient ID 205	4.3 ± 0.6	2.8 ± 0.1	.06	8.4 ± 0.4	6.3 ± 0.7	.07
ASST ^{neg} , patient ID 261	2.8 ± 0.3	3.8 ± 0.2	.06	3.4 ± 0.2	4.0 ± 0.6	.06
alGE (100 ng/mL)	51.3 ± 1.2	41.3 ± 1.5	.02	72.3 ± 1.1	65.4 ± 1.2	.02

Data are presented as a percentage of CD63⁺ basophils (medians ± SEMs). Stimulations were performed in triplicates. *P* values are from 2-tailed Mann-Whitney tests. alGE, Anti-IgE antibody; ASST^{neg}, negative ASST result; ASST^{pos}, positive ASST result; HC, healthy control.

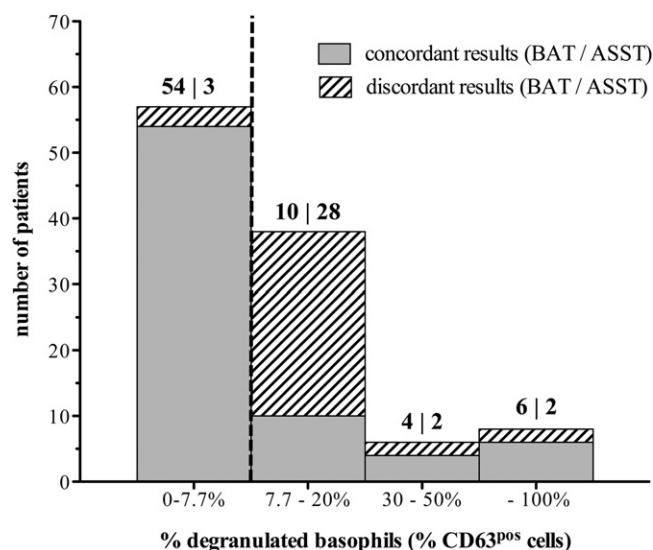


FIG 5. Real life: putting the system to the test. Test results of 110 patients from the clinical routine with direct comparison of BATs and ASSTs. Concordant results of both tests are presented as solid gray bars, and discordant results are presented as striped bars. The cutoff point is set at 7.7%, as defined by receiver operating characteristic analysis.

with pronounced dermographism might exert a relevant influence on the result of intradermal skin testing. Importantly, for obvious reasons, neither positive nor negative control sera can be injected into the patient's skin, and therefore there are no true controls possible in the ASST. Those limitations of the ASST as the diagnostic gold standard hamper the calculation of test accuracy, and the result is likely to be biased on patient selection. However, because we have not observed false-positive

results in healthy control sera, our test protocol seems to be specific and to not overestimate the rate of autoreactive components in patient sera. This was also supported by the absence of basophil activation with sera of patients with cold urticaria. As a typical form of physical urticaria, cold urticaria is thought to be associated with bacterial and viral infections and independent of autoreactive serum components.⁴² Overall, there are several advantages over the ASST: no risk of accidental infection, no influence of antihistamines on the test result, quantifiable results, and a potential in providing treatment monitoring.

The exact nature of the degranulating factor or factors in patient sera remains an open question. Autoantibodies of IgG or IgM isotypes have been postulated.^{43,44} Other probably important factors in the pathogenesis of CU, such as coagulation or complement split products or even cytokines, cannot be safely excluded.⁴⁵⁻⁴⁸ CU, especially in its most severe and refractory form, might have more than 1 pathogenetic factor. Our optimized assay seems to be able to detect most, if not all, of them in a robust and correct way. Hence it might allow for a separate analysis and dissection of the pathogenetic cascade, ultimately leading to the chronic appearance of urticarial lesions. More investigations are needed to dissect the different activation cascades and lead to optimized and individualized treatment.

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Clinical implications: The BAT might be a replacement for the ASST, lacking the risk of accidental infection and the influence of antihistamines on the result. Quantifiable results and the potential use in treatment monitoring are additional advantages.

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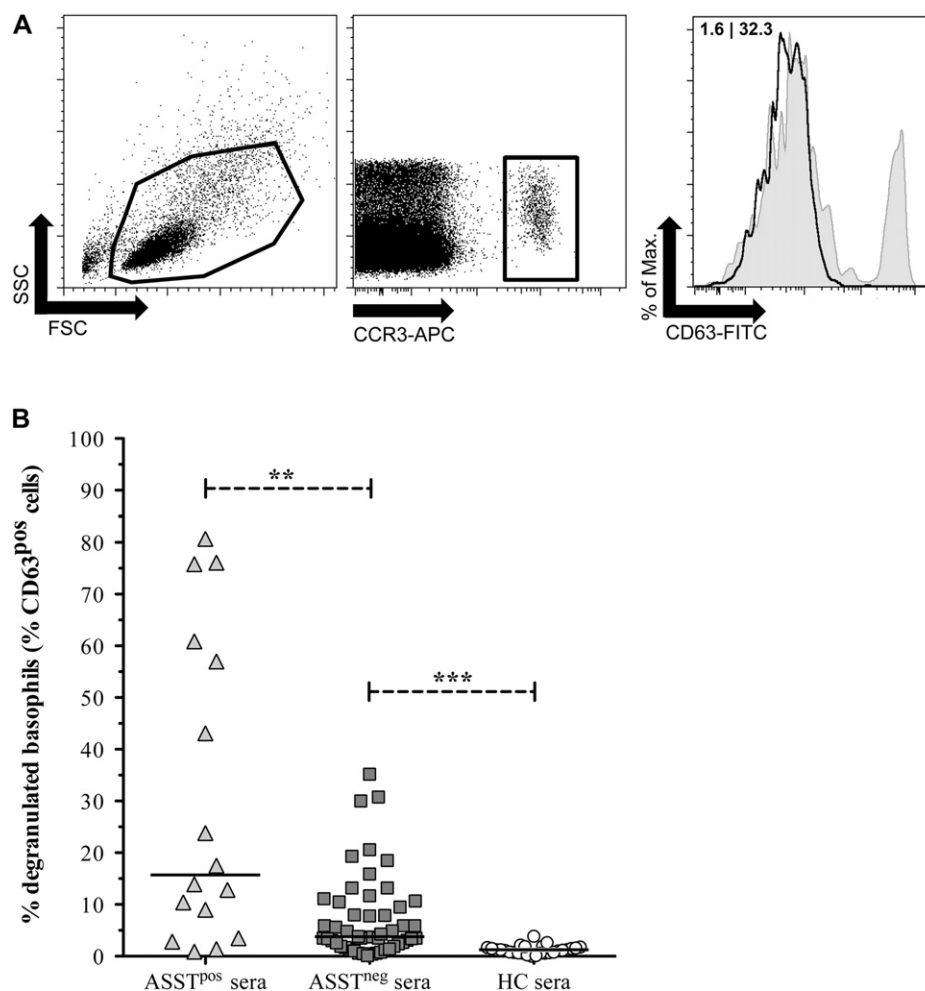


FIG E1. CD63 upregulation on donor basophils after coincubation with sera. **A**, Basophil identification according to CCR3 expression. CD63 upregulation after coincubation with the sera of a patient with a positive ASST result (*shaded histogram*) or a negative control. Numbers in the upper left corner indicate the percentage of CD63 upregulation (negative control/positive ASST result). APC, Allophycocyanin; FITC, fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter. **B**, CD63 upregulation after coincubation of 16 sera from patients with positive ASST results (ASST^{pos}) and 51 patients with negative ASST results (ASST^{neg}) and 27 healthy control (HC) sera expressed with 1 basophil donor (donor 1). Data are presented as the percentage of CD63-expressing cells (medians $\pm$ SEMs). Donor basophils with sera from patients with negative ASST results showed significantly higher activation marker upregulation compared with healthy control sera ($P < .0001$). P values are from 2-tailed Mann-Whitney tests.

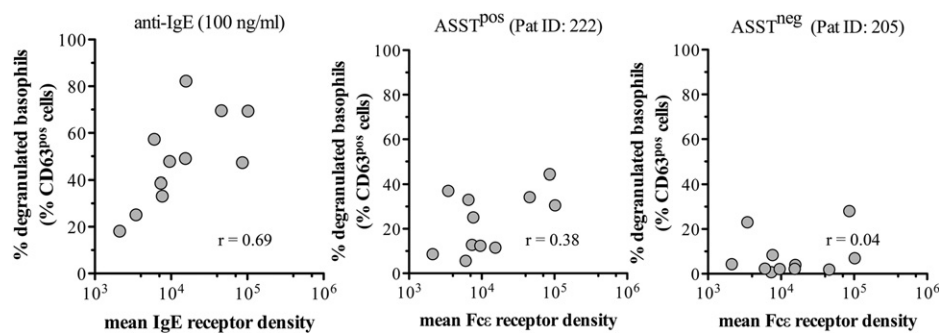


FIG E2. Mean IgE receptor density does not explain donor variability. The mean IgE receptor density of each basophil donor was compared with its corresponding CD63 upregulation after stimulation with anti-IgE, serum from a patient with a positive ASST result (ASST^{pos}; patient ID: 222), and serum from a patient with a negative ASST result (ASST^{neg}; patient ID: 205).

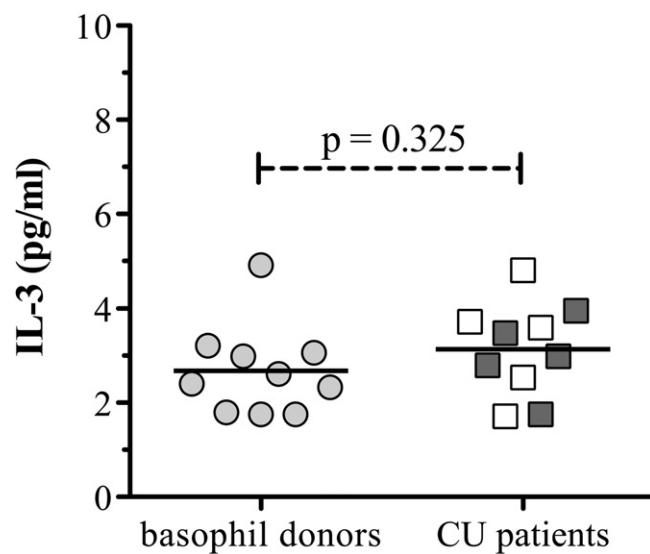


FIG E3. IL-3 detection in sera of patients and healthy control subjects. The mean IL-3 level measured in healthy control subjects was 2.68 ± 0.95 pg/mL, and that measured in patients with CU was 3.13 ± 0.97 pg/mL. There was no statistically significant difference between these 2 groups ($P = .325$). P values are from 2-tailed Mann-Whitney tests.

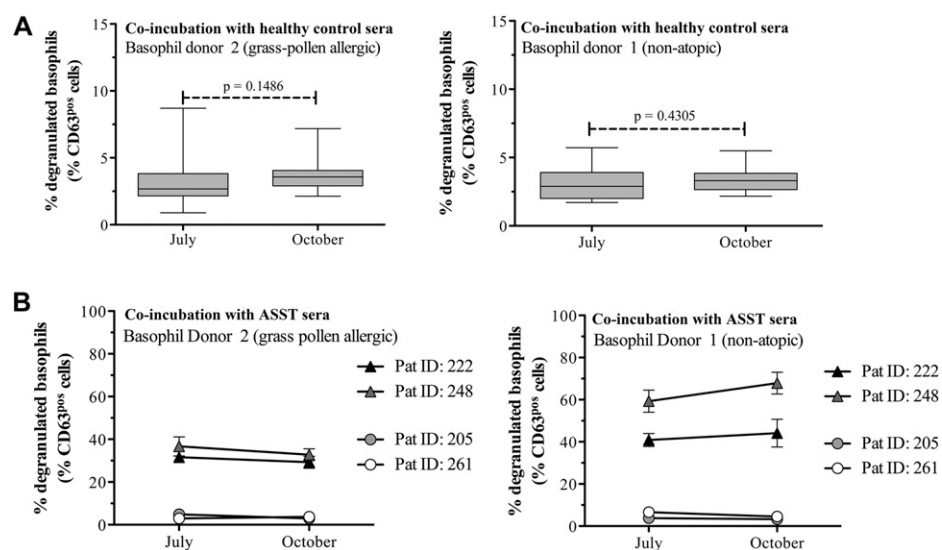


FIG E4. Intraindividual variability over time. **A** and **B**, Co-incubation with healthy control sera ($n = 10$; Fig E4, A) or sera derived from 4 patients with CU (2 with positive ASST results; Fig E4, B). Data of 2 representative basophil donors are shown (donor 2 = atopic donor) under optimal IL-3 conditions for each donor. P values are from 2-tailed Mann-Whitney tests.

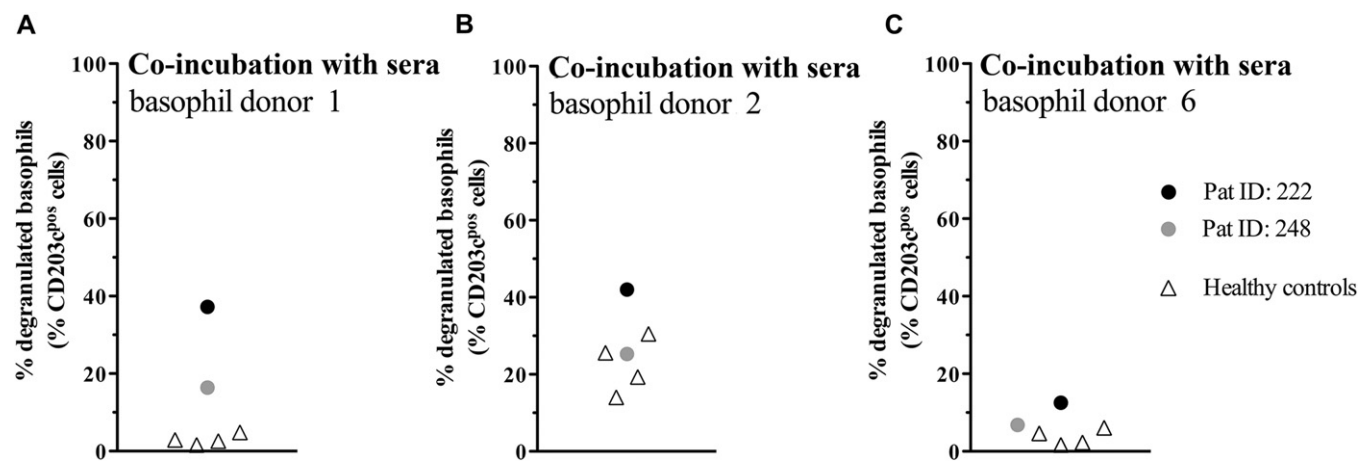


FIG E5. CD203c levels measured after coincubation of basophils with healthy control sera (n = 4) and sera of 2 patients with positive ASST results. Data of 3 representative basophil donors are shown.