

Clinical and Laboratory Investigation

Basophil CD63 expression assay on highly sensitized atopic donor leucocytes—a useful method in chronic autoimmune urticaria

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Summary

Background The autoimmune subclass of chronic idiopathic urticaria (CU) has been characterized by the occurrence of biologically relevant IgG antibodies against the IgE molecule or the α chain of the high-affinity Fc ϵ receptor (Fc ϵ RI α) on basophils and mast cells. These antibodies are usually detected by autologous serum skin testing and confirmed by histamine release studies, immunoblotting, or enzyme-linked immunosorbent assay, but not always.

Objectives To detect autoantibodies to the Fc ϵ RI α in sera of CU patients by a modified serum-induced basophil activation test measured by flow cytometry (FCM) and to evaluate the relationship between the *in vitro* functional test, the autologous serum skin test (ASST), and the serum levels of IgE, eosinophil cationic protein (ECP) and antithyroid antibodies.

Methods Sera of 30 patients with CU and 26 patients with systemic autoimmune diseases (systemic lupus erythematosus, dermatomyositis) were tested for CD63 activation marker expression on basophils by FCM. Leucocytes from two highly sensitized atopic donors (D_{A1}, D_{A2}) and one non-atopic donor (D_{NA}) were incubated with patients' sera and double-labelled with anti-IgE and anti-CD63 antibodies. Subsequently, the percentage of CD63-expressing basophils was determined by using FCM. In all CU patients an ASST was carried out and the serum IgE, and ECP levels and antithyroid antibodies were evaluated.

Results Twelve patients had a positive ASST and 14 patients a positive CD63 expression assay. There was a strong correlation between the ASST and CD63 assay. Sera from patients with systemic autoimmune diseases did not raise positive CD63 expression on basophils. There was a moderate negative correlation between the occurrence of atopic serum markers (IgE, ECP) and the ability of sera to induce CD63 expression on basophil cells of D_{A2} ($P < 0.05$). The female sex was preponderant and antithyroid antibodies were more frequent.

Conclusions Our new technical observation demonstrates that basophils of highly sensitized atopic donors can be successfully used without priming with IL-3 for the *in-vitro* flow cytometric diagnosis of CU. With this investigation the characterization of the autoimmune origin of CU is based on an objective *in vitro* technique.

Key words: autologous serum skin test, basophil activation, CD63 expression, chronic urticaria, flow cytometry

Chronic idiopathic urticaria (CU) is a common skin disorder with unknown aetiology.¹ A group of patients with CU is characterized by circulating autoantibodies against IgE molecule and/or particularly the α chain of high-affinity IgE receptor (Fc ϵ RI α).^{2,3} Their disease represents an autoimmune type of urticaria. The high-affinity IgE receptor is expressed first of all on basophils and mast cells and crosslinking of this receptor with a multivalent antigen leads to activation of these cells as a consequence of an allergic reaction.⁴ In addition, binding circulating autoantibodies in CU sera to unoccupied Fc ϵ RI α can also trigger basophil or mast cell activation as part of an autoimmune reaction.^{2,5} Activation of basophils leads to the release of several mediators, such as histamine, leucotrienes (LT), interleukin (IL)-4 and IL-13, and to an altered expression of surface marker antigens.^{6,7} It has been shown that a CD63 molecule appears *de novo* on the membrane of basophils after activation with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) or anti-IgE antibody.⁸ Some data reflect a correlation between the CD63 expression and the degranulation of basophils.^{8,9} For the diagnosis of allergy, Sainte-Laudy *et al.* have published the application of flow cytometric basophil activation tests based on the measurement of the basophil CD63 expression after allergen challenge.¹⁰ Recently, for the detection of autoimmune chronic urticaria, a patient's sera-induced basophil CD63 expression assay was interpreted,¹¹ although the specificity of this method was not considered to be satisfactory. With this method the donor cells were primed with IL-3. IL-3 is one of several cytokines that are capable of priming and potentiating histamine- and LT-C4 (LTC4)-release and CD63 expression on basophils. We performed a CU serum-induced activation test using basophils of active atopic donors. In atopic individuals the basophil cells exist in higher numbers¹² and in a more activated state^{13,14} compared with non-atopic control subjects. Serum levels of IgE in allergic subjects tend to be elevated, and IgE positively influences the expression of Fc ϵ RI on basophils.¹⁵ Based on these characteristic features of basophils of allergic individuals, we hypothesized that basophils obtained from nontreated atopic patients could be used for CD63 expression assay without *in vitro* priming with IL-3. Our goal was to identify anti-Fc ϵ RI autoantibodies in CU sera by a serum-induced basophil CD63 activation test measured by flow cytometry (FCM) using basophils from highly sensitized atopic donors. The diagnostic reliability of the technique was investigated by comparing the results with the autologous serum skin test

(ASST), the total serum IgE and eosinophil cationic protein (ECP) levels, and the frequency of autoantibodies to thyroid in the patients' sera.

Patients and methods

Patients

Sera of 30 patients (21 females and nine males; mean age 41.73 years, range 13–74; SD 18.26) with chronic idiopathic urticaria were studied. Investigations included patients with recurrent weals of more than 6 weeks' duration, occurring at least four times a week. Patients with clinical evidence of physically induced urticaria, urticarial vasculitis, focal infection or drug and food allergy were excluded. A few patients had taken immunosuppressive drugs, but they stopped the therapy at least 2 months before being tested. None of our patients took doxepin. Antihistamine treatment was stopped at least 48 h before serum samples were collected, and none of the patients was taking corticosteroids or immunosuppressive drugs at the time of venepuncture. All patients underwent ASST, *in vitro* total IgE and ECP determination, antithyroid antibody detection and CD63 expression assay. Normal, non-atopic subjects ($n = 12$), patients with dermatomyositis (DM) ($n = 10$) and patients with systemic lupus erythematosus (SLE) with skin manifestation ($n = 16$) served as controls. Healthy volunteers underwent an ASST which proved negative.

Sera were heat-inactivated for 30 min at 56 °C before the experiments to eliminate IgE.

Donors

Cells of two highly sensitized atopic donors (D_{A1} and D_{A2}) and one non-atopic healthy individual (D_{NA}) were used.

Atopic donors suffered from extrinsic atopic dermatitis and allergic rhinitis and had specific IgE for seasonal inhalant allergens. Their serum IgE levels were the following: 842, 1464 and 24 kU L⁻¹, respectively. They were without antihistamine treatment for at least 1 week before the experiment.

Skin testing with autologous serum

The test was performed following standard methods:^{16,17} fresh autologous serum, sterile saline solution and the positive control were injected intradermally into the volar forearm skin. A positive skin test

was defined as a serum skin test weal that was both red and ≥ 2 mm more in diameter than the saline test at 30 min.

CD63 surface expression assay

Preparation and staining of donor cells. Ethylenediamine tetraacetic acid (EDTA)-anticoagulated whole blood (24 mL) of donors was sedimented on 6% Macrodex (Baxter, München, Germany) for 45 min at 37 °C. The white cell fraction was aspirated and washed twice with cold HEPES-EDTA buffer [20 mmol L⁻¹ N-(2-hydroxyethyl)piperazine-N'-(4-butanesulphonic acid) (HEPES), 130 mmol L⁻¹ sodium chloride (NaCl), 5 mmol L⁻¹ potassium chloride (KCl), 5 mmol L⁻¹ EDTA, 1 g L⁻¹ bovine serum albumin (BSA), pH 7.4] and resuspended in Ca- and Mg-ion containing HEPES buffer to 10⁷ mL⁻¹ (20 mmol L⁻¹ HEPES, 130 mmol L⁻¹ NaCl, 5 mmol L⁻¹ KCl, 2 mmol L⁻¹ calcium chloride, 1 mmol L⁻¹ magnesium chloride, 1 g L⁻¹ BSA, pH 7.4). These cells were then incubated with undiluted sera or sera diluted 1 : 5 and 1 : 10 for 30 min at 37 °C. The reaction was stopped by cooling the tubes on ice. The cells were then stained with goat antihuman IgE-FITC (fluorescein-isothiocyanate) and R-phycoerythrin (PE)-conjugated antihuman CD63 monoclonal antibody (CD63-PE) (both from Caltag Laboratories, Burlingame, CA, U.S.A.) for 60 min at 4 °C. Remaining erythrocytes were lysed with Coulter Immunoprep lysing solution, washed and measured on Becton Dickinson FACS Calibur flow cytometer. Setting an FL1 threshold, cells presenting brightly fluorescent anti-IgE FITC were gated and CD63+ cells were determined on FL2 (Fig. 1). At least 500–1000 basophils were counted.

In order to establish basal values without stimulation, buffer alone was added to another tube. Values above the mean of CD63+ cells + 2 SD induced by 12 negative healthy control sera were considered as positive (cut-off values for different donors were the following: D_{A1}, 7.1%; D_{A2}, 12.3%; D_{NA}, 5.1%).

Stimulation of donor cells with different agents

To investigate the reactivity of donor cells, different stimulating agents were added: anti-IgE monoclonal antibody (0.5 µg mL⁻¹, Clone: Dε2, Immunotech, Marseille, France), CU serum previously tested by immunoblotting for autoantibodies to the high-affinity IgE receptor α-chain, FMLP (10⁻⁵ mol L⁻¹, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) and

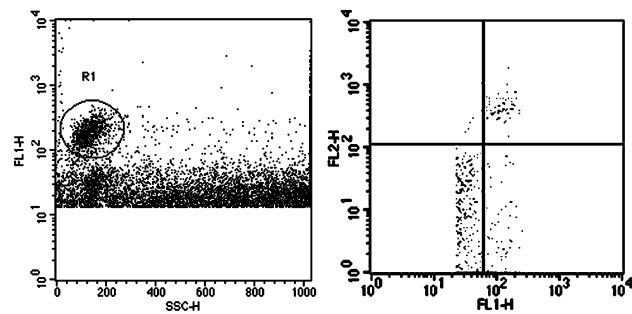


Figure 1. Determination of basophil CD63 surface expression by flow cytometry [(using pulse-height processing (H))]. (a) Acquisition gate around IgE-FITC (fluorescein-isothiocyanate)-positive basophils (gate R1) was defined in a side scatter (SSC) (abscissa) vs. FL1 (ordinate) dot plot. In most experiments the FL1 threshold was further increased to eliminate the irrelevant cell population. (b) Representative increased surface expression of CD63 on atopic donor basophil cells after incubation with a chronic idiopathic urticaria serum IgE-FITC and R-phycoerythrin-conjugated antihuman CD63 monoclonal antibody. Double-positive cells are presented in the upper right quadrant on the FL1-FL2 histogram.

monocyte chemotactic protein-1 (MCP-1, 1 µg L⁻¹, Pharmingen OPTeia kit, San Diego, CA, U.S.A.).

Serological measures by western blotting

FcεRIα-specific autoantibodies were determined as published by Fiebiger *et al.*¹⁸ Serum total IgE was measured on a Behring Nephelometer 100 Analyser (Dade Behring Marburg GmbH, Marburg, Germany) and a value > 100 kU L⁻¹ was considered as elevated. ECP content of sera was determined by the PHARMACIA CAP system (Pharmacia Diagnostics BA, Uppsala, Sweden), and samples exceeding 12 µg L⁻¹ were considered as elevated. Antithyroglobulin and antithyroid peroxidase antibodies (aTG and aTPO, respectively) were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (Hycor Biomedical GmbH, Kassel, Germany).

Statistical analysis

For normally distributed data, Student's *t*-test was used for comparisons between two groups and the Mann-Whitney rank sum test was used if the samples were taken from populations with non-normal distribution and/or unequal variance. Multiple comparisons among the CD63 expressions induced by CU sera at different dilutions on atopic and non-atopic donor cells were performed by using one-way ANOVA after logarithmic transformation of data, after which *post hoc*

comparisons were made with the Student–Newman–Keuls test.

Correlation between CD63 expression and ASST, IgE, ECP, aTG and aTPO levels were calculated using Spearman rank order correlation.

Statistical analysis was performed with SIGMAStat for Windows, Version 2.03 software. A *P*-value of <0.05 was regarded as significant. Results are expressed as mean $\pm$ SD unless otherwise indicated.

Results

Stimulation of donor cells

Antihuman IgE monoclonal antibody and a CU serum positive for anti-Fc ϵ RI α antibody confirmed by immunoblotting (IB+) induced significantly higher CD63 expression on the basophils obtained from two highly sensitized atopic donors (D_{A1} , D_{A2}), compared with the donor cells of one non atopic subject (D_{NA}) (Student's *t*-test, $P < 0.001$) (Fig. 2). There were no characteristic differences among different donors in CD63 expression after stimulation with an immunoblot negative (IB–) CU serum, FMLP or MCP-1 ($n = 5$ experiments).

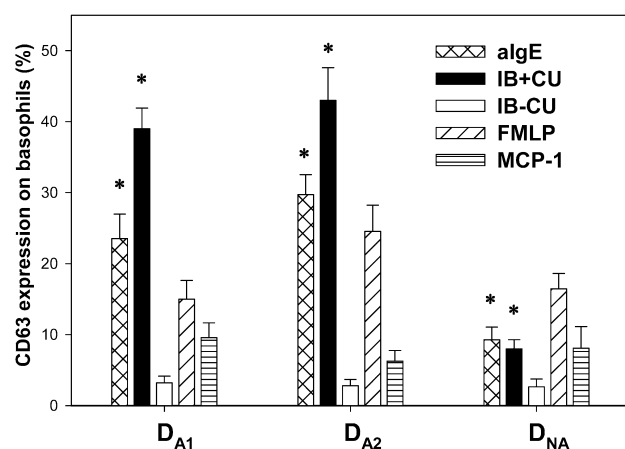


Figure 2. Capacity of different agents to raise CD63 surface expression on donor basophil cells. Mean percentage $\pm$ SD ($n = 5$ experiments) of CD63 on atopic (D_{A1} , D_{A2}) and non-atopic donor (D_{NA}) basophils after stimulation with anti-IgE monoclonal antibody, undiluted IB+ CU and IB– CU sera, FMLP (10^{-5} mol L^{-1}), and MCP-1 ($1 \mu g L^{-1}$). The CD63 expression was significantly higher in D_{A1} and D_{A2} compared with D_{NA} after stimulation with anti-IgE and IB+ CU sera (Student's *t*-test, $*P < 0.001$ for each). There were no characteristic differences in CD63 appearance on different donors after stimulation with IB– CU sera, and with non-IgE receptor stimuli (FMLP and MCP-1). aIgE, Anti-IgE; IB+ CU, immunoblot-positive chronic idiopathic urticaria; IB– CU, immunoblot-negative CU; FMLP, N-formyl-methionyl-leucyl-phenylalanine; MCP-1, monocyte chemoattractant protein-1.

(We used IB+ and IB– CU serum only for testing their capability to stimulate donor cells, because the quantity of the sterile immunoblot tested sera was limited.)

Basophil-activating effect of chronic idiopathic urticaria sera

Heat-inactivated CU sera with either positive or negative ASST results were used in undiluted and diluted (1 : 5 and 1 : 10) forms in basophil activation assay. On different donors, stimulation with ASST+ CU sera showed similar results to the stimulation with IB+ CU sera, namely, that the mean percentage of CD63-expressing cells was significantly higher in atopic donors after stimulation with ASST+ CU sera compared with non-atopic donor cells ($P < 0.001$ for D_{A1} and D_{A2}) (Fig. 3).

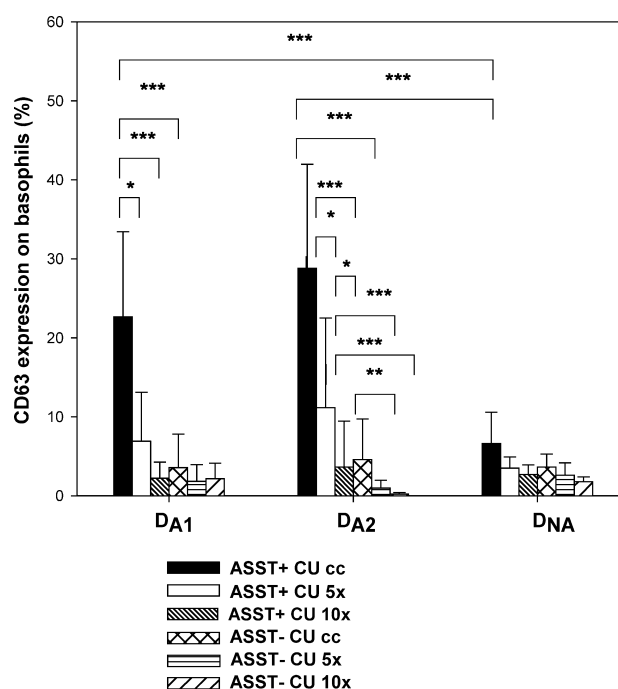


Figure 3. Basophil activation of two atopic donors (D_{A1} , D_{A2}) and one non-atopic donor (D_{NA}) induced by CU sera and expressed as mean percentage of CD63 $\pm$ SD. CU sera that have either positive (ASST+, $n = 12$) or negative (ASST–, $n = 18$) ASST results were used as undiluted and diluted (1 : 5 and 1 : 10) forms. The mean percentage of CD63-expressing cells was significantly higher in atopic donors after stimulation with ASST+ CU sera compared with non-atopic donor cells ($P < 0.001$). On both atopic donor cells incubation with ASST+ CU sera resulted in significantly higher CD63 expression compared with incubation with ASST– CU sera. Activity of ASST+ CU sera was dose-dependent. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. This figure demonstrates one representative experiment. CU, Chronic idiopathic urticaria; ASST, autologous serum skin test

On atopic donor cells incubation with undiluted ASST+ CU ($n = 12$) sera resulted in significantly higher CD63 expression ($P < 0.001$) compared with incubation with ASST- CU ($n = 18$) sera. Activity of ASST+ CU sera was dose-dependent in these experiments (Fig. 3), and significant differences between ASST+ and ASST- sera were also detected in the dilutions 1 : 5 ($P < 0.05$ for D_{A1} and D_{A2}). The difference was significant ($P < 0.05$) only in 10-fold dilutions on D_{A2} (ANOVA test after $\log_{10}$ transformation of data, followed by a *post hoc* Student–Newman–Keuls test). When individual patients were investigated, the results of the CD63 assay showed parallel positivity (coincidence) on cells of the two atopic subjects (Table 1).

Table 1. Distribution of basophil CD63 expression and ASST positivity induced by CU sera

				CD63	
Skin test	P	Sex	aTG/aTPO	D _{A1}	D _{A2}
CD63+ CU sera group					
ASST–	1	F	–/–	+	+
	2	F	–/–	+	+
	3	F	–/–	+	+
ASST+	4	F	–/–	+	+
	5	F	–/–	+	+
	6	F	–/–	+	+
	7	F	–/–	+	+
	8	M	–/–	+	+
	9	F	+/-	+	+
	10	F	+/+	+	+
	11	F	–/–	+	+
	12	F	+/+	+	+
	13	F	+/-	+	+
	14	F	–/–	+	+
CD63– CU sera group					
ASST–	1	F	–/–	–	–
	2	F	–/–	–	–
	3	M	–/–	–	–
	4	F	–/–	–	–
	5	M	–/–	–	–
	6	F	–/–	–	–
	7	F	–/–	–	–
	8	F	–/–	–	–
	9	F	–/–	–	–
	10	M	–/–	–	–
	11	M	–/–	–	–
	12	M	–/–	–	–
	13	M	–/–	–	–
	14	M	–/–	–	–
ASST +	16	M	–/–	–	–

ASST, Autologous serum skin test; CU, chronic idiopathic urticaria; P, patient; F, female; M, male; aTG, antithyroglobulin antibody; aTPO, antithyroid peroxidase antibody.

Comparison of the results of autologous serum skin test and CD63 assay

Fourteen CU patients (14/30; 46%) were considered as positive in the CD63 expression assay on two atopic donor cells and 12 CU patients (12/30; 40%) had positive ASST (Table 1).

In the CD63 assay positive CU patient group, three patients had negative ASST, while one case was found positive for ASST in the CD63– group.

There was a significant correlation between the ASST and the FCM test (Spearman rank correlation, $P < 0.001$ for both atopic donor cells; $r = 0.801$ for D_{A1} and $r = 0.819$ for D_{A2}).

Relationship between the serum IgE, eosinophil cationic protein and antithyroid antibody levels and the CD63-inducing activity of chronic idiopathic urticaria sera

The median values of total IgE levels were significantly lower both in ASST+ (16.5 kU L^{-1}) and CD63+ (19 kU L^{-1}) CU groups compared with ASST– (103 kU L^{-1}) and CD63– (151 kU L^{-1}) groups (Mann–Whitney test, $P < 0.05$ for both) (Fig. 4).

Similarly, the medians of the serum ECP levels were significantly lower in ASST+ ($4 \mu\text{g L}^{-1}$) and CD63+ ($4.5 \mu\text{g L}^{-1}$) CU groups compared with ASST– ($11 \mu\text{g L}^{-1}$) and CD63– ($12 \mu\text{g L}^{-1}$) CU groups (Mann–Whitney test, $P < 0.05$ for both) (Fig. 4).

There was a moderate negative correlation between the occurrence of atopic serum markers (IgE, ECP) of CU serum and the ability of CU sera to induce CD63 expression on atopic donor cells (IgE: $r = -0.465$ for D_{A1} and $r = -0.438$ for D_{A2} ; ECP: $r = -0.435$; $P < 0.05$ for D_{A1} and $r = -0.456$ for D_{A2} and $P < 0.05$ for each, Spearman rank order correlation).

The occurrence of aTG and aTPO was more frequent in the ASST+ (4/12 and 2/12 vs. 0/18 for both autoantibodies in the ASST– group) and CD63+ groups (4/14 and 2/14 vs. 0/16 for both in the CD63– group) (Table 1). We found a moderate correlation between aTPO and CD63 expression on one of the atopic donor cells ($r = 0.409$ and $P < 0.05$ on D_{A2} , Spearman rank order correlation).

Association of CD63-inducing effect of chronic idiopathic urticaria sera with disease characteristic

There was no connection between duration of CU (37.17 ± 29.48 months in ASST+ CU and

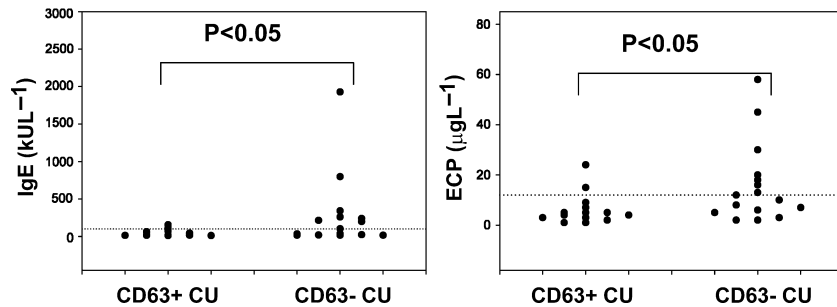


Figure 4. Distribution of serum total IgE and ECP levels in the CD63+ and CD63- CU sera. Both the median of IgE and ECP levels were significantly lower in the sera of the CD63+ CU patient group ($n = 14$) compared with the sera of the CD63- CU patient group ($n = 16$) (Mann-Whitney test; $P < 0.05$). Dashed lines represent the normal values of IgE and ECP, respectively. ECP, Eosinophil cationic protein; CU, chronic idiopathic urticaria

20.11 ± 27.15 in ASST- CU) and serum positivity for CD63 assay. The prevalence of female sex was higher in the CD63+ and ASST+ (autoimmune type) CU patient group.

CD63 expression in different groups of sera

Sera of non-atopic healthy controls ($n = 12$) and sera of patients with two skin-related autoimmune diseases, DM ($n = 10$) and SLE ($n = 18$) with skin manifestations, did not induce CD63 expression on atopic donor cells (D_{A2}) (Fig. 5).

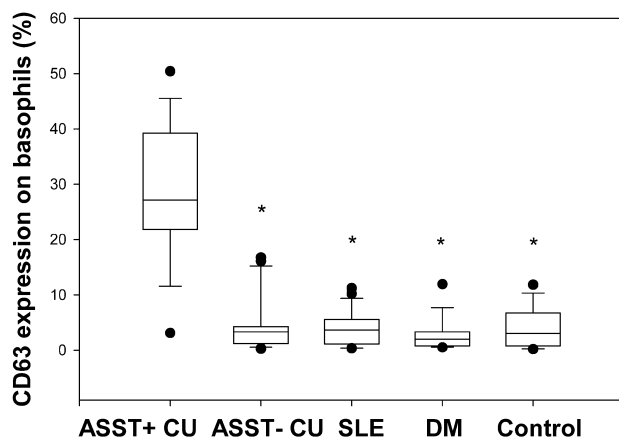


Figure 5. CD63 expression after incubation of atopic donors' basophils (D_{A2}) with heat-inactivated sera obtained from various subjects: CU serum with positive ASST ($n = 12$), CU serum with negative ASST ($n = 18$) and sera of patients with SLE ($n = 16$), DM ($n = 10$) and non-atopic healthy controls ($n = 12$). The median, 25th and 75th percentiles, and minimum and maximum CD63 expressions are presented. Sera of patients with DM, SLE and of healthy controls did not evoke CD63 expression on basophils. The Mann-Whitney test was applied to compare the CD63 expression induced by ASST- CU, SLE, DM and control groups to ASST+ CU sera. * $P < 0.001$ compared with 12 ASST+ CU sera. CU, Chronic idiopathic urticaria; ASST, autologous serum skin test; SLE, systemic lupus erythematosus; DM, dermatomyositis.

Discussion

The CD63 molecule is a tetraspan granule protein that appears *de novo* on the surface of the basophil membrane during activation.⁶ In this study we identified a subgroup of CU patients whose sera seemed to have a capability to induce basophil activation that resulted in the expression of a CD63 molecule.

The concept of using highly atopic donor cells originated from the knowledge that allergic subjects have increased numbers of basophils¹² and enhanced levels of serum IgE, and that the latter was correlated with the number of FcεRI on basophils.¹⁹ Furthermore, some evidence suggests that basophils from highly allergic individuals are in a primed state.²⁰ These basophils have an increased capacity for the release of histamine and other factors^{13,21} and an increased sensitivity for activation through FcεRI or other traditional receptor-dependent mechanisms.²² Among the several cytokines that are capable of potentiating or priming basophils,⁶ IL-3 is widely used in *in vitro* basophil activation assays.²³ Applying basophils from highly sensitized atopic donors avoids the preliminary priming of cells with this cytokine.

To assess the sensitivity of atopic and non-atopic donor basophils for activation via the FcεRIα, we compared their CD63 expression after different stimuli. The activation of atopic basophils through the FcεRIα was significantly higher when specific stimulators were used, e.g. anti-IgE monoclonal antibody or CU sera positive for anti-FcεRIα antibody by immunoblotting compared with non-atopic basophils. Based on these results, we applied highly sensitized atopic donor basophils in further experiments, as CU sera exert their effects through the FcεRIα.

On the other hand, when responsiveness to activation pathways not dependent on IgE receptor were

probed with FMLP and MCP-1, CD63 expression did not differ characteristically on atopic and non-atopic basophils. The chemoattractant oligopeptide FMLP activates the cells through the FMLP receptor that is G-protein-linked and bears structural homologies to other chemokine receptors.²⁴ The MCP-1 is a potent inducer of mediator release from basophils²⁵ as well, and its effect is mediated via the G protein-linked receptor CCR2.²⁶

For the demonstration of autoantibodies in CU sera, Wedi *et al.*¹¹ first published the serum-induced CD63 basophil activation test after IL-3 priming on whole blood. They showed that the serum of a subgroup of CU patients with positive ASST induced CD63 expression on basophils and had histamine-releasing activity.

Compared with histamine release (HR), there were relatively more sera that induced CD63 expression. However, they did not find significant correlation between HR and CD63 expression. Moreover, the activity of CU sera in elevating HR or CD63 expression was characteristic neither for ASST+ CU nor for CU patients in general. Applying whole blood of the atopic donor, Wedi *et al.* demonstrated basophil CD63 expression in 70% of ASST+ CU patients, and in 45% of ASST- patients.¹¹ A positive CD63 expression among atopic and non-atopic control subjects without urticaria was demonstrated also. Our data confirm their observation regarding the capability of ASST+ CU sera in inducing CD63 expression on basophils. In our case, there was a strong correlation between the CD63 expression and the ASST in CU patients. We detected CD63 expression in 91% of ASST+ patients and in 17% of ASST- patients. Our findings are also in accordance with the results of Sabroe *et al.*¹⁶ who found that positive skin test responses to autologous sera were associated with a functional test, the serum-evoked histamine release assay. They found positive skin test responses with histamine-releasing anti-FcεRI autoantibodies, but not with nonhistamine-releasing anti-FcεRI antibodies.

The differences between our results and those of Wedi *et al.*¹¹ are not surprising. We theorize that the higher sensitivity of our assay originates, in one respect, from using dextran-sedimented and washed leucocytes instead of whole blood, thereby avoiding the interference with serum factors such as soluble allergen-specific IgE or IgG²⁷ or serum components that increase activation in controls.²⁸ Moreover, priming with IL-3 can induce IL-4 production in basophils,²⁹ which further increases the number of serum factors in the whole blood system.

In an earlier observation, no biologically active autoantibody against FcεRI was detected in serum from healthy controls by histamine release assays and *in vivo* skin testing,¹ but with the use of ELISA and Western blotting nonhistamine-releasing, immunoreactive anti-FcεRI autoantibodies have been demonstrated in other non-urticarial autoimmune diseases, including bullous pemphigoid, pemphigus vulgaris, DM and SLE.⁵ In our experiments neither the sera of non-atopic healthy controls nor sera obtained from patients with the two systemic autoimmune diseases, DM and SLE, evoked positive CD63 expression. Wedi *et al.*¹¹ found increased CD63 expression in 30% of non-atopic controls and 20% of atopic non-urticarial subjects. We believe that our system has a higher specificity, probably because it avoids using IL-3 and uses washed leucocytes instead of whole blood.

In the present study we examined the relationship between the level of serum total IgE and ECP, and the ability of CU sera to induce basophil surface CD63 expression. We obtained a moderate inverse correlation between the serum total IgE level and serum-induced CD63 expression, and the median level of IgE was significantly lower in the CD63 and ASST+ group of CU sera. Our results are consistent with the findings³⁰ that CU patients with functional autoantibodies against FcεRI, as identified by serum-evoked histamine release, have significantly lower serum IgE levels than patients without antibodies. In the method of Wedi *et al.*,¹¹ who applied CU serum-induced CD63 expression on whole blood, the tendency was similar, but the negative correlation between IgE level and capacity for CD63 upregulation did not reach statistical significance. This could be due to the above detailed differences between the methods. ECP is a cytotoxic molecule released from the secretory granules of activated eosinophils³¹ and increased levels have been detected in the sera of patients with allergic diseases.³² In our method CU patients identified as positive with CD63 assay presented with significantly decreased levels of ECP compared with the CD63- group. Similarly, with total IgE, moderate negative correlation was also found between the CD63 expression and median levels of serum ECP. These results are well in agreement with the findings of Sabroe *et al.*,³³ who detected significantly more activated eosinophil cells in weals of biopsies from CU patients without anti-FcεRI autoantibodies than from patients with autoantibodies.³⁰ However, our data do not allow us to reach any major conclusions concerning the role of serum IgE and ECP in the pathomechanism of the

subset of CU patients whose sera induce CD63 expression on basophils.

The autoimmune form of chronic urticaria is characterized by a higher percentage of autoantibodies against thyroid antigens,³⁴ although variable occurrences have been reported.^{35,36} We found a higher prevalence of circulating autoantibodies to thyroid in the group with CU sera-inducing positive CD63 expression (4/14, 29%) and ASST (4/12, 33%), while in the ASST and CD63– group we did not detect any such antibodies. This finding is similar to those published by other authors.^{37,38} Nevertheless, a correlation between aTPO and CD63 expression was also demonstrated in our experiments. There is no evidence to support the pathogenic role of antithyroid antibodies in CU, probably because they are connected in parallel to autoimmune events. Our attempt to classify the autoimmune subset of patients on the basis of our functional test was bolstered by the presence of antithyroid antibodies.

Generally, women have a greater susceptibility to autoimmune disorders.^{39,40} In a large CU population study,³⁸ patients were mostly women and positive ASST results were found predominantly in them. Consequently, we detected a higher percentage of females, 71% (10/14) in ASST+ and 93% (13/14) in CD63+ patient groups, compared with the patients of either ASST– [56% (10/18)] or CD63– groups [44% (7/16)].

Our results indicate that cells obtained from highly sensitized atopic patients are good targets for demonstrating functional autoantibodies against high-affinity IgE receptors. As with some literature-based predictions,^{14,15,19} we were able to demonstrate that these cells could be used without IL-3 activation. The elimination of the *in-vitro* activation process results in a stable, reproducible method. The strong correlation between the positive CD63 assay and the ASST indicates that the CD63 marker expression assay is a useful functional method for the determination of autoantibodies against high-affinity IgE receptor. Serological data, such as those on the detection of IgE, ECP levels and autoantibodies to thyroid, support our method based on the FCM experiments, which identify indirectly the group of patients with CU of possible autoimmune origin.

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