Original article

Serum-induced basophil CD63 expression by means of a tricolour flow cytometric method for the in vitro diagnosis of chronic urticaria

Chronic idiopathic urticaria (CIU), defined as the occurrence of daily pruritic wheals for at least 6 weeks, is a common skin disorder in which pathophysiological mechanism often remains unknown, despite extensive laboratory investigations (1, 2). A subset of patients with CIU may have an autoimmune basis for their condition, suggested by a positive skin test response to autologous serum (ASST) (3, 4). It is now well accepted that up to half of patients with CIU (35–55%) have functional circulating IgG autoantibodies directed against the α-chain of the high-affinity IgE receptor (FcεRIα) or, less commonly, against IgE itself, which are able to induce in vitro histamine release from basophils and mast cells via a direct cross-linking of adjacent IgE or IgE receptors (5–7). Although several attempts have been made to develop specific and sensitive in vitro tests for the detection of anti-FcεRIα autoantibodies in sera of patients with autoimmune chronic urticaria (AICU), no routine laboratory test is yet available for in vitro diagnosis of AICU and time-consuming functional assays (histamine release from healthy donor basophils and mast cells) are required (8–11). As regard, basophil activation can induce the release of several soluble mediators (histamine, leucotrienes, cytokines and chemokines) as well as the expression of membrane bound activation markers (12, 13). CD63, a surface molecule (glycoprotein-53) member of the tetraspan family, is the only marker expressed de novo with a high density on activated basophils (14) and its up-regulation have been shown to well correlate with histamine release from basophils after stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP), anti-IgE antibody or allergens (15–17). Thus, CD63 expression has been proposed as a reliable tool to monitor basophil activation and several literature data account for the application of flow cytometric analysis of
in vitro activated basophils assessed by CD63 in allergy diagnosis (16, 17). Recently, two independent studies performed by Wedi et al. (18) and Gyimesi et al. (19) investigated a flow cytomtric basophil activation test for the detection of AICU. The authors, using different methods of basophil isolation and activation, demonstrated that sera from patients with CU and positive ASST are able to induce in vitro CD63 expression on atopic donor basophils identified by flow cytometry (FCM) using monoclonal antibody (MoAb) directed against IgE.

In order to confirm the relationship between the in vivo response to ASST and in vitro serum-induced basophil CD63 expression we performed a study in a large series of CU patients using a novel tricolour flow cytomtric method based on CD63, CD123 and HLA-DR expression. It is well documented that, by means of FCM, peripheral blood basophils can be selectively identified as a distinctive single cell population using MoAbs directed against the alpha chain of interleukin-3 receptor (anti-IL-3Rα/CD123), staining predominantly basophils and monocyte-derived dendritic cells (20), and the HLA-DR antigen which is virtually absent on basophils (21–23). Thus this method allows to accurately quantify CD63+ activated cells on a selected population of CD123+ HLA-DR- basophils from whole blood. Furthermore we statistically established a cut-off able to discriminate ASST+ CU from ASST− CU patients giving the best values of sensitivity and specificity by means of a receiving operating characteristic (ROC) curve. Three patients with severe unremitting CU and a positive ASST underwent cyclosporine A (CyA) treatment and modulation of serum-induced CD63 expression was evaluated in response to therapy.

Patients and methods

Patients, sera and donors

We enrolled for the study 64 patients (46 females and 18 males ageing from 32 to 54 years) with diagnosis of CU defined as recurrent whealing occurring at least twice a week for more than 6 weeks. Physically or drug-induced urticaria, urticaria vasculitis and allergic (IgE-mediated) urticaria were considered as criteria for exclusion. When assumed, antihistamine treatment was stopped at least 1 week before the study and none of the patients was taking any other drug when serum samples were collected for ASST and CD63 expression assay.

The severity of urticaria was estimated according to the body surface area (BSA) affected by wheals at the assessment time and scored as follows: 0 = no wheals; 1 = <20% BSA; 2 = from 20% to 50% BSA; 3 = >50% BSA. The severity of itching was graded from 0 to 3, where 0 = none, 1 = mild, 2 = moderate, 3 = severe. The wheal and itch scores were added together, so that the maximum urticaria activity score was 6 (Tables 1 and 2).

Sera from 10 non-atopic healthy subjects served as control samples. All sera, stored at −20°C until investigations, were thawed to room temperature (RT) and heat inactivated (30 min at 56°C) before their use to eliminate IgE and inactive complement. For in vitro serum-induced basophil activation test one non-atopic healthy donor (DNA; circulating IgE 32 kU/l) and one atopic donor (DA) suffering from allergic rhinitis (serum specific IgE for seasonal allergens: 920 kU/l) were selected and they were both out of any antihistamine treatment when heparinized whole blood was collected.

Autologous serum skin test

Upon informed consent each subject enrolled in the study underwent an intradermal test with 0.05 ml of fresh autologous serum performed as described by Sabroe et al.; autologous serum, sterile saline solution (0.9% W/v NaCl) as negative control and histamine (1 mg/ml) as positive control were injected into the volar forearm healthy skin and the ASST reactions were scored as positive when a red serum-induced wheal with a diameter of >1.5 mm than the saline-induced response at 30 min could be observed. Twenty-two of 64 CU patients (34.3%)...
were strongly positive on ASST (ASST + CU) and 42 of 64 (65.6%) were negative (ASST−CU). ASST performed on the healthy control group (n = 10) was negative in all subjects.

Flow-cytometric basophil CD63 serum-induced activation assay

Flow-cytometric quantitative expression of CD63 on activated basophils was measured on D_NA and D_A cells. Briefly, 100 µl of heparinized whole blood was directly incubated at 37°C on water bath for 30 min with 100 µl of heat-inactivated undiluted sera of CU patients and controls, making a final volume of 200 µl. Degranulation was stopped by adding 10 µl of 20 mM EDTA at RT for 5 min and cells then stained with 20 µl of CD63-FITC/CD123-PE/Anti-HLA-DR-PerCP antibody cocktail (BD FastImmuneTM CD63/CD123/Anti–HLA-DR; Becton-Dickinson, San Jose, CA) and incubated for 15 min at RT. Finally, whole blood probes were lysed (1× BD FACS lysing solution; Becton-Dickinson), washed and resuspended in 300 µl of 0.5% paraformaldehyde to be measured on a FACScan flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA) within 2 h. Setting a threshold on FL2 (red fluorescence) to eliminate most of CD123 negative cells, at least 500 CD123+ cells per probe were acquired and basophils were then identified as low side scatter (SSC), CD123+ and HLA-DR- cells with a double gating strategy. CD63 basophil surface expression measured on FL1 (green fluorescence) was quantified on the gated CD123+HLA-DR- cells (Fig. 1). The addition of wash buffer alone and fMLP (1 µmol/l) to distinct tubes served to establish respectively baseline values and positive control of CD63 expression on donors' basophils in three consecutive experiments (MV ± SD of CD63+ cells at baseline was 7.3% ± 1.2% for D_NA and 6.4 ± 0.2 for D_A; MV ± SD of CD63+ cells after fMLP stimulation was 23% ± 3% for D_NA and 45% ± 2.2 for D_A, P < 0.001).

Three of 22 ASST+ CU patients with severe relapsing disease, unresponsive to antihistamines, were selected for short-term CyA treatment (Sandimmun Neoral® at 3 mg/kg/day for 4 weeks, 2 mg/kg/day for the next 2 weeks and 1 mg/kg/day for the remaining 2 weeks). Serum samples from these patients were collected to perform ASST and serum-induced CD63 activation assay before, after 4 weeks and at the end of the therapy (8 weeks).

Statistical analysis

Statistical analysis was performed by a software package (SigmaStat 2.03 for Windows; SPSS Inc, Chicago, IL, USA) and Student’s t-test or Mann–Whitney rank sum test were alternatively used to determine significant differences between groups, depending on the type of distribution. In ASST+ CU patients treated with CyA, a Paired t-test served to establish the modulation of serum induced CD63 expression during treatment. A ROC curve analysis was used to estimate the cut-off of CD63 serum-induced assay able to discriminate ASST+ CU from ASST−CU patients, regarding optimal values of sensitivity and specificity. The area under the ROC curves (AUC) was calculated by Analyse-it 1.69 program (Analyse-it software, Ltd, Leeds, UK) to evaluate the discriminatory value of the test. Unless otherwise stated, all data are shown as mean values ± standard deviation (MV ± SD) and a probability (P) value of <0.05 was considered to be statistically significant.
Results

CD63 surface expression on donor basophils in response to sera

As shown in Fig. 2, when heat-inactivated, undiluted ASST+ CU sera \((n = 22)\) were used to stimulate donors whole blood, a significant induction of CD63 on basophils from both D\(_A\) and D\(_{NA}\) with respect to ASST− CU \((n = 42)\) and normal sera \((n = 10)\) was documented by FCM \((P < 0.001)\). However, ASST+ CU sera activity was about 1.8-fold higher in D\(_A\) than in D\(_{NA}\), being the mean percentage of CD63+ basophils significantly higher in D\(_A\) when compared with D\(_{NA}\) \((33.3 ± 14 \text{ vs } 18.9 ± 7; P < 0.001)\).

Diagnostic performance of the serum-induced CD63 expression assay

A ROC curve was derived to establish the ability of the serum-induced CD63 expression assay to discriminate ASST+ CU and ASST− CU patients when whole blood from D\(_A\) was used. The AUC for the assay was 0.971, that means an overall probability (97.1%) that ASST+ CU patients can be correctly identified by FCM measurement of CD63 expression \((P < 0.0001)\). By analysing the ROC curve, the cut-off value giving the best sensitivity and specificity (95% and 91% respectively) was found to be 15% (Fig. 3).

Modification of serum-induced CD63 expression during CyA therapy

In three ASST+ CU patients treated with oral administration of CyA serum samples were collected before (T0), after 4 weeks (T1) and at the end of therapy (T2) and evaluated for CD63 induction on D\(_A\) whole blood. When compared with T0, a significant reduction of mean percentage of CD63+ basophils was detected at T1 \((57 ± 8.5\% \text{ vs } 14.6 ± 6.4; P = 0.02)\), further decreasing at T2 below the calculated cut-off \((9.3 ± 2\%; P < 0.01)\) (Fig. 4). All treated patients showed an early clinical response (2 weeks) in terms of itch and wheals reduction, and a complete clearing of skin lesions was obtained at 4 weeks, still maintaining when CyA was tapered at lower dosage for further 4 weeks. The ASST performed at T1 and at the end of CyA therapy (T2) was negative in all patients.

Discussion

Involvement of functional autoantibodies against the high-affinity IgE receptor (FceRI) of dermal mast cells

![Figure 2](image1.png)

Figure 2. ASST+ CU sera are able to induce CD63 expression on basophils of both D\(_{NA}\) and D\(_A\) with respect to ASST− CU and healthy donors sera. ASST+ CU sera activity, however, is about 1.8-fold higher \((P < 0.001)\) when evaluated on D\(_A\) basophils when compared with the activity on D\(_{NA}\). Data are reported as mean values ±SD. ASST, autologous serum skin test, CU, chronic urticaria, D\(_{NA}\), nonatopic donor, D\(_A\), atopic donor.

![Figure 3](image2.png)

Figure 3. Diagnostic performance of the serum-induced CD63 expression assay was established by a receiver operating characteristic (ROC) curve stratifying chronic urticaria (CU) patients according to the autologous serum skin test (ASST) response; at the calculated cut-off of 15%, the assay provided the best discrimination between ASST+ and ASST− CU patients \((P < 0.0001)\) regarding optimal values of sensitivity (95%) and specificity (91%). In the table is reported the relative number of ASST+ and ASST− CU patients whose serum activity was above or below the cut-off value of CD63 expression.

<table>
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<tr>
<th>ASST CU</th>
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Our results demonstrated that a two-colour flow cytometric method using anti-IgE and anti-CD63 MoAbs. On the basis of these interesting data, we believe that the improved diagnostic performance may be due especially to the new tricolour flow cytometric method used in our study which allows an high basophil identification, as its expression is less variable through staining variations when compared with surface IgE and is independent of the allergy status of the donor (20, 23). CD123, costitutively expressed at high density on activated basophils and mirrors mediators release (15–17). Two previous studies by Wedi et al. (18) and Gyimesi et al. (19) based on different laboratory approaches demonstrated that sera from a subset of CU patients with positive ASST are able to induce CD63 expression on atopic donors basophil when identified with a two-colour flow cytometric method using anti-IgE and anti-CD63 MoAbs. On the basis of these interesting data, the goal of our study was to standardize the CU serum-induced CD63 expression assay using dextran-sedimented washed leucocytes from highly sensitized atopic donors, thus avoiding preliminary priming with IL-3. With this method they reported higher values of sensitivity and specificity in respect to Wedi et al., documenting CD63 expression in 91% of ASST+ CU patients and in 17% of ASST− CU patients, with no induction of CD63 in both healthy and disease control groups (19). On one hand, our results confirm, in agreement with Gyimesi et al., that ASST+ CU sera are able to significantly induce CD63 expression on basophils, in contrast to ASST− CU and healthy donors sera. On the other hand, using a model consisting of whole blood without IL-3 pretreatment and a different strategy of basophil identification, we statistically found values of sensitivity and specificity of the serum-induced CD63 assay higher than those observed in the previous studies. We believe that the improved diagnostic performance may be due especially to the new tricolour flow cytometric method used in our study which allows an high basophil identification, as its expression is less variable through staining variations when compared with surface IgE and is independent of the allergy status of the donor (20, 23). CD123, costitutively expressed at high density on activated basophils and mirrors mediators release (15–17). Two previous studies by Wedi et al. (18) and Gyimesi et al. (19) based on different laboratory approaches demonstrated that sera from a subset of CU patients with positive ASST are able to induce CD63 expression on atopic donors basophil when identified with a two-colour flow cytometric method using anti-IgE and anti-CD63 MoAbs. On the basis of these interesting data, the goal of our study was to standardize the CU serum-induced CD63 expression assay using dextran-sedimented washed leucocytes from highly sensitized atopic donors, thus avoiding preliminary priming with IL-3. With this method they reported higher values of sensitivity and specificity in respect to Wedi et al., documenting CD63 expression in 91% of ASST+ CU patients and in 17% of ASST− CU patients, with no induction of CD63 in both healthy and disease control groups (19). On one hand, our results confirm, in agreement with Gyimesi et al., that ASST+ CU sera are able to significantly induce CD63 expression on basophils, in contrast to ASST− CU and healthy donors sera. On the other hand, using a model consisting of whole blood without IL-3 pretreatment and a different strategy of basophil identification, we statistically found values of sensitivity and specificity of the serum-induced CD63 assay higher than those observed in the previous studies.
Taking in mind the crucial need for inter-laboratory standardization in clinical decision-making, we suggest that the easy recognition of basophils in whole blood and the reliable assessment of their activation by means of this tricolour FCM protocol could be the most useful tool for in vitro identification of a subset of patients with CU of possible autoimmune origin in which a third line therapy with immunomodulatory drugs could be contemplated (1, 2, 4). As regard, cyclosporine has been successfully employed in randomized controlled trials involving severe, unremitting AICU (25, 26) and its effectiveness may be attributed to the ability to inhibit basophil and mast cell degranulation as well as T-cell dependent antibody formation by B cells (25, 27–29). In our study we provide the first evidence that, in severe ASST+ CU patients who underwent short-term CyA treatment, serum-induced CD63 expression is significantly reduced during therapy, in accordance to clinical remission and ASST response, suggesting that the assay may also become a promising tool for monitoring the effectiveness of immunomodulatory drugs in AICU. Recently, the presence of a histamine-releasing factor specific for mast cells and not active on basophils, other than an anti-FcεRI or anti-IgE autoantibody, has been documented by Sabroe et al. (6) in a small subset of CU patients with positive ASST (about 9%) and, in these cases, a negative serum-induced basophil activation test result should be expected. Furthermore, although many functional and structural similarities between basophils and mast cells exist, a FCM detection system using mast cell lines and/or chimeric cell lines expressing the human FcεRI may be validated in the future for a larger screening of CU sera with both basophil and mast cells activating properties.

References


In vitro diagnosis of chronic urticaria