

Original article

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

Background: Functional autoantibodies against the α -chain of the high-affinity IgE receptor (Fc ϵ RI α) identify a subset of patients with chronic urticaria (CU) due to autoreactivity, as assessed by an *in vivo* positive response to autologous serum skin test (ASST). We performed a study to standardize the serum-induced basophil activation assay by flow cytometry (FCM) using a new tricolour method, assessing the diagnostic performance of this test in discriminating between ASST+ and ASST- CU patients.

Methods: Sera of 64 CU patients (22 ASST+ CU and 42 ASST- CU) and 10 healthy subjects were tested for their ability to induce basophil CD63 expression when incubated with whole blood of both atopic (D_A) and non-atopic donors (D_{NA}). Using a triple-labelled strategy with anti-CD123, anti-HLA-DR and anti-CD63 antibodies, CD63+ basophils were identified on a selected population of CD123+ HLA-DR- cells. In 3 ASST+ CU patients who underwent cyclosporine therapy, the assay was performed before and after treatment.

Results: The ASST+ CU sera resulted in a significant higher induction of basophil CD63 expression compared with ASST- CU and healthy donors sera; when whole blood from D_A was used, sensitivity and specificity of the assay were 95.5% and 90.5% respectively. ASST+ CU serum activity was significantly decreased during cyclosporine A treatment, in parallel with clinical remission.

Conclusions: Chronic urticaria serum-induced CD63 expression assay performed on D_A whole blood by means of our tricolour FCM method could be the most useful tool for identification of a subset of patients with autoimmune CU and may become a promising tool also for monitoring treatment efficacy.

**A. Frezzolini¹, A. Provini², P. Teofoli²,
D. Pomponi¹, O. De Pittà¹**

¹Laboratory of Immunology and Allergology;

²Division of Dermatology, Istituto Dermatologico dell'Immacolata, IDI-IRCCS, Rome, Italy

Key words: autologous serum skin test; basophils; CD63; chronic urticaria; cyclosporine.

Alessandra Frezzolini
Laboratory of Immunology and Allergology
Istituto Dermatologico dell'Immacolata, IDI-IRCCS
Via dei Monti di Creta, 104
I-00167 Rome
Italy

Accepted for publication 13 November 2005

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 Chronic urticaria (CU) may have an autoimmune basis for their condition, suggested by a positive skin test response to intradermal injection of autologous serum [autologous serum skin test (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 (Fc ϵ RI α) 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-Fc ϵ RI α 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 cytometric 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 cytometric 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-3R α /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 (D_{NA};

Table 1. Clinical activity scores assessed in ASST– CU patients (n = 42)

Sex	Urticaria duration (months)	Wheal score	Itch score	Urticaria activity score (wheal + itch)
M	9	1	2	3
M	12	1	2	3
F	4	2	3	5
M	5	2	2	4
F	12	2	2	4
M	9	2	1	3
F	8	2	1	3
M	25	1	2	3
F	33	1	0	1
M	12	2	3	5
F	2	1	2	3
F	2	2	2	4
M	1	1	1	2
F	6	3	3	6
M	14	3	2	5
F	10	2	3	5
F	9	2	3	5
F	9	1	2	3
F	12	1	1	2
F	4	3	2	5
F	1	2	0	2
M	3	1	1	2
F	4	3	1	4
F	4	1	0	1
F	7	1	2	3
M	12	1	1	2
F	20	2	2	4
F	2	2	1	3
F	2	1	2	3
F	14	2	1	3
F	2	2	2	4
F	5	1	3	4
M	12	1	3	4
F	14	1	2	3
F	3	3	3	6
F	7	1	0	1
F	32	2	2	4
F	7	1	1	2
F	21	2	1	3
M	30	1	2	3
F	4	1	2	3
F	6	3	3	6

circulating IgE 32 kU/l) and one atopic donor (D_A) 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%)

Table 2. Clinical activity scores assessed in ASST+ CU patients ($n = 22$)

Sex	Urticaria duration (months)	Wheal score	Itch score	Urticaria activity score (wheal + itch)
F	26	2	1	3
M	9	2	2	4
F	18	1	2	3
M	42	2	1	3
F	6	1	0	1
M	3	1	2	3
F	13	3	2	5
F	38	2	3	5
F	20	2	3	5
F	12	1	2	3
F	4	2	2	4
F	12	3	2	5
M	18	3	3	6
F	24	1	1	2
F	6	1	2	3
F	14	1	1	2
M	7	2	3	5
M	9	1	2	3
F	12	2	1	3
F*	16	3	3	6
F*	8	3	2	5
F*	34	2	3	5

*Patients selected for CyA treatment.

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 μ l of heparinized whole blood was directly incubated at 37°C on water bath for 30 min with 100 μ l of heat-inactivated undiluted sera of CU patients and controls, making a final volume of 200 μ l. Degranulation was stopped by adding 10 μ l of 20 mM EDTA at RT for 5 min and cells were then stained with 20 μ l of CD63-FITC/CD123-PE/Anti-HLA-DR-PerCP antibody cocktail (BD FastImmune™ CD63/CD123/Anti-HLA-DR; Becton-Dickinson, San Jose, CA) and incubated for 15 min at RT. Finally, whole blood probes were lysed (1 $\times$ BD FACS lysing solution; Becton-Dickinson), washed and resuspended in 300 μ l 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 μ mol/l) to distinct tubes served to establish respectively baseline values and positive control of CD63 expression on donors' basophils in three consecutive experiments ($MV \pm SD$ of CD63+ cells at baseline was $7.3\% \pm 1.2\%$ for D_{NA} and 6.4 ± 0.2 for D_A ; $MV \pm SD$ of CD63+ cells after fMLP stimulation was $23\% \pm 3\%$ for D_{NA} and $45\% \pm 2.2$ for D_A , $P < 0.001$).

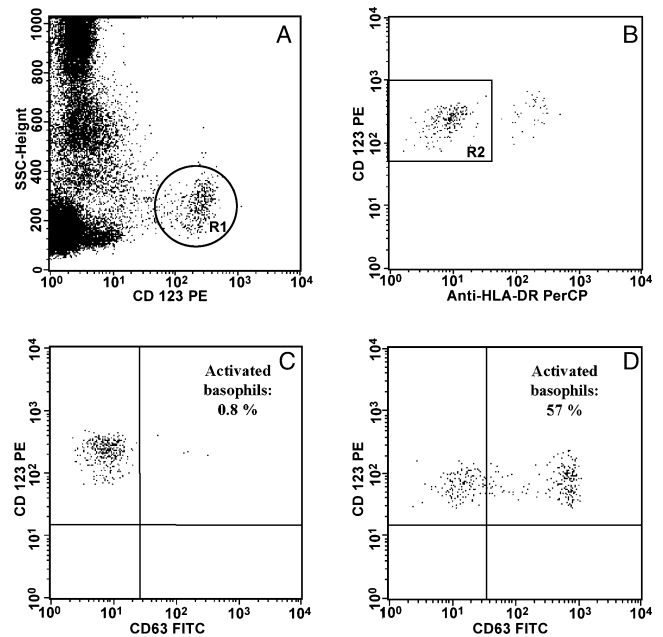


Figure 1. Identification of activated basophils by means of a whole blood tricolour flow cytometric protocol using anti-CD123, anti-HLA-DR and anti-CD63 MoAbs. Gate R1 (A) isolates the low side-scatter (SSC), CD123+ basophil population, while gate R2 (B) detects a well defined population of cells which are highly positive for CD123 but negative for HLA-DR (CD123+ HLA-DR- basophils) on a biparametric dot plot activated on R1. With a double gating strategy, the expression of CD63 is determined in the upper right quadrant of a biparametric dot plot CD123-CD63 referred to the combined gate R1 + R2. (C, D) percentage of CD63 activated basophils on D_A whole blood after incubation with representative ASST– CU (C) and ASST+ CU sera (D).

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 $\pm$ standard deviation ($MV \pm SD$) 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 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).

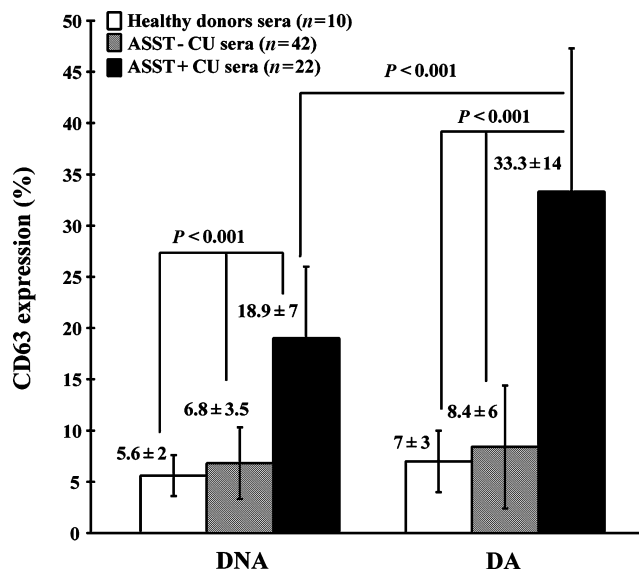


Figure 2. ASST+ CU sera are able to induce CD63 expression on basophils of both D_{NA} and D_A with respect to ASST- CU and healthy donors sera. ASST+ CU sera activity, however, is about 1.8-fold higher ($P < 0.001$) when evaluated on D_A basophils when compared with the activity on D_{NA} . Data are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

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 ($57 \pm 8.5\%$ vs 14.6 ± 6.4 ; $P = 0.02$), further decreasing at T2 below the calculated cut-off ($9.3 \pm 2\%$; $P < 0.01$) (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 (FcεRI) of dermal mast cells

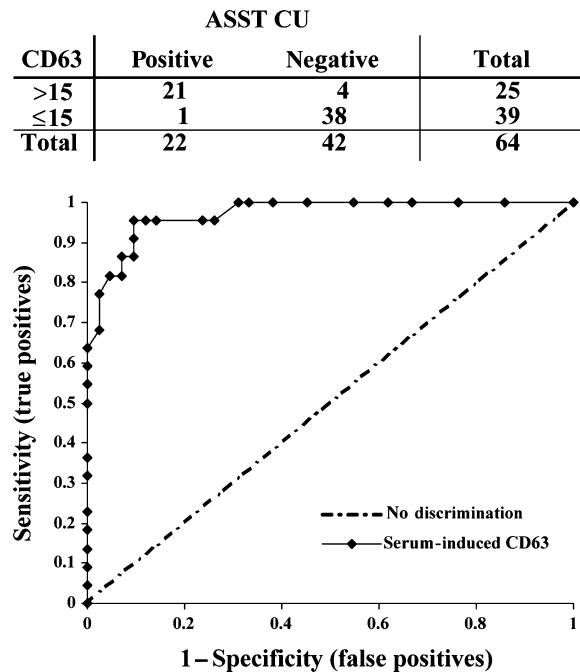


Figure 3. Diagnostic performance of the serum-induced CD63 expression assay was established by a receiver operating characteristic (ROC) curve stratifying chronic urticaria (CU) patients according to the autologous serum skin test (ASST) response; at the calculated cut-off of 15%, the assay provided 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.

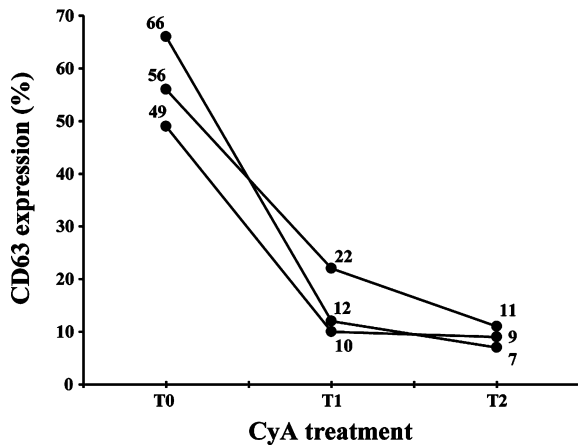


Figure 4. Serum induced CD63 expression is significantly reduced in three patients treated with short-term CyA at T1 with respect to the beginning of the therapy (T0; $P = 0.02$), further decreasing at the end of the treatment (T2; $P < 0.01$). Percentage values of single determination of CD63 at T0, T1 and T2 are reported for each patient.

and basophils has been largely documented (2, 5–7) and the term of AICU now identifies a subset of patients in which these autoantibodies are the cause of the lesions, as suggested by a positive *in vivo* response to intradermal injection of autologous serum (3, 4). Although numerous attempts have been made to devise specific and sensitive *in vitro* tests for AICU, including enzyme-linked immunosorbent assay and other immunobinding assays (6, 7, 11), the current gold standard laboratory test is the demonstration and measurement of histamine release from target basophils or dermal mast cells (4, 9). It is well known that basophils activation can be detected by flow cytometric measurement of CD63, a tetraspan granule protein that appears *de novo* on basophils membrane after degranulation (14); in resting basophils, CD63 is anchored in basophilic intracytoplasmic granules and, as a consequence of cell degranulation, it is expressed with a 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 approach demonstrated that sera of 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 on a large series of patients by means of a new tricolour flow cytometric method which allows to accurately quantify activated CD63+ cells on a selected population of CD123+ HLA-DR- basophils from whole blood, without IL-3 pretreatment (22, 23). Applying this method for the first time in a large series of CU patients, our results demonstrated that

(i) sera from ASST+ CU patients are able to induce CD63 expression on donors basophil in a significant higher manner when compared with ASST- CU patients and healthy donors sera, (ii) ASST+ CU serum activity on CD63 expression was significantly enhanced when basophils from an atopic sensitized donor were used, (iii) according to the ROC-generated cut-off value of 15%, the sensitivity and specificity of the serum-induced CD63 expression assay was 95.5% and 90.5% respectively, (iv) ASST+ CU serum induced CD63+ basophils from D_A are significantly decreased below the cut-off during CyA treatment, in parallel with the complete clearing of skin lesions and the reduced ASST response. Wedi et al. (18), using whole blood primed with IL-3, demonstrated serum-induced CD63 expression in 70% of ASST+ CU patients, as well as in 45% of ASST- CU and in 35% of control subjects (both atopic and nonatopic), thus concluding that CU serum activity inducing CD63 expression cannot be regarded as a diagnostic marker of CU. Gyimesi et al. proposed a modified protocol of 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 basophils 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 recovery even in whole blood. Indeed, the identification of basophils using prior protocols relied on a single IgE-labelling, although it is known that FcεRI expression can vary considerably on cell surfaces from one subject to another (24). CD123, constitutively expressed at high density on basophils, is known to be a useful marker for basophil identification, as its expression is less variable than surface IgE and is independent of the allergy status of the donor (20, 23). With a double gating strategy of basophil phenotyping which isolate low side scatter, CD123+ HLA-DR- cells, the expression of CD63 can be easily and accurately quantify in this selected population. The higher sensitivity of this method could also explain the ASST+ CU serum activity in inducing CD63 expression also on D_{NA} basophils, although the use of D_A whole blood is highly recommended to obtain a better results in terms of the magnitude of the serum challenge.

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 signifi-

cantly 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-Fc ϵ RI 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 Fc ϵ RI may be validated in the future for a larger screening of CU sera with both basophil and mast cells activating properties.

References

- Kozel MM, Sabroe RA. Chronic urticaria: aetiology, management and current and future treatment options. *Drugs* 2004;**64**:2515–2536.
- Kaplan AP. Chronic urticaria: pathogenesis and treatment. *J Allergy Clin Immunol* 2004;**114**:465–474.
- Sabroe RA, Grattan CE, Francis DM, Barr RM, Kobza Black A, Greaves MW. The autologous serum skin test: a screening test for autoantibodies in chronic idiopathic urticaria. *Br J Dermatol* 1999;**140**:446–452.
- Grattan CE. Autoimmune urticaria. *Immunol Allergy Clin North Am* 2004;**24**:163–181.
- Hide M, Francis DM, Grattan CE, Hakimi J, Kochan JP, Greaves MW. Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic urticaria. *N Engl J Med* 1993;**328**:1599–1604.
- Sabroe RA, Fiebiger E, Francis DM, Maurer D, Seed PT, Grattan CE et al. Classification of anti-Fc ϵ RI and anti-IgE autoantibodies in chronic idiopathic urticaria and correlation with disease severity. *J Allergy Clin Immunol* 2002;**110**:492–499.
- Fiebiger E, Hammerschmid F, Stingl G, Maurer D. Anti-Fc ϵ RI α autoantibodies in autoimmune-mediated disorders. Identification of a structure-function relationship. *J Clin Invest* 1998;**101**:243–251.
- Tong LJ, Balakrishnan G, Kochan JP, Kinet JP, Kaplan AP. Assessment of autoimmunity in patients with chronic urticaria. *J Allergy Clin Immunol* 1997;**99**:461–465.
- Ferrer M, Kinet JP, Kaplan A. Comparative studies of functional and binding assays for IgG anti-Fc (epsilon) RI alpha (alpha subunit) in chronic urticaria. *J Allergy Clin Immunol* 1998;**101**:672–676.
- Kikuchi Y, Kaplan AP. Mechanisms of autoimmune activation of basophils in chronic urticaria. *J Allergy Clin Immunol* 2001;**107**:1056–1062.
- Zuberbier T, Henz BM, Fiebiger E, Maurer D, Stingl G. Anti-Fc ϵ RI α serum autoantibodies in different subtypes of urticaria. *Allergy* 2000;**55**:951–954.
- Schroeder JT, MacGlashan DW Jr, Lichtenstein LM. Human basophils: mediator release and cytokine production. *Adv Immunol* 2001;**77**:93–122.
- Falcone FH, Haas H, Gibbs BF. The human basophil: a new appreciation of its role in immune responses. *Blood* 2000;**96**:4028–4038.
- Knol EF, Mul FP, Jansen H, Calafat J, Roos D. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J Allergy Clin Immunol* 1991;**88**:328–338.
- Sainte-Laudy J, Sabbah A, Vallon C, Guerin JC. Analysis of anti-IgE and allergen induced human basophil activation by flow cytometry. Comparison with histamine release. *Inflamm Res* 1998;**47**:401–408.
- Crockard AD, Ennis M. Laboratory-based allergy diagnosis: should we go with the flow? *Clin Exp Allergy* 2001;**31**:975–977.
- Ebo DG, Hagendorens MM, Bridts CH, Schuerwegh AJ, De Clerck LS, Stevens WJ. In vitro allergy diagnosis: should we follow the flow? *Clin Exp Allergy* 2004;**34**:332–339.
- Wedi B, Novacovic V, Koerner M, Kapp A. Chronic urticaria serum induces histamine release, leukotriene production, and basophil CD63 surface expression—inhibitory effects of anti-inflammatory drugs. *J Allergy Clin Immunol* 2000;**105**:552–560.
- Gyimesi E, Sipka S, Danko K, Kiss E, Hidvegi B, Gal M et al. Basophil CD63 expression assay on highly sensitized atopic donor leucocytes – a useful method in chronic autoimmune urticaria. *Br J Dermatol* 2004;**151**:388–396.
- Moretti S, Lanza F, Dabusti M, Tieghi A, Campioni D, Dominici M et al. CD123 (interleukin 3 receptor alpha chain). *J Biol Regul Homeost Agents* 2001;**15**:98–100.
- Olweus J, BitMansour A, Warnke R, Thompson PA, Carballido J, Picker LJ et al. Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. *Proc Natl Acad Sci USA* 1997;**94**:12551–12556.
- Heinemann A, Hartnell A, Stubbs VE, Murakami K, Soler D, LaRosa G et al. Basophil responses to chemokines are regulated by both sequential and cooperative receptor signaling. *J Immunol* 2000;**165**:7224–7233.

23. Varro R, Chen C-H. A no-wash 3-color basophil degranulation assay using the CD123+ HLA DR-phenotype for basophil classification. *Cytometry* 2000;**10**:S116.
24. Kinet JP. The high-affinity IgE receptor (Fc ϵ RI): from physiology to pathology. *Annu Rev Immunol* 1999;**17**:931–972.
25. Grattan CE, ÓDonnell BF, Francis DM, Niimi N, Barlow RJ, Seed PT et al. Randomized double-blind study of cyclosporin in chronic ‘idiopathic’ urticaria. *Br J Dermatol* 2000;**143**: 365–372.
26. Di Gioacchino M, Di Stefano F, Cavallucci E, Verna N, Ramondo S, Paolini F et al. Treatment of chronic idiopathic urticaria and positive autologous serum skin test with cyclosporine: clinical and immunological evaluation. *Allergy Asthma Proc* 2003;**24**:285–290.
27. Plath KE, Grabbe J, Gibbs BF. Calcineurin antagonists differentially affect mediator secretion, p38 mitogen-activated protein kinase and extracellular signal-regulated kinases from immunologically activated human basophils. *Clin Exp Allergy* 2003;**33**:342–350.
28. Marsland AM, Soundararajan S, Joseph K, Kaplan AP. Effects of calcineurin inhibitors on an in vitro assay for chronic urticaria. *Clin Exp Allergy* 2005;**35**:554–559.
29. Stellato C, de Paulis A, Ciccarelli A, Cirillo R, Patella V, Casolaro V et al. Anti-inflammatory effect of cyclosporin A on human skin mast cells. *J Invest Dermatol* 1992;**98**:800–804.