

ORIGINAL PAPER

Evidence of *in vivo* basophil activation in chronic idiopathic urticaria

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Summary

Background Approximately 40% of chronic idiopathic urticaria (CIU) subjects have autoantibodies to either FcεRIα or IgE. The effect of such autoantibodies on circulating basophil activation status is unknown.

Objective The expression of cell surface activation markers on basophils from CIU, non-allergic, and allergic subjects were compared. Further, the relationship between marker expression and serum factors reported in CIU, such as histamine-releasing activity (HRA) and immunoreactivity to FcεRIα were examined.

Methods Peripheral blood was obtained from CIU, allergic, and non-allergic donors and fractionated by density gradients. Enriched basophils (1–12%) were analysed by flow cytometry for expression of activation markers including CD63, CD69, and CD203c. Dilutions of serum (5–50%) were analysed for HRA on basophils from a normal donor. Serum was tested for immunoreactivity by western blotting to a standard cell lysate prepared from an RBL-SX38 cell line transfected with human FcεRIα.

Results CIU subjects ($n = 9$) and allergic subjects ($n = 8$) exhibited enhanced expression of CD63 and CD69, as compared with non-allergic subjects ($n = 7$); however, no difference was seen among groups for CD203c expression. Five CIU and two non-allergic subjects had evidence of significant serum HRA ($> 20\%$), whereas two CIU, two allergic, and three non-allergic subjects had evidence of serum immunoreactivity to FcεRIα. Serum HRA and serum immunoreactivity to FcεRIα were not associated with enhanced surface marker expression.

Conclusion Basophil activation marker expression is increased in CIU subjects and is not associated with serum factors. In addition, serum HRA and FcεRIα immunoreactivity are not unique to CIU, or related to enhanced circulating basophil marker expression.

Keywords autoantibodies, basophils, cell surface markers, cellular activation, chronic urticaria
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Introduction

In the last decade several groups have forwarded the idea that chronic idiopathic urticaria (CIU) may be the result of autoimmunity [1]. Specifically, approximately 40% of patients are reported to have serum autoantibodies of which 90% are directed against the high affinity IgE receptor alpha subunit (FcεRIα) while 10% target IgE [1, 2]. Separately, it has been noted that another 40% of CIU patients possess serum histamine releasing activity (HRA) by *in vitro* testing using donor basophils [3]. Although it is assumed that serum HRA and autoantibodies represent the same entity, there is a significant discordance between the presence of these two serum factors in CIU subjects [4, 5]. Although it has been suggested that subjects with auto-

antibodies have more severe disease [5], the presence of serum HRA did not significantly modify the leucocyte infiltrates present in biopsies of either new (< 4 h) or more established (> 12 h) lesions [6].

In recent years, a role for basophils has emerged in CIU [7]. Basophils are present in the leucocyte infiltrate in skin biopsies of CIU lesions, which resemble the cellular infiltrate found in late-phase allergen injection sites [8]. Likewise, reduced circulating basophil numbers have been correlated with heightened CIU disease activity [9]. Additionally, basophils of CIU subjects have a selective reduction in functional response to anti-IgE, but not to other stimuli [10–12]. It has also been noted that the functional defect via the IgE receptor resolves as CIU remits [10]. Several groups recently have shown that cell

surface marker expression is useful to detect basophil activation [13–15]. For example, CD63, a member of the transmembrane-4 superfamily, is rapidly mobilized on the basophil surface by IL-3, anti-IgE, and allergen, as well as other degranulation stimuli [13, 16]. In contrast, CD69, is slowly induced after basophil exposure to IL-3, and is thought to require *de novo* synthesis. *In vivo*, CD69 is also increased on basophils from the BAL of asthmatics [16]. Most recently, cell surface expression of CD203c (ectonucleotide pyrophosphatase (E-NPP3)) has been shown to be enhanced by anti-IgE and allergen [17–20]. Further, its expression is unique to basophils, mast cells and their progenitors [21]. Although these same basophil markers have been applied as an *in vitro* measure to support the clinical diagnosis of allergy, issues remain in regards to their sensitivity and specificity relative to more traditional tests such as skin tests, specific IgE levels measured by RAST or basophil histamine release assays [15, 18, 22].

Although reported for over a decade, direct evidence that circulating serum autoantibodies or HRA in CIU is linked to altered basophil or mast cell function *in vivo* is limited [23]. While recent studies have suggested the use of CIU sera-induced CD63 expression on basophils of normal donors as a potential screening tool for autoimmune urticaria [24–27], we are interested in directly examining the *in vivo* levels of activation markers on the basophils of CIU subjects. In the present study, we compared the *in vivo* expression of basophil activation markers among non-allergic, allergic, and CIU subjects to test the hypothesis that basophil activation status would be greatest in CIU subjects. In addition, we examined the relationship between serum factors and the levels of these basophil activation markers. Specifically, we measured serum HRA, as well as immunoreactivity to FcεRIα.

Materials and methods

Study subjects

All study subjects were recruited from the Johns Hopkins Asthma and Allergy Center, via postings. CIU subjects ($n = 9$, 1 M/8 F, mean age = 37.6 years) were characterized based on allergist diagnosis as well as active status of disease. Allergic subjects ($n = 8$, 6 M/2 F, mean age = 33.3 years) were characterized based on positive skin test and a history of allergic rhinitis or asthma. Non-allergic subjects ($n = 7$, 2 M/5 F; mean age = 40.7 years) were characterized based on absence of the previously mentioned criteria. After informed consent, subjects underwent venipuncture via a protocol approved by the Western Institutional Review Board.

Basophil isolation

Venous blood samples were drawn into syringes containing 5 mM EDTA [26] to prevent coagulation. Blood mononuclear

cells were obtained by density-gradient sedimentation, using Accuspin separation tubes (Sigma-Aldrich, St. Louis, MO, USA) and a discontinuous double Percoll gradient, as described [28]. Basophils (purity 1–12%) were enumerated by Alcian Blue staining [29]. For serum histamine-releasing assays, venous blood from non-CIU donors was subjected to dextran sedimentation to obtain mixed leucocytes with basophil purity <1% [30]. A separate serum tube was collected from all donors for immunoblotting assays.

Dual colour flow cytometry

Enriched basophil suspensions were labelled for direct and indirect immunofluorescence and flow cytometry. Antibodies used include: irrelevant mouse IgG1 control (MOPC-21; BD Biosciences, Mountain View, CA, USA), FITC-conjugated polyclonal goat anti-human IgE and its control FITC-conjugated normal goat IgG (Kirkegaard and Perry, Gaithersburg, MD, USA) and PE anti-mouse IgG (Biosource; Camarillo, CA, USA). PE-conjugated mouse IgG1, mouse anti-CD63 IgG1 (CLB-gran12), mouse anti-CD69 IgG2B (TP1.55.3), and PE-conjugated mouse anti-CD203c IgG1 (97A6) were purchased from Immunotech (Hialeah, FL, USA). In select subjects, platelet contamination of enriched basophil preparations was monitored by the use of a mouse anti-CD42b IgG1 (SZ2), also from Immunotech. Cells were incubated with monoclonal antibodies specific to each surface marker for 30 min at 4 °C in the presence of FITC-conjugated anti-human IgE. Cells were then washed and, if necessary, labelled with PE-conjugated anti-mouse for an additional 30 min. Samples were analysed on a Berton Dickinson FACS Caliber flow cytometer employing Cell Quest software. Cells were initially gated based on FITC-IgE-positive cells to select the basophil population and then analysed for specific activation markers [31]. With rare exception, marker expression was unimodal. Data are expressed as net median fluorescence intensity (actual MFI – MFI of irrelevant IgG control). In pilot studies, basophils were also labelled for flow cytometry using a whole blood lysis approach [18] in parallel with Percoll isolation.

Serum histamine-releasing activity

In preliminary studies, eight normal basophil donors were screened for high-range (upper quartile) release to anti-IgE and CIU sera [24]. A single donor was selected, whose basophils were then activated by dilutions of the subjects' sera (50, 25, 5%) as well as a crosslinking anti-IgE as a positive control. Histamine content was quantitated in cell-free supernatants using an automated fluorometric assay [32]. Results are shown as the percentage of total histamine content present in a lysate of a leucocyte aliquot after subtracting spontaneous release. In addition,

sera was also tested with RBL-SX38 cells (transfected with human FcεRIα) as a separate measure of serum HRA that may involve factors targeting unoccupied FcεRIα.

Serum immunoreactivity for FcεRIα

Diluted sera (1 : 250) was used to immunoblot a standard lysate of RBL-SX38 cells transfected with human FcεRIα as described [3, 28]. Cell lysates from a single passage of RBL-SX38s were separated by SDS-PAGE gel electrophoresis and transferred to PVDF membranes. Membranes were next immunoblotted with each individual serum, followed by use of a secondary anti-human IgG-HRP (Amersham-Pharmacia, Piscataway, NJ, USA). As a positive control, a single lane of each gel was immunoblotted with a commercial antibody against FcεRIα (polyclonal rabbit anti-human FcεRIα subunit, Upstate Biotechnology, Lake Placid, NY, USA), followed by anti-rabbit IgG-HRP (Amersham-Pharmacia, Piscataway, NJ, USA) to allow for comparisons of relative band intensity between gels (Fig. 1) [28]. Analysis of band density was conducted using the Bio-Rad imaging system (Bio-Rad Laboratories, Segrate, Italy). Using this software, a three-dimensional model of each band was generated. The volume of each band was calculated, and adjusted for local background. Positive serum immunoreactivity was based on two criteria: an adjusted volume greater than 1 U, as well as an adjusted volume greater than 10% of the respective gel-positive control.

Statistical design

The statistical analysis utilized STATVIEW 5 package for Macintosh and Student's *t* test for normally distributed data such as cell surface marker expression. A *P*-value of less than 0.05 was considered to be significant.

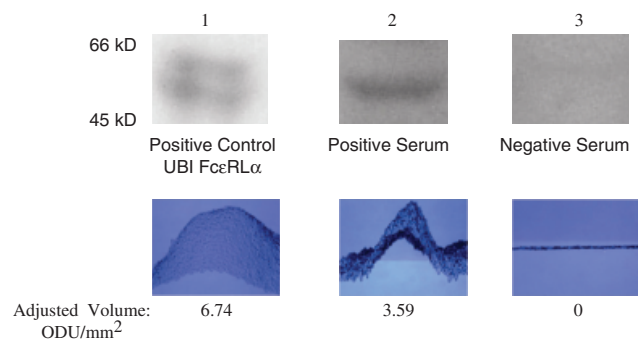


Fig. 1. Representative immunoblot of human sera to detect FcεRIα immunoreactivity. Immunoblot (lane 1) using a commercial antibody against FcεRIα, showing typical band density of surface FcεRIα at 60 kDa. An example of a positive serum immunoblot (lane 2) and a negative serum immunoblot (lane 3). Beneath each lane is a digital image of band density, as well as the respective volume analysis.

Results

Activation marker expression

We first examined *ex vivo* basophil activation marker expression in our study subjects using dual colour flow cytometry (Fig. 2). In pilot studies, we observed higher levels of activation marker expression on basophils examined using the whole-blood lysis approach as compared with Percoll isolated, and therefore all subsequent experiments used the latter approach. As shown in Fig. 3a, basophils from CIU and allergic subjects had significantly heightened expression of CD63 in comparison with non-allergics ($P < 0.02$). Furthermore, there was a trend for higher CD63 expression in basophils from CIU subjects relative to allergic subjects. Of note, levels of CD63 detected on basophils were independent of the presence of platelets which are also known to bear CD63 (data not shown). In contrast, CD69 expression was significantly elevated on basophils from allergics, as compared with non-allergic subjects ($P < 0.04$). In the case of CIU, CD69 expression was variable, with five of nine subjects having little expression ($P = 0.12$ vs. non-allergics). However, no difference was seen in CD203c expression among subject groups.

For comparison purposes, we optimally activated basophils obtained from an allergic subject and measured activation marker expression (Fig. 3b). *In vitro* stimulation of basophils with doses of grass allergen known to trigger

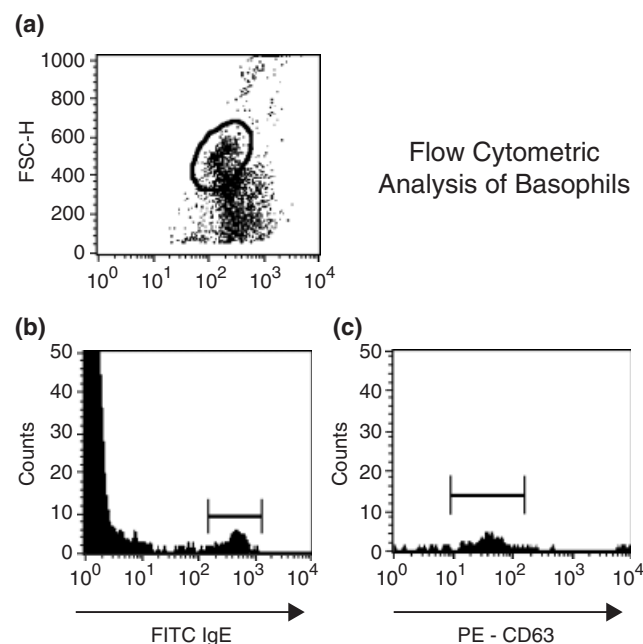


Fig. 2. Representative flow cytometric basophil analysis. (a) Typical cell scatter and gate to capture basophils. (b) Gated cells from (a) examined for intensity of labelling with FITC anti-human IgE. (c) Detection of surface marker CD63 on cells selected for high expression of human IgE in (b).

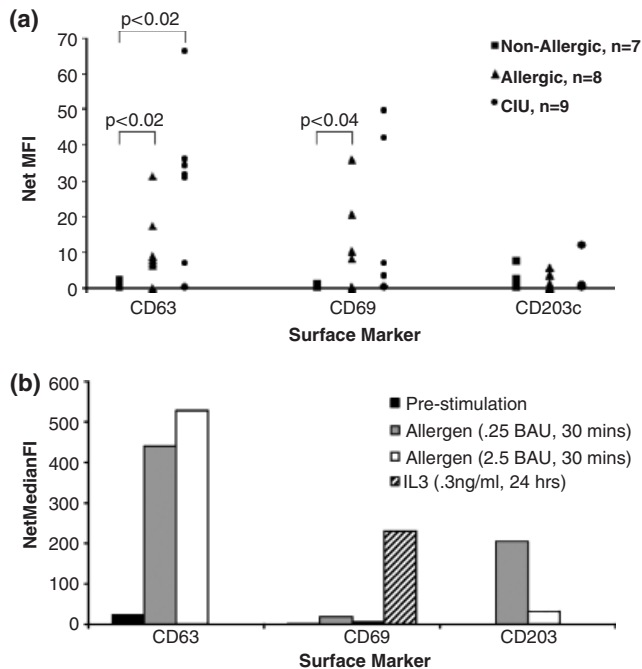


Fig. 3. (a) Basophil activation marker expression in study subjects. Basophils from chronic idiopathic urticaria and allergic subjects had significantly heightened expression of CD63 in comparison to non-allergics. CD69 was elevated in allergics as compared with non-allergics (all comparisons to non-allergics using a Student's *t*-test.) (b) Basophils stimulated for maximal surface marker activation, via allergen and IL-3. *In vitro* stimulation with grass allergen led to significant increase in expression of CD63 and CD203c as compared with pre-stimulation, whereas IL-3 incubation of basophils led to maximal increase in CD69. CD63 and CD203c expression not measured following IL-3 incubation.

histamine release (0.25 and 2.5 ng/mL, 30 min at 37 °C) led to a significant increase in expression of CD63 (> 400 net MFI) and CD203c (> 200 net MFI), as compared with pre-stimulation net MFI. However, allergen exposure led to modest shifts in CD69, whereas IL-3 (0.3 ng/mL, 24 h) led to maximal increase (> 200 net MFI) as reported [16]. Thus, the level of CD63 expression observed on basophils of allergics or CIU subjects was 2.5–5% of the maximal expression achieved by *in vitro* activation (Fig. 3b), respectively, whereas, expression of CD69 was 5% of the maximal expression achieved by *in vitro* IL-3 exposure (Fig. 3b) in both groups.

Serum histamine-releasing activity

Given the observed differences in basophil activation marker expression, we next compared serum HRA between CIU, allergic, and non-allergic subjects. Serum was considered positive for HRA if it induced greater than 20% histamine release above spontaneous release, a criterion similar to that used by others [2]. As shown in Fig. 4, a total of seven subjects were considered positive: two non-allergic and five CIU subjects. Surprisingly, none of the

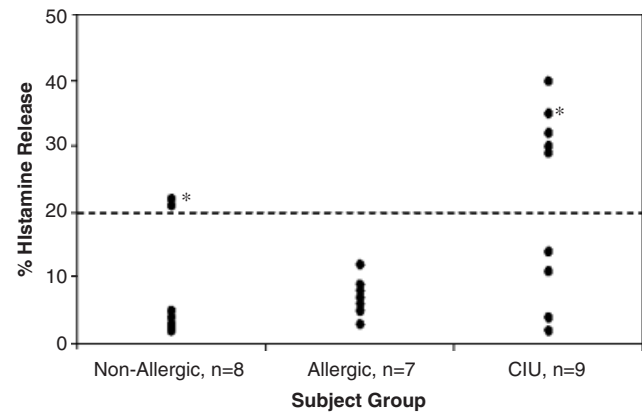


Fig. 4. Serum histamine releasing activity (HRA). Basophils were incubated with dilutions of the subjects' sera, ranging from 5% to 50%. Serum was considered positive if histamine release was greater than 20% above spontaneous release (dashed line). A total of seven subjects were positive: two non-allergic and five chronic idiopathic urticaria subjects. None of the allergic subjects had serum HRA. *Denotes subjects positive for both HRA and serum immunoreactivity to FcεRIα.

allergic subjects were positive for serum HRA. Serum studies using RBL-SX38 with unoccupied IgE receptors did not clearly discriminate HRA among donors (data not shown).

We next examined whether basophil activation marker expression was associated with the presence of serum HRA, within each subject group. There was no significant difference in marker expression between HRA-positive and -negative subjects in either the non-allergic subject group or the CIU group (data not shown).

Serum immunoreactivity for FcεRIα

We next compared serum immunoreactivity for FcεRIα between CIU, allergic, and non-allergic subjects. As shown in Fig. 5, a total of seven subjects were serum positive: three non-allergic, two allergic, and two CIU subjects. Of note, no significant difference in immunoblot intensity was seen among subjects. As expected from earlier studies, there was little overlap between immunoblot-positive and HRA-positive subjects with only two subjects positive for both serum factors [4]. We next examined activation marker expression relative to serum immunoreactivity within each subject group. In all subject groups, there were no significant differences in marker expression between positive and negative subjects, relative to the presence of serum immunoreactivity to FcεRIα (data not shown).

To further clarify the activity of identified serum factors, we examined sera positive for either HRA, immunoreactivity to FcεRIα, or both, for *in vitro* activation of basophil marker expression (Fig. 6). All categories of sera led to modest increases in CD203c levels (~40 net MFI) on donor basophils after 30 min of exposure, whereas most

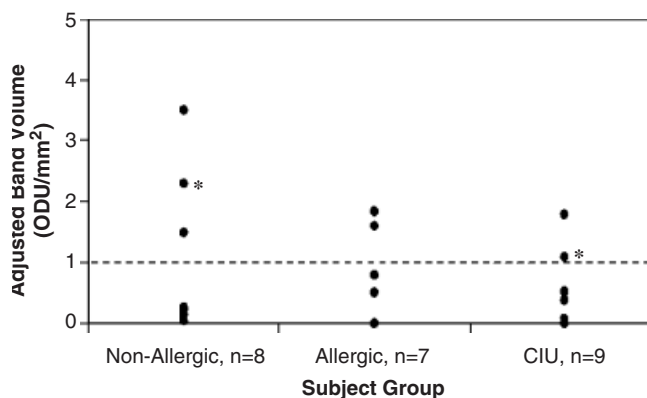


Fig. 5. Serum immunoreactivity to FcεRIα. A total of seven subjects were positive: three non-allergic, two allergics, and two chronic idiopathic urticaria subjects. Dashed line indicates minimal band volume necessary to meet volume criteria for positivity. *Denotes subjects positive for both histamine releasing activity and serum immunoreactivity to FcεRIα.

sera classes led to a reduction in CD63 expression. No changes were noted in CD69 expression after serum incubation.

Discussion

We have found that CD63 expression is increased on the circulating basophils from CIU and allergic subjects, and that both groups have significantly higher expression than non-allergic subjects. In contrast, CD69 expression levels were similar on basophils from CIU and allergics and elevated as compared with non-allergics. However, no difference in expression of CD203c was seen among the three subject groups. In all cases, levels of activation marker expression were modest relative to levels achieved with maximal *in vitro* stimulation, suggesting that the level of circulating basophil activation *in vivo* is far less than expected with specific stimulation via FcεRIα activation. Interestingly, higher basophil marker expression levels have been reported by other groups [18] using a whole-blood lysis approach, which we observed increased levels beyond that seen on basophils isolated in parallel via Percoll. Although we cannot exclude activation of basophils via our selected isolation approach, we utilized this procedure to better reflect the status of basophil activation status *in vivo*. We have also not found a correlation between basophil CD63 levels and CD42 presence, indicating that platelet adhesion on basophils is not leading to falsely elevated CD63 expression. The underlying mechanism behind enhanced activation marker expression is unclear, but certainly in the case of CD69 and CD63, it may be linked to the presence of cytokines such as IL-3 which could be expressed in the sera of both allergic and CIU subjects. It has been noted that both CIU

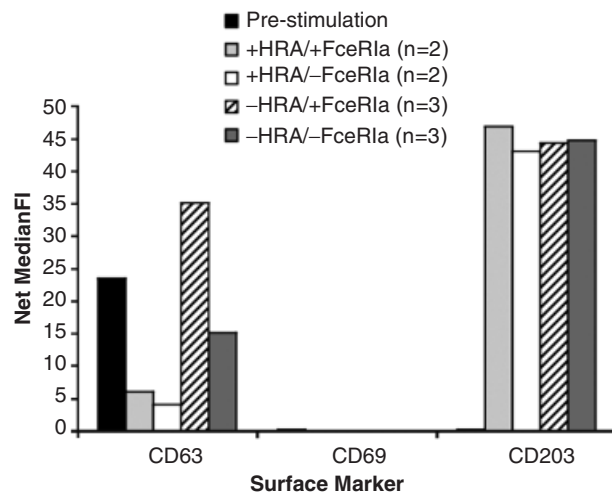


Fig. 6. *In vitro* effects of sera on activation marker expression. Various serum categories (serum histamine releasing activity positive $n = 2$, positive FcεRIα immunoblot (IB) $n = 3$, both $n = 2$, or neither $n = 3$) were incubated with a normal donor's basophils for 30 min. As compared with baseline, all sera caused an elevation in CD203c levels, whereas a mixed effect on CD63 expression was seen. No shifts in CD69 were noted.

and atopic subjects share similar levels of elevation in serum IL-4 as compared with healthy controls [33].

Consistent with the modest levels of basophil activation markers in our subject groups, we also found little evidence that the presence of circulating autoantibodies to FcεRIα as detected by western blotting, altered basophil marker expression. This is a rather surprising finding given the proposed role for these autoantibodies in CIU pathogenesis [23]. We also failed to see a bimodal distribution in activation marker expression that would suggest that only a subset of basophils was being activated via autoantibody presence. Likewise, the presence of serum HRA was not associated with elevated marker expression in CIU and non-allergic subjects, and absent in allergic subjects. The lack of serum HRA in allergic subjects may explain why some investigators have reported the exclusive presence of serum HRA in CIU subjects vs. others subjects presenting to an allergy clinic [2]. However, the presence of serum HRA was not a distinguishing feature of CIU in our study as also observed by others [34].

Among the explanations for the lack of association between the presence of FcεRIα autoantibodies and basophil activation marker expression is the idea that these autoantibodies can only bind to unoccupied receptors on the basophil surface or a 'conditional' autoimmune state [35, 36]. However, in past population studies of receptor densities on basophils, it was noted that the number of open receptors is fairly constant despite a wide range of receptor expression [24]. This observation argues against the existence of *in vivo* states that would increase the numbers of unoccupied receptors to permit pathogenic activation of either basophils or mast cells. However, such an altered state of increased unoccupied receptors has

been described in the early months of treatment with the therapeutic anti-IgE, omalizumab [37]. Furthermore, the existence of FcεRIα autoantibodies has also been related to cross-reactivity with tetanus toxoid and therefore may offer a further challenge to clearly link these autoantibodies specifically to CIU [38, 39]. These groups have found naturally occurring FcεRIα autoantibodies in healthy subjects [38, 39], as we have also observed in this study. Other groups have also shown the presence of such anti-FcεRIα antibodies in other inflammatory, autoimmune skin diseases, suggesting a broad presence of such autoantibodies [40].

As an aside, we noted allergen dose dependence for CD203c expression on basophils, supporting the use of a wide range of allergen doses in assays involving activation markers to detect basophil activation. We also saw that *in vitro* exposure of basophils to all categories of sera (based on HRA or presence of anti-FcεRIα antibodies) led to enhanced CD203c expression and a variable effect on CD63 expression. From our studies of basophil activation markers, we have observed high baseline activation marker expression in highly allergic individuals with a subsequent drop in marker expression following stimulation. This may explain the unexpected decrease in sera-induced CD63 expression observed in Fig. 6. Although we focused on the expression of activation markers on basophils of CIU subjects, recently, several groups have suggested the use of serum-induced basophil CD63 expression as a screening test for autoimmune urticaria based on serologic factors [25–27, 34]. However, the disparity in serum effects on CD63 begins to highlight some of the issues with broad application of these assays for *in vitro* diagnosis of clinical allergy or serologic factor status.

In conclusion, we have found that expression of basophil activation markers is increased in CIU and is similar to that observed in allergic subjects. We also found that elevated basophil marker expression was not associated with CIU serologic factors. In addition, serum HRA and FcεRIα immunoreactivity are not unique to CIU subjects or related to enhanced basophil marker expression.

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