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Basophil activation test in children with autoimmune chronic spontaneous urticaria: Is it ready for clinical practice?

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ABSTRACT

Introduction: Chronic spontaneous urticaria (CSU) is characterized by recurrent itchy wheals, angioedema or both, that persist for longer than six weeks. In children, up to 40% of chronic spontaneous urticaria is due to mast cells and basophils-activating autoantibodies, mostly directed against the IgE high-affinity receptor subunit (FcεRI). Indirect basophil activation test (BAT) has been proposed in the diagnosis of autoimmune urticaria.
Materials and methods: Sera from sixteen patients, aged from 2 to 15 yrs, with CSU were evaluated through indirect BAT by flow cytometry using a commercial kit (Flow CAST®, BUHLMANN Laboratories, Schonenbuch, Switzerland) according to the manufacturer's instructions.
Results: Indirect BAT test gave a much better diagnosis in our cohort than the gold standard ASST. Six children (37.5%) showed a positive indirect BAT while we could perform ASST in only 3 patients with just one patients showing a positive ASST. The specificity of BAT positive results was confirmed by the absence of significant difference between the BAT results obtained from negative controls vs negative sera of the patient ($p = 0.65$) on the basophil donors, indicating that the serum is not activating basophil per se.
Conclusions: This pilot study suggests the utility of BAT to identify the subtype of autoimmune CSU in children in clinical practice.

1. Introduction

Chronic spontaneous urticaria (CSU) is characterized by recurrent itchy wheals and angioedema that appear without any identifiable cause (Zuberbier et al., 2018). The condition is defined as recurrent urticaria, occurring for at least 6 weeks. In children, approximately 35–40% of chronic spontaneous urticaria is considered due to autoimmune mechanisms (Sahiner et al., 2011). CSU has been found to be associated with several autoimmune diseases, like thyroid diseases, rheumatoid arthritis, type I diabetes mellitus, Sjörgen syndrome, celiac disease, and systemic lupus erythematosus (Confino-Cohen et al., 2012). An autoimmune background for CSU is supported by the observation of immediate wheals and flare responses after an autologous serum skin test (ASST) in CSU patients (Sabroe et al., 1999). These patients show circulating auto-antibodies, directed towards the high-affinity IgE receptor subunit (FcεRI) and IgE itself (Jirapongsananuruk et al., 2010; Fiebiger et al., 1995; Hide et al., 1993).

Currently, the *in vivo* ASST is recommended to identify the subtype of autoimmune urticaria (AIU) in the diagnostic workup of CSU (Zuberbier et al., 2018). However, ASST can not be used in case of concomitant treatment with antihistamines, that is quite frequent in clinical practice.

Indirect basophil activation test (BAT), intended as the in vitro stimulation of heterologous basophils from peripheral blood donors mediated by the serum of CSU patients, followed by the flow cytometric determination of the basophils activation, has been proposed as an useful tool for the assessment of autoimmune chronic urticaria to overcome the ASST limitations (Hoffmann et al., 2015). BAT offers several advantages: it is less invasive than ASST and does not carry the risk of accidental infection by injections of autologous serum (Sabroe et al., 1999). As well as ASST for mast cells, indirect BAT allows the identification of patients expressing auto-antibodies able to activate basophils, including anti-FcεRI, anti-FcεRII and anti-IgE, even if it is not able to exactly distinguish which autoantibody is present in the serum.

Abbreviations: CSU, chronic spontaneous urticaria; AIU, autoimmune chronic urticaria; BAT, basophil activation test; ASST, autologous serum skin test; anti IgE, autoantibodies; FcεRI, high-affinity IgE receptor subunit; CD63, ANA, anti-nuclear antibodies

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Table 1
Subjects clinical characteristics.

Subject	Triptase	CD63%	Age(y)	IgE level (KU/lt)	autoimmunity tests	Allergic sensitization (skin test and/or sIgE)	***PU	ASST
1	NEG	1,30	15	554,36	Neg	Birch; grasses	Negative	**N/A
2	NEG	3,20	15	524,86	Neg	grasses	negative	**N/A
3	POS	Neg	14	1946,9	Neg	*ND	negative	**N/A
4	POS	Neg	6	2383,8	*ND	*ND	negative	**N/A
5	NEG	Neg	7	318,47	*ND	grasses, ambrosia	negative	**N/A
6	POS	14,58	10	1190	ANA Pos 1:80; Ab anti-IgE	grasses, birch	negative	Neg
7	NEG	3,12%	6	231,8	negative	dust mites, grasses	negative	**N/A
8	POS	Neg	5	1450,6	negative	negative	negative	**N/A
9	POS	8,38%	8	1517	pos (Ab anti-IgE)	*ND	negative	Neg
10	NEG	5,59%	12	202,95	ANA 1:320	*ND	negative	**N/A
11	NEG	5,55%	14	261,81	ANA Pos 1:80	negative	heat urticaria	POS
12	NEG	1,42%	3	226,64	negative	negative	negative	**N/A
13	NEG	4,20%	7	239,59	negative	ascaris, anisakis, alternaria	negative	**N/A
14	NEG	3,20%	6	118,1	negative	peach, prune, cherry, dust mites, birch	negative	**N/A
15	POS	5,90%	7	1369,97	*ND	negative	negative	**N/A
16	POS	6,80%	6	1455,66	*ND	Negative	negative	**N/A

* ND: not determined.
** N/A: not applicable.
*** PU: physical urticaria.

Indeed, a positive BAT can indicate that CSU in patients has an autoimmune basis (Hide et al., 1993; Hoffmann et al., 2015; Gruber et al., 1988).

Several BAT have been assessed in adult CSU patients, and CD63 and CD203c are held to be the most reliable markers (Yasnowsky et al., 2006; Irinyi, 2013). While several studies have evaluated the employment of BAT in adult patients (Hoffmann et al., 2015; Curto-Barredo et al., 2016; Rauber et al., 2017; Gentinetta et al., 2011), very few data exist on children. To our knowledge, a single study by Netchiporouk et al. evaluated BAT test performance, aiming to standardize it in Canadian CSU children cohort (Netchiporouk et al., 2016). In that study the authors evaluated a clinical cut-off for sera from CSU pediatric patients on one basophil donor and subsequently evaluated the reproducibility of the results with randomly chosen sera from different patients group on a second healthy non atopic donor.

Our study is a real life study to investigate the potential benefit of the indirect BAT in the daily practice for the diagnosis of AIU in children with CSU. The goal of our pilot study is to generate proof of concept data that might be worthy of further and deeper investigation with clinical validations.

2. Materials and methods

This study was carried out between April 2015 and September 2017 at two University Hospital in Northern Italy. Subjects were recruited and evaluated at the Pediatric Allergy and Immunology Unit of San Matteo Hospital in Pavia and at the Pediatric Allergy Unit of Vittore Buzzi Children's Hospital in Milan.

We analyzed sera from pediatric CSU patients through indirect BAT in flow cytometry using a commercial kit (Flow CAST®, BUHLMANN Laboratories, Schonenbuch, Switzerland) according to the manufacturer's instructions. Briefly, the presence of auto-antibodies in patients sera able to activate basophils was evaluated incubating 50 µl of patient serum with 50 µl of EDTA whole blood from 3 independent heterologous donors of basophils. The stimulation was performed for 15 min at 37 °C in a stimulation buffer included in the kit, containing calcium and IL3, and a staining reagent including anti CD63-FITC and anti CCR3-PE antibodies. The flow cytometry analysis was performed calculating the percentage of activated basophils, determined as the percentage of CD63 positive cells in the population of CCR3pos/SSClow according to the manufacturer's instruction. Basal activation and positive control activation by a monoclonal antibody anti FcεRI receptor included in the kit was checked on each of the independent basophil donors. We included sera from sixteen patients, aged from 2 to 15 years, all suffering from CSU of unknown etiology. Donor basophils were obtained from healthy, non-atopic donors.

BAT tests were considered positive accordingly to the technical cutoff of the Flow CAST® test indicated by the manufacturer: (i) the percentage of CD63pos basophils > 5%, and (ii) the stimulation index SI that must be equal or higher than 2 (SI is defined as the percentage of CD63pos basophils induced by the serum patient divided by the percentage of CD63pos induced by the negative control). The patient serum was considered positive in the indirect basophil activation when at least one donor was positive.

The majority of subjects were also evaluated according to our clinical practice for allergy (serum total IgE and specific IgE levels) using ImmunoCAP® test system (Thermo Fisher Scientific) and concurrent autoimmune diseases by auto-immunity tests, including anti-thyroid autoantibodies (anti-thyroglobulin and thyroid microsomal antibodies), that were detected by radioimmunoassay, and anti-nuclear antibody (ANA) detected by an indirect fluorescent antibody technique.

Skin prick test reactivity to common aero and food allergens were also determined.

ASST was performed according to European guidelines, in subjects not undergoing therapy with antihistamines.

Written informed consent was obtained from the children's parents or legal guardians prior to conducting any study-related investigation. The research protocol was approved by the Local Ethic Committee of the Hospitals (N° 27980).

3. Results

The clinical features and main results of ASST and BAT are summarized in Table 1.

Despite the indication of the guidelines, in our cohort ASST could not be performed in the majority of subjects (80.5%) due to concomitant treatment with antihistamines. We could perform ASST only in three patients for whom the absence of concomitant medications allowed reliable results of the test. Two of them resulted negative and one positive by this method.

To overcome this limitation, all the patients were assessed by the indirect BAT to identify the presence of autoantibodies able to activate donor basophils from three independent healthy donors. Six children (37.5%) of our cohort showed a positive indirect BAT on at least one donor basophils. We used 5% of CD63pos basophil as cutoff to define positive sera, that is the technical cutoff of the commercial kit used to set a standardized assay for real clinical use of BAT results. No significant difference was observed between the BAT results obtained from

negative controls on the basophil donors vs patient sera that gave negative basophil activation ($p = 0.65$), indicating that the serum is not activating basophil *per se* and that each activation observed was specific. Considering our results as a pilot experiment, the technical cutoff should be further explored with serum from healthy pediatric donors to exclude any non-specific effect of serum factors.

Due to the low number of patients, it was not possible to make a correlation between indirect BAT and ASST. Nevertheless, the patients with negative ASST showed a positive result with the indirect BAT, while the only one who had a positive ASST was positive also to the basophil test.

All patients were also screened for the presence of other autoantibodies to check possible concurrent autoimmune diseases. We tested 12 patients of our small cohort for autoimmunity and we found only three patients that resulted positive for antinuclear antibodies. Noteworthy, all these three patients were also positive for BAT; among them, one was found ASST positive and one was found ASST negative, respectively. One of the BAT positive patient was negative for autoimmune test while on 2 BAT positive patients we did not perform the autoimmunity tests.

We didn't find any correlation between allergy status in terms of sIgE and skin tests and BAT results.

4. Discussion

It's important to emphasize the necessity of a proper diagnosis of AIU, as it represents the most severe form of chronic urticaria. Similarly to mast cells, basophils express IgE receptors on their surface. Cross-linking by IgE and an antigen or an antibody results in cell activation and secretion of cytokines, chemokines, and other mediators (Schroeder, 2011). It has been demonstrated that CD63, a cell surface marker, is not expressed on resting basophils, but it is up-regulated on degranulating basophils (Knol et al., 1991). Indeed, indirect BAT test using CD63 expression has been recently proposed to be used in the diagnostic workup of different allergic diseases, including CSU (Hoffmann et al., 2015; Szegedi et al., 2006). Furthermore, the possible role of seruminduced BAT in the follow-up of patients on allergen immunotherapy and anti-IgE treatment or in monitoring the resolution of allergy has been addressed (Hoffmann et al., 2015).

For which regards CSU, some studies have evaluated the employment of BAT in adult patients (Hoffmann et al., 2015; Curto-Barredo et al., 2016; Rauber et al., 2017; Gentinetta et al., 2011), showing its utility to identify patients with more active disease (Curto-Barredo et al., 2016) and to determine distinct subtypes in adult with CSU (Rauber et al., 2017). Conversely, very few and preliminary data exist about the employment of BAT test in children with CSU. To our knowledge, a single study by Netchiporouk et al. evaluated BAT test performance, aiming to standardize it in Canadian CSU children cohort (Netchiporouk et al., 2016).

Considering our cohort, ASST, that is the actual gold standard, was not applicable in the majority of the patients due to concomitant treatment with antihistamines. In this respect, we could perform ASST in 3 patients only.

Conversely, we could screen all patients of our cohort suffering from CSU applying BAT test. By the use of indirect BAT we could identify 37.5% of patients having AIU. Our result is quite in agreement with the literature data showing a percentage of up to 40% of autoimmune urticaria in children (Sahiner et al., 2011; Konstantinou et al., 2013). Even with the limitation of the small sample, our findings suggest that BAT may be a useful test also in the pediatric population, for which we experience more difficulties in performing ASST in clinical practice.

Indirect BAT may be a more feasible and sensitive test helping in the diagnosis of children suffering from AIU among those with CSU, identifying subjects with circulating autoantibodies able to activate basophils and directed toward the IgE receptor. However, like ASST, indirect BAT is not able to exactly distinguish which autoantibody activate basophils, anti-FcεRI, anti-FcεRII or anti-IgE.

Even if with limited numbers, our findings also suggest that BAT and ASST could give different results for patients (eg patients that can be positive only for ASST or BAT, or both) that could be integrated for a better diagnosis. However, an extended evaluation is necessary to define if this finding can be associated with a specific clinical phenotype.

Noteworthy, in our cohort BAT test resulted positive in the subjects showing positive autoimmunity tests, like ANA. Conversely, it resulted negative in the majority of subjects not showing them. This finding permits to speculate that AIU might be considered an epiphenomenon of a wider breakdown of the tolerance, that may affect multiple autoimmunity mechanism, including the IgE receptor on basophils and mast cells.However, the small sample do not allow us to draw conclusions about this issue, that should be further investigated.

At a methodological level, our study should be considered a pilot study.

The strenght of our study is that it is the second study showing data from children suffering from CSU, beside the study by Netchiporouk et al (Netchiporouk et al., 2016). In that study, the authors proposed a more sensitive cut-off at 1.8% in children, using the same standard assay.

Conversely, we adopted the technical cutoff of 5% CD63 pos basophils based on the standardized assay indicated by the manufacturer, as lower cut-off may not discriminate betweennonspecific and specific basophil activation. We observed no significant difference between the BAT results obtained from negative controls on the basophil donors vs sera of patient with negative result ($p = 0.65$), indicating the absence of non-specific activation mediated by serum factors and the validity of the cutoff adopted. Nevertheless, an extended evaluation of the technical cut-off with negative sera from healthy donor or from patient with urticaria from known etiology should be performed to increase the robustness of the data obtained for children sera. In contrast with the findings of Netchiporouk et al, we observed positive BAT test only on some of the heterologous basophil donors and not in all of them, with a casual behavior, and it was not possible to find one donor better than another. This finding is in agreement with the findings of Gentinetta et al. (2011) that suggested that modulating the quantity of IL3 in the stimulation can bypass this effect. We believe that in real clinical practice this approach is not feasible since it would imply major changes in the assay test system at any testing day with a continuous re-evaluation of the technical cutoff. We suggest that the use of multiple donors in indirect BAT could be the best way to overcome this bias effect of the basophil donor. Further investigation should be necessary to further explore this initial finding.

Our pilot study shows that BAT may be considered a useful functional tool in the diagnostic work-up of CSU, helping in identifying the subtype of autoimmune CSU in children. It is warranted to evaluate BAT performance on a larger number of children suffering from CSU to identify a clinical cut-off, prior to implementation in the routine clinical practice.

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