

Usefulness of the CD63 basophil activation test in detecting *Anisakis* hypersensitivity in patients with chronic urticaria: diagnosis and follow-up

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Summary

Background. The basophil activation test (BAT) has been recently described as a useful *in vitro* tool for diagnosis of allergy to *Anisakis* species in patients with acute urticaria.

Aim. To evaluate the relationship between sensitization to *Anisakis simplex* and chronic urticaria (CU), using flow cytometry analysis of *in vitro* BAT.

Methods. *A. simplex* sensitization was evaluated in patients with CU ($n = 57$) and in atopic ($n = 22$) and healthy controls ($n = 20$) by means of skin prick test (SPT), specific IgE and *Anisakis*-induced BAT using a triple-labelled strategy with anti-CD123, anti-human leucocyte antigen DR and anti-CD63 antibodies. During a follow-up period of 6 months in 10 patients with CU who accepted a fish-free dietary regimen, the diagnostic performance of the *in vivo* and *in vitro* methods was calculated, and changes in specific IgE and BAT were evaluated with respect to clinical response.

Results. A significant association between CU and *A. simplex* sensitization was found, with an overall prevalence of 75.4% in patients with CU (43/57) compared with 18% (4/22) and 10% (2/20) of the atopic and healthy controls, respectively ($P < 0.0001$). BAT (cut-off $> 13\%$) had the highest sensitivity and specificity, with significantly better ability than specific IgE testing for the identification of *A. simplex* sensitization in patients with CU. During the 6-month follow-up, clinical improvement was seen in all patients, and specific IgE and BAT results decreased to normal values in 6/10 (60%) and 10/10 (100%) patients, respectively.

Conclusions. BAT can be considered a reliable new *in vitro* method to evaluate *A. simplex* hypersensitivity in patients with CU, supplementing standardized procedures in both diagnosis and follow-up.

Introduction

Anisakis simplex is a parasite of fish, with a worldwide incidence. It is responsible for both human infection and IgE-mediated hypersensitivity reactions after intake of *A. simplex*-infested fish.^{1–3} Diagnosis of *A. simplex*

allergy is currently based on clinical history and *in vivo* skin prick test (SPT), confirmed by *in vitro* detection of serum-specific IgE by means of fluoroenzyme immunoassay and/or immunoblotting.^{3–6} However, the usefulness of these techniques in the diagnosis of a true allergic response to *A. simplex* is a subject of debate, due to crossreactivity between *Anisakis* antigens and other ascaridoid nematodes, microorganisms or parasites probably responsible for false-positive results.^{7–9} Extensive data support the use of the *in vitro* allergen-induced basophil activation test (BAT) in allergy diagnostic investigations, based on flow cytometry quantification of surface markers such as CD63 and/or CD203c,

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which are expressed or upregulated on activated basophils and which mirror histamine release. The diagnostic value of BAT has been reported in allergies to pollen, house dust mite, venoms, drugs and food,^{10–12} and in autoimmune chronic urticaria (CU).^{13–15} A recent study proposed *A. simplex*-induced BAT as a new additional laboratory tool in diagnosing *A. simplex* allergy, supplementing specific IgE determination.¹⁶ It has been reported that acute urticaria and anaphylactic shock are often associated with IgE-mediated hypersensitivity to *A. simplex*, with symptoms developing within a few minutes to hours after eating fresh raw or undercooked fish.^{2,3,17,18} On the other hand, conflicting data exist concerning the association of *A. simplex* sensitization with recurrent CU,^{2,19} although a recent study documented the relevance of *A. simplex* as an aetiological factor in a high proportion of patients with CU in a Spanish population who were evaluated for the presence of specific IgE antibodies.²⁰

The aim of the present study was to determine the prevalence of *A. simplex* sensitization in patients with clinical history of CU by means of *Anisakis*-induced BAT and to compare the diagnostic performance of this *in vitro* method with the well-established diagnostic tests of *in vitro* serum allergen-specific IgE and *in vivo* SPT. In a subset of patients with CU ($n = 10$) who experienced clinical improvement after 6 months of dietary-based treatment, we further compared the results of specific IgE and of BAT before and after the diet, to reveal the possible modification of the *in vitro* immunological parameters and their relationship with clinical response.

Methods

The study was approved by the institutional ethics committee. Informed consent was not required.

Subjects

In total, 57 ($n = 57$) adult patients (8 men, 49 women; mean $\pm$ SD age 42 ± 11 years, range 24–54) with a clinical diagnosis of CU, defined as recurrent wealing occurring at least twice a week for > 6 weeks, were enrolled. All patients regularly consumed fish and about 75% (43/57) associated their symptoms of urticaria with fish consumption, either cooked or raw, especially with sardines, mackerel and cod, which are common dishes in their areas of residence (central and southern Italy). An extensive and detailed medical history investigating all possible eliciting factors was taken and a physical examination to establish the nature of the CU lesions (duration, number, size, distribution) performed

in all patients.^{21,22} Patients with associated physical urticaria and clinical evidence of urticarial vasculitis were excluded. Laboratory investigations including full blood count, erythrocyte sedimentation rate, C-reactive protein level, urinalysis, chemistry panel and thyroid function tests gave negative results in all patients. At the first visit, disease severity was estimated for each patient, using the Urticaria Activity Score (UAS), recording on a 3-point scale the number and average size of lesions and the itch severity.²² Thus, the UAS ranged from 0 to 6 for each patient with CU, with a mean of 3.5 ± 1.3 . All patients had to be free of antihistaminic drugs or systemic corticosteroids for at least 2 weeks before blood sampling.

The control group comprised 12 healthy ($n = 20$) subjects with no previous history of any allergy and/or urticaria, and 22 controls with atopy sensitized to common aeroallergens such as moulds, animal dander, mites or pollens (positive SPT and serum-specific IgE result to at least one allergen). Positive SPT and/or IgE titre > 0.35 kU/L were chosen as the criteria for *A. simplex* sensitization.

Skin prick test

SPT was performed for all subjects, using a commercially acquired larval crude extract of *A. simplex* (2 mg/mL; ALK-Abelló, Lainate, Italy). Histamine 10 mg/mL and saline solutions were used as positive and negative controls, respectively. The response was recorded after 15 min. Subjects with a weal diameter ≥ 3 mm larger than that of the negative control were regarded as positive.

Anisakis simplex specific IgE determination

Specific IgE to *A. simplex* were determined on serum samples (CAP-FEIA; UniCAP; Pharmacia Upjohn, Uppsala, Sweden) and results ≥ 0.35 kU/L were considered positive. Fish allergy was excluded by analysing specific IgE in all subjects. Furthermore, in patients with CU, serum samples were also tested for specific IgE to some allergens related to some of the crossreactivities reported for *A. simplex*, such as house dust mite (*Dermatophagoides pteronyssinus*) and shrimp, but IgE serum levels were < 0.35 kU/L for both allergens.

Anisakis-induced basophil activation test

Flow-cytometry expression of CD63 on activated basophils was measured on heparinized whole blood within 24 h of sampling. Briefly, 100 μ L of blood

sample were incubated at 37 °C in a water bath for 15 min with 20 µL of allergen (2 µg/mL final concentration of *A. simplex* crude extract; ALK-Abelló) in stimulation buffer containing 10 ng/mL interleukin-3.

Dosing experiments were initially performed using BAT with serial increasing dilutions of *A. simplex* extract (0.1, 1, 2, 5, 10 µg/mL) in five subjects allergic to *A. simplex* and in six healthy controls. The concentration that produced the greatest reactivity was 2 µg/mL, thus this was chosen for all future experiments. The addition of wash buffer alone and 2 µmol/L formyl-methionyl-leucyl-phenylalanine to distinct tubes served to establish, respectively, baseline values and positive control of CD63 expression. After stopping degranulation, cells were stained with 20 µL of CD63-FITC/CD123-PE/anti-HLA-DR-PerCP antibody cocktail (BD FastImmune; Becton-Dickinson, San Jose, CA, USA) and incubated for 20 min at room temperature. Samples were lysed, washed and resuspended in 300 µL of 0.5% paraformaldehyde to be measured in a flow cytometer (FACScan; Becton-Dickinson Immunocytometry System, San Jose, CA, USA) within 2 h, identifying CD63+ CD123+ HLA-DR- basophils as described previously.¹³

Evaluation of specific IgE and *Anisakis*-induced basophil activation test during dietary-based follow-up

Of the 57 patients with CU, 10 patients with specific IgE > 0.35 kU/L and positive BAT results were put on a fish-free diet for 6 months to evaluate the possible modification of specific IgE and CD63 and their

association with clinical response after 3 (T1) and 6 months (T2).

Statistical analysis

The χ^2 test, Fisher exact test and Mann-Whitney *U*-test were used for comparison of patients with CU and controls according to the categorical or quantitative nature of the variables. The Spearman correlation coefficient was used for correlation between the *in vivo* and *in vitro* tests. Receiver operating characteristic (ROC) curve analysis was performed to calculate the optimum cut-off (percentage of CD63+ cells) value corresponding to the best specificity and sensitivity, and the area under the curve (AUC) was calculated for both specific IgE and BAT to compare the best efficiency of the *in vitro* tests. Statistical analyses were performed with the Analyse-it programme (version 1.39; Analyse-it Software Ltd, Leeds, UK). All data are shown as mean $\pm$ SD, and *P* < 0.05 was considered significant.

Results

Prevalence of *Anisakis* sensitization in chronic urticaria

We found a significant association between CU and *A. simplex* sensitization when evaluated with the *in vivo* (SPT) and *in vitro* tests (specific IgE and BAT), with an overall prevalence of 75.4% in patients with CU (43/57) compared with 18% (4/22) of atopic controls and 10% (2/20) of healthy donors (Fisher exact test: *P* < 0.0001). Results for each test are summarized in Table 1.

Table 1 Prevalence of *Anisakis simplex* sensitization in patients with chronic urticaria (CU) and atopic and healthy controls by means of *in vivo* (SPT) and *in vitro* tests (specific IgE and BAT).

Sensitization to <i>Anisakis simplex</i>	Subjects Patients with CU		Atopic controls		Healthy controls		Test	
	<i>n</i>	Mean $\pm$ SD	<i>n</i>	Mean $\pm$ SD	<i>n</i>	Mean $\pm$ SD	Sensitivity, %	Specificity, %
SPT (weal size, mm)								
Positive (≥ 3)	36	4.81 $\pm$ 2.15	3*	2.08 $\pm$ 0.5**	2*	2.5 $\pm$ 0.34**	63	86†
Negative (< 3)	21		19		18			
Specific IgE, kU/L						0.41 $\pm$ 0.15**		
Positive (≥ 0.35)	35	18.5 $\pm$ 14.5	4*	0.85 $\pm$ 0.2**	2*		61	82†
Negative (< 0.35)	22		18		18		61	90‡
BAT, % CD63 cells								
Positive ($\geq 13\%$)	38	48 $\pm$ 25	0*	9 $\pm$ 2**	0*	6 $\pm$ 3**	67	100†
Negative (< 13 %)	19		22		20		66	100‡

BAT, basophil activation test; CU, chronic urticaria; SPT, skin prick test. Comparison between patients with CU and both atopic and healthy controls: **P* < 0.0001 (Fisher's exact test); ***P* < 0.0001 (Mann-Whitney *U*-test). Patients with CU vs. †atopic controls; ‡healthy controls.

Diagnostic performance of the tests in evaluating *Anisakis* sensitization in chronic urticaria

The diagnostic performance of all three tests was calculated in terms of sensitivity and specificity (Table 1) by ROC analyses, with a cut-off value for *Anisakis*-induced BAT of 13% CD63+ cells. Although all three tests had similar sensitivity for the patient group compared with both control groups, a specificity of 100% was reached only when *A. simplex* sensitization was determined by BAT. AUC calculated to compare the two *in vitro* tests was 0.79 for the specific IgE test and 0.87 for BAT ($P = 0.019$), indicating that BAT was significantly better than the specific IgE test in correctly identifying *A. simplex* sensitization in patients with CU. A significant correlation was also found between *Anisakis*-induced BAT and both SPT ($r = 0.87$; $P < 0.0001$) and specific IgE serum levels ($r = 0.78$; $P < 0.0001$) (Fig. 1).

Modification of serum levels of specific IgE and *Anisakis*-induced basophil activation test during fish-free diet

In 10 of 57 patients with CU who excluded fish from their diet for a 6-month period, specific IgE and *Anisakis*-induced

CD63 expression were evaluated before (T0), after 3 months (T1) and after 6 months (T2) of the diet, in parallel with clinical assessment of CU by UAS. Specific IgE mean serum levels were significantly reduced at T1; in 3 of the 10 (30%) patients, values were < 0.35 kU/L. At the end of the diet (T2) a further reduction was seen, with specific IgE at normal levels in 6/10 patients (60%) (Fig. 2a). Compared with T0, a significant reduction in mean percentage CD63+ basophils was detected (cut-off value $< 13\%$) at T1 in 7/10 patients (70%), further decreasing at T2 below the calculated cut-off in all subjects (Fig. 2b). All patients had an early clinical response (UAS 4.3 ± 1.2 at T1 vs. 1.4 ± 1 at T2; $P < 0.0001$) and complete clearing of skin lesions at T2.

Discussion

It is now well documented that *in vitro* BAT can be a reliable laboratory tool to detect allergen-specific responses^{10,12} and to identify autoimmunity in patients with CU.^{13–15} It was recently reported that BAT can also be useful for diagnosis of *Anisakis* allergy using both *Anisakis* crude extract and recombinant allergen (rAni s4).^{16,23} *A. simplex* has been described as a relevant

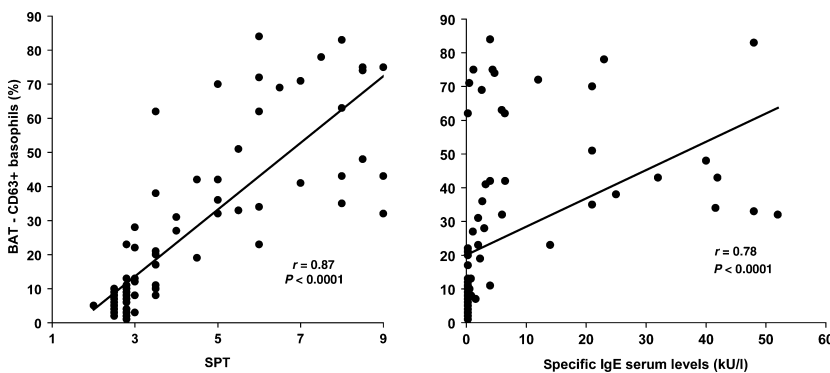


Figure 1 Significant correlation between results obtained with basophil activation test, skin prick test and specific IgE as calculated by Spearman rank correlation coefficient (r).

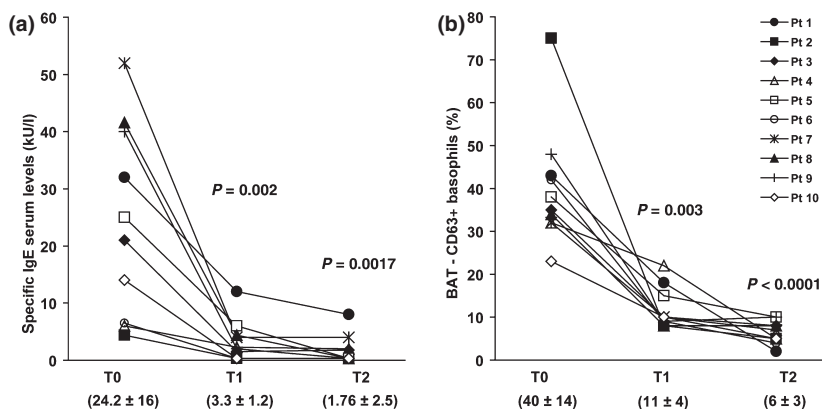


Figure 2 Significant modification of (a) specific IgE serum levels and (b) basophil activation test in 10 patients with chronic urticaria during a 6-month follow-up on a fish-free diet. Single paired determination for each patient (Pt) and mean $\pm$ SD are reported. T0, before diet; T1, after 3 months; T2, after 6 months.

aetiological factor in acute urticaria and anaphylaxis, and more recently, a high frequency of *A. simplex* sensitization has been documented in patients with CU, based on positive SPT and specific IgE antibodies against *A. simplex*.²⁰ To our knowledge, this is the first study aimed at evaluating the prevalence of *A. simplex* sensitization in patients with CU by means of *Anisakis*-induced BAT, and comparing the diagnostic performance of this method with both *in vitro* serum allergen-specific IgE and *in vivo* SPT. In agreement with recently published data,²⁰ we found a high proportion of patients with CU were sensitized to *A. simplex* as measured by all three tests, with an overall prevalence of 75.4% in patients with CU compared with a sensitization rate of 18% in atopic controls and 10% in healthy donors. When we compared the diagnostic performance of the tests, *Anisakis*-induced BAT was found, at the calculated cut-off of 13% CD63-expressing cells, to have the best sensitivity and specificity in detecting *A. simplex* sensitization in patients with CU, and the significant difference found between the AUC of BAT and the specific IgE test confirmed that BAT had the higher overall diagnostic accuracy. Our results complement the findings of Gonzalez-Muñoz *et al.*,¹⁶ who first described the usefulness of *Anisakis*-induced BAT for the diagnosis of anisakidosis in patients with acute urticaria associated with *Anisakis* allergy, with a higher sensitivity than we found in our study. As we used the same laboratory protocol for *Anisakis*-induced BAT as that used by Gonzalez-Muñoz *et al.*, the difference possibly relates to the patient populations under study (acute vs. chronic urticaria). Although allergic reactions are rarely a cause of CU, we hypothesize that *A. simplex* hypersensitivity may have a causative effect in the subset of patients with CU in whom we found significant clinical improvement after a fish-free diet, as measured by UAS reduction and immunological parameters (reduced specific IgE and BAT values). To date, no clear guidelines exist concerning dietary restrictions for patients with *A. simplex* hypersensitivity, although some authors have reported clinical improvement in sensitized patients after avoiding fish or eating only frozen or well-cooked fish, in parallel with a significant decrease in total and specific IgE serum levels during follow-up.^{19,24,25}

Our study provides the first evidence that *Anisakis*-induced CD63 expression is significantly reduced during dietary treatment, in parallel with the clinical improvement in all patients at the end of follow-up. BAT had a better correlation with clinical response compared with specific IgE levels, and may be a new *in vitro* laboratory tool to monitor the effectiveness of dietary treatment in patients with *A. simplex* hypersensitivity.

In conclusion, our study confirms that measurement of CD63 by BAT is a reliable new *in vitro* method to evaluate *A. simplex* hypersensitivity, with better efficiency in detecting *A. simplex* sensitization in patients with CU and better evaluation of immunological changes in patients after dietary restriction.

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