

Original article

Validation of basophil histamine release against the autologous serum skin test and outcome of serum-induced basophil histamine release studies in a large population of chronic urticaria patients

Background: Endogenous histamine-releasing factors (HRFs) are involved in 30–60% of patients with chronic urticaria (CU). Evidence for their existence comes from *in vivo* studies of autoreactivity with the autologous serum skin test (ASST), *in vitro* immunoassays demonstrating autoantibodies against the immunoglobulin E (IgE) or the high affinity IgE receptor (FcεRI) and serum-induced histamine release (HR) from basophils and mast cells. We have examined the correlation between the ASST and a new basophil histamine-releasing assay (the HR-Urticaria test) in a group of well-characterized CU patients and subsequently determined the frequency of HR-Urticaria-positive sera from a larger population of CU patients.

Subjects: Group 1 consisted of 28 patients with CU (16 were ASST-positive) 20 patients with atopic dermatitis, 24 patients with allergy to birch and nine healthy controls. Group 2 consisted of 873 unselected CU patients.

Methods: White blood cells containing 1–2% basophils from a healthy nonatopic donor were incubated with patients sera in the presence of interleukin (IL)-3. Histamine was measured by the glass fibre method.

Results: Using the ASST as the true outcome, the HR-Urticaria test showed a sensitivity and specificity of 75% in group 1 using a cut-off value for HR of > 16.5%. None of the controls was positive in the HR-Urticaria test. In group 2, we found no difference in the frequency of positives between male (34.6%, $n = 254$) and female adults (35.1%, $n = 576$) but twice as many females as males were tested.

Conclusions: Our studies have shown that the HR-Urticaria test has a good sensitivity and specificity for endogenous HRFs demonstrated by the ASST in patients with CU and that about one-third of unselected patients with CU have a positive result.

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Approximately 30–60% of patients with idiopathic spontaneous chronic urticaria (CU) (1) react with a wheal-and-flare response to intradermally injected autologous serum (2), indicating the presence of histamine-releasing factors (HRF) in blood (3, 4). Only about 50% of CU patients with a positive autologous serum skin test (ASST) seem to have functional autoantibodies on basophil assays.

The understanding that CU may be caused by endogenous vasoactive factors, including autoantibodies, is important for patients with severe disease as their reduction in quality of life has been compared with that of patients awaiting coronary by-pass surgery (5). An early diagnosis of autoimmune urticaria should optimize the care of these patients and improve the outcome of their disease. Bindslev-Jensen et al. (6) recommended that

patients with suspected CU should be investigated with the ASST and that those with a positive ASST result should be confirmed by basophil histamine release (HR) studies.

With the use of enzyme-linked immunosorbent assay (ELISA) systems (7, 8) autoantibodies against FcεRI have been demonstrated in patients with CU, atopic dermatitis, dermatomyositis, pemphigus vulgaris, bullous pemphigoid, angio-oedema, urticarial vasculitis, and healthy controls (HC) but only in CU patients do they seem to be functional by means of ASST and HR from basophils or mast cells. Furthermore, there is strong evidence to suggest that the basophil is involved in CU. Both the capability of basophils to release histamine in response to immunological stimuli and the absolute number of basophils in peripheral blood from patients

with CU are decreased when compared with HC (9). The CU patients with functional autoantibodies are found to have more severe systemic symptoms and larger wheals than the patients without autoantibodies (10) and a longer duration of disease (11).

We have compared a standardized laboratory test using basophils for HR against the ASST as a 'gold standard' of endogenous histamine-releasing activity, in contrast to previously published reports of the ASST being compared against functional autoantibodies defined by *in vitro* HR studies on basophils, because a low-molecular weight nonautoantibody 'mast cell-specific factor' may be present in the serum of some CU patients (12, 13) who have negative basophil HR assays. Although the ASST appears to be a clinically useful marker of endogenous vasoactive factors, including autoantibodies, which are relevant to understanding urticaria pathogenesis and its treatment it is time-consuming and not accessible to all clinicians.

The aim of this work has been to standardize an *in vitro* basophil assay for use in the diagnosis of autoimmune urticaria and investigate the frequency of positive results in a group of patients with unselected CU.

Material and methods

Patients

Group 1. Sera from a small group of CU patients presenting with the ordinary pattern of disease ($n = 28$) were examined initially for histamine-releasing activity. An ASST was positive in 16 of them. An additional group of sera from nine HC, 20 patients with atopic dermatitis and 24 patients with allergy to birch (no ASST performed) were tested with the HR-Urticaria test as well. All sera in the initial study group were submitted to the laboratory blinded with respect to diagnosis and outcome of the ASST.

Group 2. Sera from 873 unselected consecutive patients presenting to specialist clinics with CU were tested for serum-induced HR in the HR-Urticaria test. About 830 were adults and 43 were children (Table 1).

Methods

Autologous serum skin test. Venous blood was drawn into sterile tubes without clotting accelerator (Vacutainer®, Becton Dickinson,

Franklin Lakes, NJ, USA), and allowed to clot at room temperature for 30 min. Serum was separated by centrifugation at 500 *g* for 15 min and divided into aliquots for use in the ASST and in basophil HR assays. Samples (50 µl) of autologous serum and 0.9% sterile saline were injected intradermally into volar forearm skin with at least 3 cm between injection sites. Areas known to have been involved in spontaneous weals in the last 24 h were avoided. The ASST was considered positive if a pink weal response 1.5 mm or greater than an adjacent saline control was seen at 30 min (14).

Serum-induced basophil histamine release – HR-Urticaria test. Serum-induced basophil HR was performed as previously described (15). In brief, basophil leucocytes were incubated with serum for 60 min at 37°C with 40 µl serum diluted 1 : 4 or 1 : 8 (final concentration). To compensate for nonspecific HR because of changed protein concentration, incubations were performed in the absence and presence of 0.0125 M ethylenediaminetetraacetic acid (EDTA). Serum and released histamine was removed and the cells lysed using 20 µl of 7% perchloric acid. After lysis of the cells 200 µl PIPES were added and the samples were centrifuged at 2000 *g* for 10 min and the histamine content in the filtrate was measured using the glass fibre method (RefLab, Copenhagen, Denmark) (16).

Histamine release was expressed as a percentage of total histamine content. Spontaneous HR from the cells was < 5% when incubated with serum at final dilutions of 1 : 8 or 1 : 4 in the total reaction volume.

Basophils from two normal nonatopic subjects were initially tested for HR induced by anti-immunoglobulin E (IgE) and sera from urticaria patients. Considerably variability of response was observed, but generally the same ranking of sera was observed. One donor whose basophils responded to both anti-IgE and to many of the urticaria sera was chosen for the studies reported here. This donor was nonatopic with an IgE level of 8 kU_A/l.

Assessment of sensitivity and specificity. The assay developed and described here (the HR-Urticaria test) uses the human basophil as a model for the reaction seen in the skin of patients. In order to standardize the *in vitro* assay, the ASST outcome was used as a 'gold standard' to determine the sensitivity and specificity of the basophil assay at different cut-off values for histamine release. The calculations of sensitivity and specificity are based upon the results from ASST and the HR-Urticaria test using only the well-characterized CU patients from group 1. Sensitivity was calculated as (ASST- and HR-Urticaria test-positives)/(ASST-positives) and specificity was calculated as (ASST- and HR-Urticaria test-negatives)/(ASST-negatives).

Results

Group 1

Basophils from the healthy donor described in Methods were incubated with sera from the well-characterized CU patients or HC and HR was determined. Mean (SD) HR for the HC at a 1 : 4 final serum dilution was 12.6% (2.8) yielding a threshold value of 18.2% (mean ± 2 SD) for significant HR in the Urticaria-HR assay. Using this cut-off value, the sensitivity and specificity for basophil HR were 56.3 and 83.3% by comparison with the ASST.

Maximum sensitivity and specificity of the HR-Urticaria test was achieved by assuming a threshold of HR

Table 1. Sex and age distribution of 873 consecutive patients

Age (years)	Positive, % (n)	
	Female	Male
0–16 ($n = 43$)	43.5 (23)	35.0 (20)
17+ ($n = 830$)	35.1 (576)	34.6 (254)
Total (873)	35.2	

N designates the total number of patients in the indicated groups. A chi-squared test was performed on the data and showed no difference in the frequency of positives between the groups ($P = 0.655$).

between 16.5 and 17.5% (closed circles, Fig. 1) for 1 : 4 serum dilution. By contrast, the sensitivity of the assay was considerably less with 1 : 8 serum dilution (open triangles, Fig. 1) although the specificity at this dilution was consistently high. Based on these results a threshold cut-off value of 16.5% was taken for all further studies of CU sera at a 1 : 4 dilution to discriminate between those with and without histamine-releasing activity. This value was comparable with the threshold value derived from the mean $\pm$ 2 SD release of HC sera on the same basophil donor.

Patients were grouped into those with and without a positive ASST. Twelve of 16 patients with a positive ASST and four of 12 with a negative ASST but none of the HC elicited HR above 16.5% (Fig. 2). None of the sera from the group of atopic dermatitis patients or birch pollen allergic patients elicited HR above 16.5% (results not shown). All patients had a greater response to 1 : 4 than 1 : 8 serum dilution in the test (data not shown).

Group 2

A total of 873 CU sera received consecutively by our laboratory were tested by the HR-Urticaria test (Table 1). In this unselected population there was no difference in the frequency of positives between male and female adults ($P = 0.897$) or the entire group ($P = 0.719$). However, females predominated in a ratio 2 : 1. In the group of children between 0 and 16 years of age there seemed to be an over-representation of females but it was not statistically significant ($P = 0.725$).

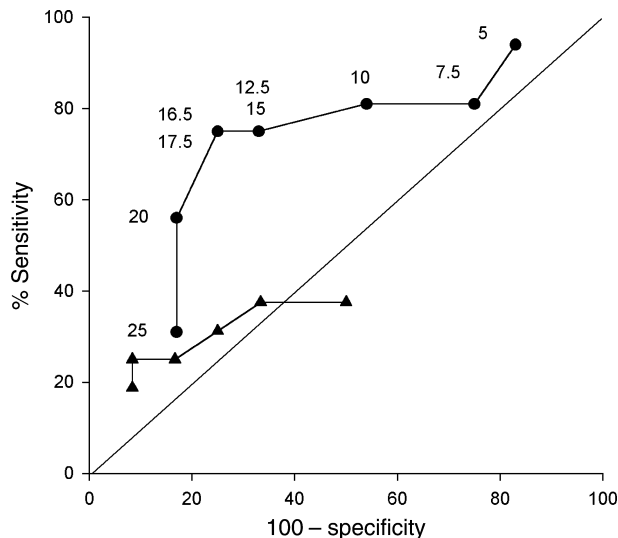


Figure 1. Receiver operating characteristic (ROC) curves based on results with autologous serum skin test (ASST) and histamine release from group 1, $n = 28$. Curves shown are 1 : 8 serum dilution ($\blacktriangle$) and 1 : 4 serum dilution ($\bullet$) induced maximum histamine release.

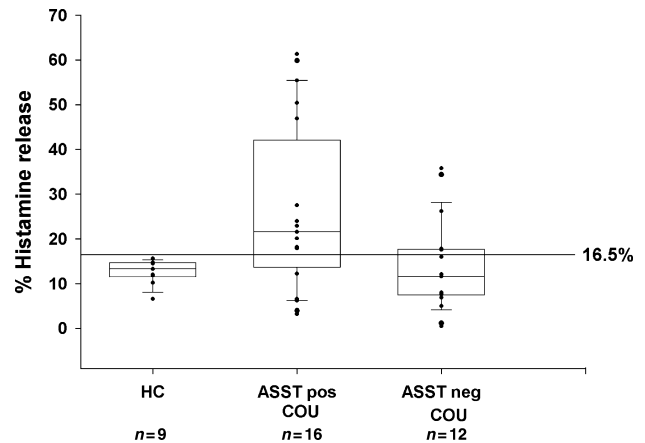


Figure 2. Activation of human basophils by sera of chronic urticaria (CU) patients with either positive or negative autologous serum skin test (ASST) and healthy controls (HC). Maximum percentage histamine release is indicated in the box and whisker plot after incubation with both 1 : 4 and 1 : 8 dilutions of sera.

In Fig. 3 the accumulated frequency of test-results for both serum dilutions is shown as a function of histamine release. As indicated in Fig. 3 there is a 'concentration dependency' in the basophil HR elicited when using the two serum dilutions. When using the 1 : 4 serum dilution, 35.2% of the CU sera elicited a basophil HR above 16.5%. At 1 : 8 serum dilution, only 10% of the tested individuals showed significant histamine release. In this unselected dermatological material with no clear distinction between positive and negative test-results, the

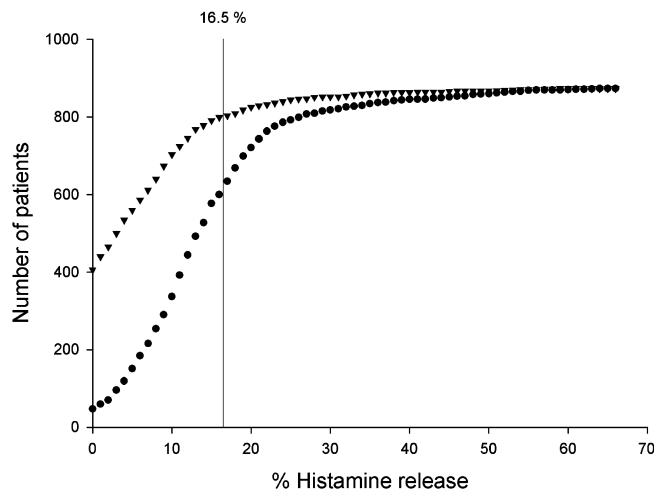


Figure 3. Distribution of histamine release results from 873 patients using different serum dilutions. The number of patients on the ordinate axis represents the accumulated number of tested patients eliciting a histamine release below the indicated histamine release. Curves shown are 1 : 8 serum dilution ($\blacktriangle$) and 1 : 4 serum dilution ($\bullet$) induced maximum histamine release.

distribution of test-results seems to be continuous (Fig. 3).

Discussion

There is evidence to suggest that most functional autoantibodies bind to the FcεRI in the presence of IgE (noncompetitive) but a minority compete with IgE for the receptor and will therefore not be detected on basophils primed with IgE (4). It follows that sera to be tested should be incubated with both stripped and native basophils to be certain that all autoantibodies can be detected in CU. However, it appears that priming with interleukin (IL)-3 lowers the threshold for activation of the basophil so that the level of free FcεRI is sufficient for activation, perhaps through costimulation with other HRFs.

It has been reported that natural anti-FcεRI antibodies can interfere with basophil HR induced by autoimmune urticaria sera (17), this can be due to methodological differences as the amount of IL-3 used to prime the basophils varies from 100 (17) to 0.5 ng/ml used in our method. It has been shown that IL-3 can induce HR in concentrations down to 10 ng/ml (18).

We present here a cellular assay able to distinguish between patients with and without functional HRFs with a high sensitivity and specificity. We have defined the threshold of the HR-Urticaria test by maximizing sensitivity and specificity on a population of well-characterized CU patients. This value is nearly the same as the threshold derived from the mean $\pm$ 2 SD of HR with HC sera. Using a 16.5% cut-off value on a group of HC we found no individuals with positive HR. In the group of patients with CU, four of 16 patients with a positive ASST failed to elicit HR above 16.5% and four of 12 patients with a negative ASST elicited HR above 16.5%. The difference between the two groups was significant ($P = 0.0313$).

Up to now we have tested 873 sera sent to our laboratory with a clinical diagnosis of CU (Table 1) but detailed clinical findings on these patients were not available so this relatively unselected group may include patients with physical and vasculitic urticarias. Applying

the results found in the well-characterized group of patients tested using the ASST we found no difference in the distribution of positives between male and females, however, the frequency of women tested to men were found to be 2 : 1 in keeping with the expected gender difference in CU. When testing for HRFs it seems necessary to use at least a 1 : 4 serum dilution (Fig. 3), otherwise the number of positives will be underestimated. Using the 1 : 8 serum dilution, less than a third of the sera found positive using 1 : 4 serum dilution also tested positive. However, it may be useful to use different serum dilutions in order to evaluate the amount of HRFs.

In this study, we have compared the basophil HR test against the ASST test as a 'gold standard'. The discordance between a positive ASST and a negative HR-Urticaria test may be explained by a low titre of HRFs or the presence of 'mast cell-specific' HRFs. A negative ASST and a positive HR-Urticaria test might be explained by some inherent variability of the ASST if, for instance, the skin test had been placed in an area of previous wealing that was relatively refractory to further stimulation.

In conclusion, serum-induced HR using the commercial HR-Urticaria test confirmed the findings of other groups that the outcome of basophil mediator release assays and the ASST are related but not identical. In addition, our studies demonstrated a reasonably high frequency of sera-inducing HR (35%) in patients with CU. In the present study, only one basophil donor has been used during the 1-year study period. We have however, seen in preliminary studies that other basophil donor cells can be used provided that they respond to a selected panel of positive sera. Further studies are however, needed to demonstrate if other factors than autoantibodies against the IgE or FcεRI are involved in the release process, such as cytokines and/or complement split products (C3a and C5a) or as yet other unidentified HRFs.

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References

1. Zuberbier T. Urticaria. *Allergy* 2003;**58**:1224–1234.
2. Niimi N, Francis DM, Kermani F, O'Donnell BF, Hide M, Kobza-Black A et al. Dermal mast cell activation by autoantibodies against the high affinity IgE receptor in chronic urticaria. *J Invest Dermatol* 1996;**106**:1001–1006.
3. Sabroe RA, Greaves MW. The pathogenesis of chronic idiopathic urticaria. *Arch Dermatol* 1997;**133**:1003–1008.
4. 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.
5. O'Donnell BF, Lawlor F, Simpson J, Morgan M, Greaves MW. The impact of chronic urticaria on the quality of life. *Br J Dermatol* 1997;**136**:197–201.
6. Bindslev-Lensen C, Finzi A, Greaves M, Camarasa J, Ortonne JP, Schopf E et al. Chronic urticaria: diagnostic recommendations. *J Eur Acad Dermatol Venereol* 2000;**14**:175–180.
7. Fiebiger E, Hammerschmid F, Stingl G, Maurer D. Anti-FcεRIα autoantibodies in autoimmune-mediated disorders. Identification of a structure-function relationship. *J Clin Invest* 1998;**101**:243–251.

8. Zuberbier T, Henz BM, Fiebiger E, Maurer D, Stingl G. Anti-FcεRIα serum autoantibodies in different subtypes of urticaria. *Allergy* 2000;**55**:951–954.
9. Sabroe RA, Francis DM, Barr RM, Black AK, Greaves MW. Anti-FcεRI auto antibodies and basophil histamine releasability in chronic idiopathic urticaria. *J Allergy Clin Immunol* 1998;**102**(4 Pt 1):651–658.
10. Sabroe RA, Seed PT, Francis DM, Barr RM, Black AK, Greaves MW. Chronic idiopathic urticaria: comparison of the clinical features of patients with and without anti-FcεRI or anti-IgE autoantibodies. *J Am Acad Dermatol* 1999;**40**:443–450.
11. Toubi E, Kessel A, Avshovich N, Bamberger E, Sabo E, Nusem D et al. Clinical and laboratory parameters in predicting chronic urticaria duration: a prospective study of 139 patients. *Allergy* 2004;**59**:869–873.
12. Sabroe RA, Fiebiger E, Francis DM, Maurer D, Seed PT, Grattan CE et al. Classification of anti-FcεRI and anti-IgE autoantibodies in chronic idiopathic urticaria and correlation with disease severity. *J Allergy Clin Immunol* 2002;**110**:492–499.
13. Fagiolo U, Kricek F, Ruf C, Peserico A, Amadori A, Cancian M. Effects of complement inactivation and IgG depletion on skin reactivity to autologous serum in chronic idiopathic urticaria. *J Allergy Clin Immunol* 2000;**106**:567–572.
14. Sabroe RA, Grattan CE, Francis DM, Barr RM, Kobza BA, Greaves MW. The autologous serum skin test: a screening test for autoantibodies in chronic idiopathic urticaria. *Br J Dermatol* 1999;**140**:446–452.
15. Brunetti L, Francavilla R, Miniello VL, Platzer MH, Rizzi D, Lospalluti ML et al. High prevalence of autoimmune urticaria in children with chronic urticaria. *J Allergy Clin Immunol* 2004;**114**:922–927.
16. Skov PS, Mosbech H, Norn S, Weeke B. Sensitive glass microfibre-based histamine analysis for allergy testing in washed blood cells. Results compared with conventional leukocyte histamine release assay. *Allergy* 1985;**40**:213–218.
17. Pachlopnik JM, Horn MP, Fux M, Dahinden M, Mandallaz M, Schneeberger D et al. Natural anti-FcεRIα autoantibodies may interfere with diagnostic tests for autoimmune urticaria. *J Autoimmun* 2004;**22**:43–51.
18. Tedeschi A, Salmaso C, Di Donato M, Lorini M, Miadonna A. Granulocyte-macrophage colony-stimulating factor and interleukin-3 cause basophil histamine release by a common pathway: downregulation by sodium. *Immunology* 1999;**96**:164–170.