

The autologous serum skin test: a screening test for autoantibodies in chronic idiopathic urticaria

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Summary

One-third of patients with chronic idiopathic urticaria (CIU) have circulating functional autoantibodies against the high affinity IgE receptor FcεRI, or IgE. The intradermal injection of autologous serum causes a weal and flare reaction in many patients with CIU, and this reaction forms the basis of the autologous serum skin test (ASST). We have determined the parameters of the ASST which define the optimal sensitivity and specificity for the identification of patients with autoantibodies. Two physicians (R.A.S. and C.E.H.G.) performed ASSTs in a total of 155 patients with CIU, 40 healthy control subjects, 15 patients with dermatographism, nine with cholinergic urticaria and 10 with atopic eczema. Patients were classified as having functional autoantibodies by demonstrating *in vitro* serum-evoked histamine release from the basophils of two healthy donors. There were significant differences ($P < 0.001$) in the mean weal diameter, weal volume, weal redness and flare area of the intradermal serum-induced cutaneous responses at 30 min between patients with CIU with autoantibodies and either those without autoantibodies or control subjects. The optimum combined sensitivity and specificity of the ASST was obtained if a positive test was defined as a red serum-induced weal with a diameter of ≥ 1.5 mm than the saline-induced response at 30 min. For R.A.S. and C.E.H.G., the ASST sensitivity was 65% and 71% and specificity was 81% and 78%, respectively. Using these criteria, the following subjects had positive ASSTs: none of 15 dermatographics, none of 10 atopics, one of nine cholinergics and one of 40 controls.

Key words: autoantibodies, autologous serum skin test, chronic idiopathic urticaria, high affinity IgE receptor

Chronic idiopathic urticaria (CIU) is defined as widespread short-lived (< 24 h) weals occurring daily or almost daily for at least 6 weeks, where a predominant physical cause has been excluded.¹ CIU is extremely disabling in its severe form,² and can be difficult to treat. Mast cell degranulation is of central importance in the pathogenesis of CIU,³ but until recently it was unusual to identify underlying specific activating factors.⁴ There is now strong evidence for an autoimmune basis to the disease in some patients, and circulating functional histamine-releasing autoantibodies reactive against either the α subunit of the high affinity IgE receptor (FcεRI α) or IgE have been identified in about one-third of patients with CIU.^{5–9}

Trials of immunomodulatory treatments including cyclosporin A, plasmapheresis and intravenous immunoglobulin have been performed in patients with severe CIU unresponsive to antihistamines.^{10–12} However, it

has not been established whether these treatments are more effective in patients with autoantibodies than in those without. A rapid and reliable clinical test to differentiate between patients with and without circulating functional autoantibodies would be of value in evaluating the efficacy of immunomodulatory treatment. Autoantibodies in patients' serum can be detected by serum-induced histamine release from the basophils of healthy donors, or by Western blot analysis.^{6–8} Recently, an enzyme-linked immunosorbent assay (ELISA) test for autoantibodies has been developed.¹³ Neither Western blot nor ELISA distinguish functional histamine-releasing autoantibodies from non-functional autoantibodies. At present, these tests are done only in a few specialist laboratories, are time-consuming to perform, and are not available to the majority of clinicians. The induction of a weal and flare response by the intradermal injection of autologous serum in some

patients was the original observation¹⁴ which led to the identification of circulating autoantibodies in CIU, and provides the basis of the autologous serum skin test (ASST; Fig. 1). We have determined the parameters of the ASST which give the optimum sensitivity and specificity for detecting patients with autoantibodies.

Subjects and methods

Subjects

ASSTs were performed by two physicians, R.A.S. and C.E.H.G. The former obtained 68 patients with CIU, nine patients with cholinergic urticaria and 15 with dermographism from specialist urticaria clinics at St John's

Institute of Dermatology. Diagnoses of cholinergic urticaria and dermographism were confirmed by standard testing.¹⁵ Ten patients with atopic eczema, all satisfying the Hanifin and Rajka criteria,¹⁶ were recruited from staff or from the specialist contact dermatitis clinics at St John's Institute of Dermatology. Forty healthy control subjects were recruited from staff at St Thomas' Hospital. C.E.H.G. independently obtained 87 different patients with CIU from specialist urticaria clinics at St John's Institute of Dermatology and general dermatology clinics at the West Norwich Hospital. All patients with CIU (R.A.S. and C.E.H.G.) had active disease at the time of testing.

In all subjects antihistamines were withdrawn at least 3 days (R.A.S.) or 2 days (C.E.H.G.) prior to skin tests, except for doxepin and astemizole, which were stopped 2 and 6 weeks beforehand, respectively. No subject was taking immunosuppressants for at least 2 months before testing. All subjects were aged 18 years or above. Ethical approval was obtained from St Thomas' Hospital Research Ethics Committee and from Norwich District Ethics Committee.

Skin test technique

Venous blood was taken into sterile glass tubes without clotting accelerator (Vacutainer®, Becton Dickinson, NJ, U.S.A.), 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 histamine release assays. Samples (50 µL) of autologous serum, histamine 10 µg/mL (Martindale Pharmaceuticals, Romford, Essex, U.K.) and 0.9% sterile saline were separately injected into the volar aspect of the forearm. R.A.S. and C.E.H.G. left gaps of at least 5 cm and 3 cm, respectively, between injection sites. Areas known to have been involved in spontaneous weals in the last 24 h were avoided.

Weal and flare responses were measured at 30 min and, where shown, at 60 min. Weal and flare areas were calculated from the measurement of two perpendicular diameters (d_1 and d_2) according to the formula $\pi((d_1 + d_2)/4)^2$. Weal volume was calculated by multiplying weal area by half the change in skin fold thickness, measured with a low-tension spring-loaded thickness gauge (Mitutoyo, Japan). Weal and flare measurements for serum-induced responses were corrected by subtraction of the equivalent values for the saline-induced response in each subject. The redness of the weal response was observed at 30 min and scored 0 if the colour of the serum response resembled that of the saline response which could be either pink or skin



Figure 1. A positive autologous serum skin test, at 30 min.

coloured; 1 if the response to serum was pink when that to saline was skin coloured; and 2 if the response to serum resembled that of the mature histamine-induced weal (red).

Basophil histamine release assay

Serum-evoked histamine release from basophils of two healthy donors was used to identify subjects with circulating functional anti-Fc ϵ RI α and/or anti-IgE autoantibodies. The methods (using a 1:2 dilution of serum) and basophil donors were the same as described previously.^{5,6,17}

Statistical analysis

The Mann–Whitney *U*-test was used for unpaired data, and the Wilcoxon signed rank test was used for paired data. Sensitivity (%) was calculated according to the formula: number of patients who were both autoantibody positive and ASST positive $\times$ 100/total number of patients who were autoantibody positive. Similarly, specificity (%) was calculated according to the formula: number of patients who were both autoantibody negative and ASST negative $\times$ 100/total number of patients who were autoantibody negative.¹⁸

Results

Detection of subjects with autoantibodies

Serum from 26 of 68 (38%) patients with CIU recruited by R.A.S., and 28 of 87 (32%) patients recruited by

C.E.H.G., caused histamine release of $\geq 5\%$ from basophils from one or both donors, indicating the presence of autoantibodies to Fc ϵ RI α and/or IgE. Serum from 40 healthy control subjects, 15 patients with dermatographism, nine patients with cholinergic urticaria and 10 patients with atopic eczema did not contain histamine-releasing autoantibodies.

Sensitivity and specificity of the autologous serum skin test: clinician R.A.S.

Weal diameter. The mean $\pm$ SD saline-corrected serum-induced weal diameter at 30 min was significantly greater for CIU patients with autoantibodies (2.0 ± 1.8 mm; $n = 26$) than for patients without autoantibodies (0.4 ± 1.5 ; $n = 42$; $P = 0.0005$) or healthy control subjects (-0.2 ± 1.4 ; $n = 40$; $P < 0.0001$; Fig. 2a). There was overlap, however, between the distributions of values of the groups. Figure 3a shows the relationship between test sensitivity and specificity for the detection of patients with autoantibodies using a positive ASST defined as serum-induced weal diameters ranging from 0.5 to 2.5 mm greater than the saline control. The intersection of the plots suggests a difference of 1.5 mm between serum- and saline-induced weal diameters as the criterion for a positive ASST with no bias towards either sensitivity (69%) or specificity (71%). However, 30% of tests were either apparently false positives or false negatives using weal diameter alone to define a positive ASST.

Weal volume. The mean $\pm$ SD saline-corrected serum-induced weal volume was significantly greater for CIU

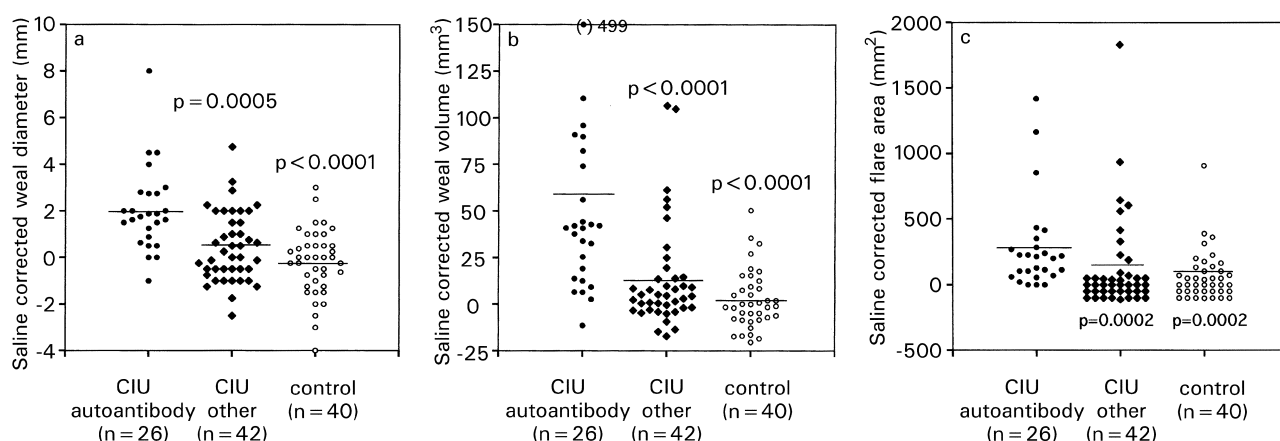


Figure 2. Saline-corrected serum-induced (a) weal diameters, (b) weal volumes, and (c) flare areas for 26 chronic idiopathic urticaria (CIU) patients with autoantibodies, 42 CIU patients without autoantibodies and 40 control subjects, in autologous serum skin tests measured at 30 min by R.A.S. — Indicates the mean value in each group.

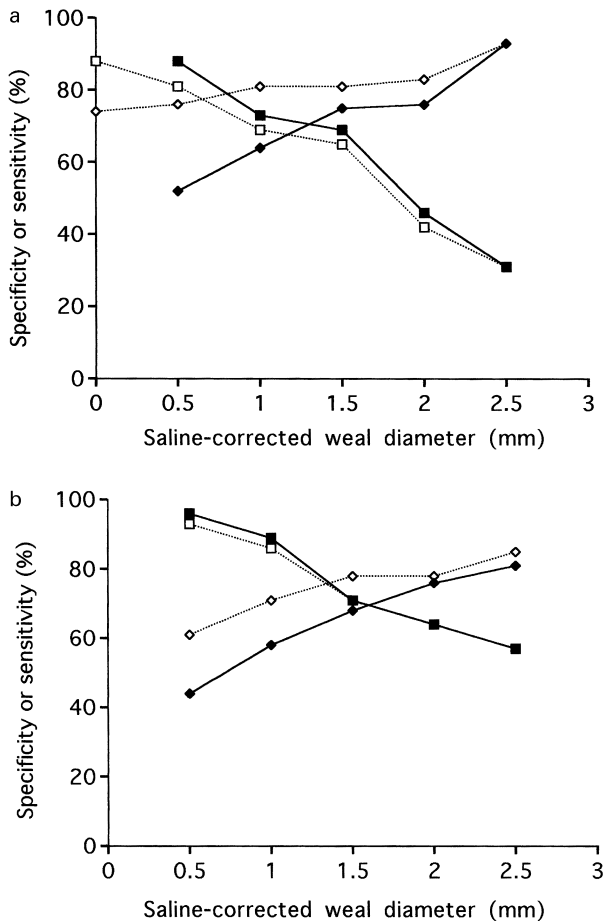


Figure 3. The sensitivity and specificity (%) of the autologous serum skin test (ASST) for detecting subjects with autoantibodies in (a) 68 patients with chronic idiopathic urticaria (CIU) (R.A.S.) and (b) 87 patients with CIU (C.E.H.G.). Values are given for the sensitivities and specificities of ASSTs defined as positive by saline-corrected serum-induced weal diameters from 0 to 2.5 mm alone (complete lines and filled symbols), and for tests defined as positive by weal diameter and redness scoring 2 (dotted lines and unfilled symbols). Tests were measured at 30 min. ■, Sensitivity using weal diameter alone; ♦, specificity using weal diameter alone; □, sensitivity for weal diameter and redness; ◇, specificity for weal diameter and redness.

Table 1. Summary of the optimum combined sensitivities and specificities of the autologous serum skin test for the detection of autoantibodies when defining tests as positive using single or combined parameters of the serum-induced saline-corrected cutaneous responses measured in patients with chronic idiopathic urticaria by the physicians R.A.S. ($n = 68$) and C.E.H.G. ($n = 87$) at 30 min

Parameter measured	R.A.S.		C.E.H.G.	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Weal diameter (≥ 1.5 mm)	69	71	71	68
Weal volume (≥ 10 mm ³)	81	73	N/A	N/A
Weal redness (score 2)	88	69	93	51
Weal diameter (≥ 1.5 mm) plus redness (score 2)	65	81	71	78
Weal volume (≥ 10 mm ³) plus redness (score 2)	81	78	N/A	N/A

N/A, results not available.

patients with autoantibodies (59 ± 95 mm³; $n = 26$) than for patients without autoantibodies (13 ± 28 ; $n = 42$; $P < 0.0001$) or healthy control subjects (3 ± 16 ; $n = 40$; $P < 0.0001$; Fig. 2b). A serum-induced weal volume of ≥ 10 mm³ than the saline-induced weal diameter gave the highest combined sensitivity and specificity of 81% and 73%, respectively, over a range from 5 to 25 mm³ for defining a positive test.

Flare area. The mean $\pm$ SD saline-corrected serum-induced flare area was significantly greater for CIU patients with autoantibodies (280 ± 350 mm²; $n = 26$) than for patients without autoantibodies (140 ± 345 ; $n = 42$; $P = 0.0002$) or healthy control subjects (87 ± 173 ; $n = 40$; $P = 0.0002$; Fig. 2c). A serum-induced flare area of ≥ 50 mm² than the saline-induced flare area gave the highest combined sensitivity and specificity of 85% and 74%, respectively, for the detection of patients with autoantibodies. However, a high proportion (35% or 14 of 40) of tests performed in control subjects was false positive using these criteria.

Weal redness. The mean $\pm$ SD weal redness (score 0, 1 or 2) was significantly greater for CIU patients with autoantibodies (1.8 ± 0.6 ; $n = 26$) than for patients without autoantibodies (0.9 ± 0.8 ; $n = 42$; $P < 0.0001$) or healthy control subjects (0.8 ± 0.7 ; $n = 40$; $P < 0.0001$). If a positive test was defined as a serum-induced weal with a redness score of 2, irrespective of the weal size, the sensitivity and specificity for the detection of autoantibody-positive patients was 88% and 69%, respectively (Table 1).

Combination of weal redness with weal diameter or weal volume. Using a combination of weal redness (score 2) and weal diameter to define a positive ASST increased the specificity with only a minimal loss in sensitivity at all saline-corrected weal diameters from 0.5 to 2.0 mm

(Fig. 3a). Defining a positive ASST as a serum-induced weal that was both red (score 2) and had a diameter of ≥ 1.5 mm than the saline-induced weal, gave a sensitivity of 65% and a specificity of 81% for detecting patients with autoantibodies (Table 1). Similarly, defining a positive ASST as a serum-induced weal which was both red (score 2) and had a volume of ≥ 10 mm³ than the saline-induced weal, increased specificity to 78% from 73% for volume difference alone but with no loss of sensitivity (Table 1).

Intertest variation. When ASSTs were repeated at different sites (right and left arms) at the same time, the mean $\pm$ SD difference between the test sites in serum-induced saline-corrected weal diameters was 0.1 ± 1.4 mm for 34 patients with CIU, and 0.4 ± 1.0 mm for 15 control subjects. ASSTs repeated on different days in 18 control subjects had a mean difference in serum-induced saline-corrected weal diameter of -0.13 ± 1.9 mm. There was no significant difference between the measurements taken either at two separate sites or on 2 separate days.

Sensitivity and specificity of the autologous serum skin test: clinician C.E.H.G.

The intersection of the sensitivity and specificity plots (Fig. 3b) at a 1.5 mm difference between serum- and saline-induced weal diameters suggests this value as the criterion for a positive ASST evenly balanced for sensitivity (71%) and specificity (68%). Combined weal diameter data for R.A.S. and C.E.H.G. are shown in Table 2. If a positive test for C.E.H.G. was defined as a red serum-induced weal at 30 min, irrespective of weal size, the sensitivity was 93%, but the specificity was poor, at only 51% (Table 1). Like R.A.S., a combination of a serum-induced weal which was both red (score 2) and

had a diameter of ≥ 1.5 mm than the saline-induced weal, improved specificity to 78% from 68% without loss of sensitivity compared with using weal diameter alone (Fig. 3b, Table 1).

Comparison of readings at 30 and 60 min. Weal diameter measurements were compared at 30 and 60 min in ASSTs in patients with CIU ($n = 84$, C.E.H.G.). If tests were taken as positive when the serum-induced weal had a diameter of ≥ 1.5 mm than the saline-induced weal, at 30 min the sensitivity of the ASST was 71% and specificity 68%, and at 60 min the sensitivity improved to 93% but the specificity fell to 48%.

Autologous serum skin tests in patients with other diseases and healthy control subjects

When a positive ASST was defined as a red serum-induced weal (score 2) with a diameter of ≥ 1.5 mm than the saline-induced weal diameter, there were no positive tests among 15 patients with dermatographism and 10 patients with eczema (tests performed by R.A.S.). One of nine patients with cholinergic urticaria had a positive test, although this patient had a past history of angio-oedema. When applied to the 40 healthy control subjects, only one subject (2.5%) had a false positive ASST. Similar results were found if a positive ASST was defined as a red serum-induced weal (score 2) with a volume of ≥ 10 mm³ than the saline-induced weal volume, except four of 40 healthy subjects (10%) had a false positive ASST.

Discussion

The intradermal injection of autologous serum in some patients with CIU produces a weal and flare response. This simple observation suggested that there are

Table 2. Relationship between test sensitivity and specificity for the detection of autoantibodies (AB) using positive autologous serum skin tests (ASSTs) defined as serum-induced weal diameters ranging from 0.5 to 2.5 mm greater than the saline-induced weal diameter at 30 min in 155 patients with chronic idiopathic urticaria (R.A.S. and C.E.H.G. patients combined)

	ASST + if weal diameter ≥ 0.5 mm		ASST + if weal diameter ≥ 1 mm		ASST + if weal diameter ≥ 1.5 mm		ASST + if weal diameter ≥ 2 mm		ASST + if weal diameter ≥ 2.5 mm	
	AB +	AB -	AB +	AB -	AB +	AB -	AB +	AB -	AB +	AB -
ASST +	50	53	44	40	38	31	30	24	24	14
ASST -	4	48	10	61	16	70	24	77	30	87
total AB (+ or -)	54	101	54	101	54	101	54	101	54	101
Sensitivity	50/54 (93%)		44/54 (81%)		38/54 (70%)		30/54 (56%)		24/54 (44%)	
Specificity	48/101 (48%)		61/101 (60%)		70/101 (69%)		77/101 (76%)		87/101 (86%)	

circulating histamine-releasing factors in the serum of these patients, and this led to the identification of functional anti-FcεRI autoantibodies in CIU.⁵ A positive ASST may also indicate the presence of functional anti-IgE autoantibodies,¹⁹ and other histamine-releasing factors cannot be excluded.^{6,14,20} The identification of an autoimmune aetiology in CIU in some patients has stimulated the use of immunotherapy in patients with severe disease unresponsive to antihistamine therapy,^{10–12} but it is not known if patients with autoantibodies respond better to immunotherapy than those without. At present, autoantibodies are detected by histamine release assays, Western blot analysis or ELISA, which are tedious methods requiring specialist facilities and are not available to most clinicians. Therefore, we have investigated in detail the conditions required to gain the optimum combined sensitivity and specificity of the ASST for detecting patients with autoantibodies.

In this study basophil histamine release assays were taken as the gold standard for the identification of patients with autoantibodies. We used well characterized basophils from the same two healthy donors as used in previous work,^{5,6} and there was very little variability between assays.¹⁷ The distribution of the data obtained was similar to that previously published.⁶ Basophil histamine release assays detect functional autoantibodies, and may miss non-functional autoantibodies. However, we have found good correlation between Western blot analysis data and the results of basophil histamine release assays (unpublished data).

We demonstrated that serum-induced weal diameters, weal volumes, weal redness and flare areas were significantly greater in patients with CIU with autoantibodies than either those without autoantibodies or healthy control subjects. However, there was overlap in the values for each parameter measured between CIU patients with autoantibodies and patients without autoantibodies or control subjects. Defining a positive ASST by any single parameter, or by combinations of parameters, gave sensitivities and specificities of between 51% and 93% for detecting patients with autoantibodies (Table 1).

In choosing a parameter or combined parameters to define test positivity, an ASST biased to higher sensitivity or specificity can be chosen. When using the test for clinical purposes, where it is important to avoid giving inappropriate treatment to patients without a known autoimmune basis for their disease, we feel that a test with high specificity is required. On this basis, we recommend defining a positive ASST as one with a

serum-induced weal which is both red and has a diameter of ≥ 1.5 mm than the saline-induced response at 30 min. Using these criteria, the sensitivity and specificity of the ASST for detecting autoantibodies was 65% and 81% for R.A.S., and 71% and 78% for C.E.H.G., respectively, and false positive results in healthy subjects and control disease groups were minimal. If a test with higher sensitivity is required, then using weal volume ≥ 10 mm³ together with weal redness (score 2) to define a positive ASST may be better, although this increased the false positive rate in healthy subjects (four of 40).

Although unconventional, weal redness was used rather than flare area because of the high false positive rate in healthy control subjects using this criterion alone. Additionally, if a positive test was defined as one with a serum-induced response with both a weal diameter of ≥ 1.5 mm and flare area of ≥ 50 mm² than the saline-induced response at 30 min, the sensitivity and specificity were 58% and 83%, respectively, for R.A.S., and 68% and 80%, respectively, for C.E.H.G., and there were four false positive tests among 40 control subjects. We therefore suggest the use of weal redness rather than flare area. A positive skin test in the absence of *in vitro* evidence of autoantibodies may occur if autoantibodies are present only at a low concentration not detectable in basophil histamine release assays using serum at a 1 : 2 dilution and using a histamine release of $\geq 5\%$ of the total histamine content to indicate the presence of autoantibodies. Alternatively, non-autoantibody mast cell-specific histamine-releasing factors may be present.^{6,20,21} Conversely, an apparently negative ASST may occur in the presence of autoantibodies according to *in vitro* tests if the serum is injected into a site rendered refractory by a recent weal.

Dermographic subjects represent a special group. They do not have autoantibodies according to *in vitro* tests, but manipulation of skin to estimate skin thickness prior to injection and the actual injection may both cause a weal and flare response, regardless of the substance injected. Thus, a dermographic response may incorrectly be taken as positive if the saline-induced response is not taken into account. Conversely, a true positive ASST due to circulating autoantibodies could be missed in a patient with both CIU and dermographism.

Minor variations in injection technique, such as in depth or volume of injection, may cause errors in measuring all parameters. Weal diameter is a simple, direct measurement. However, although the edges of weals induced by serum in a positive ASST are usually easy to define, the edge of a saline- or serum-induced

non-inflamed bleb may be difficult to detect. Calculation of weal volume requires estimation of skin fold thickness, and in some subjects, particularly those who are obese, it can be difficult to lift up a skin fold for measurement. Repeated attempts at measuring skin thickness should be avoided, as this may reduce weal thickness because of loss of interstitial or oedema fluid, and encourage subsequent non-specific dermatographic responses. Weal redness is simple to observe, but is subjective. Flare area can be difficult to measure, as the edge of the flare is not always clearly definable because of outlying macules. Redness of both weal and flare reactions is difficult to see in pigmented skin types. Overall, weal redness and weal diameter are the easiest measurements to take, and do not require special equipment.

Although a positive ASST is superficially like a histamine-induced weal and flare response, it is likely to be more complex. Preliminary studies showed that the serum-induced weal response was variable but usually reached a maximum by 1.5–2 h, and remained positive for an average of 8 h (range 4–13).¹⁴ In this study, the specificity of the ASST for the detection of autoantibodies was unacceptably low at 60 min when measuring weal diameters in patients with CIU. We therefore recommend that the ASST be read at 30 min. This is more likely to reflect the time course of a weal and flare response due to initial histamine release from mast cells, rather than to other inflammatory mediators released from mast cells or mediators from incoming cells.

In summary, the ASST, with a positive test defined as one with a serum-induced weal which is both red and has a diameter of ≥ 1.5 mm than the saline-induced response at 30 min, can be used as a reasonably predictive clinical test to indicate the presence of functional circulating autoantibodies to Fc ϵ RI and/or to IgE to aid the management of patients with CIU. This conclusion is supported by independent findings obtained by two investigators in different centres using different patients. It is a time-consuming test, and is best performed regularly by an experienced observer, with validation by *in vitro* laboratory tests at least initially for a new observer. In view of the intertest variation we routinely perform the test in duplicate (right and left arms). The ASST is useful only for patients with severe CIU under consideration for immunomodulatory treatment.

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