

Effects of complement inactivation and IgG depletion on skin reactivity to autologous serum in chronic idiopathic urticaria

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Background: Intradermal injection of autologous serum elicits a wheal-and-flare response in about 60% of patients with chronic idiopathic urticaria (CIU). This reactivity has been attributed to the presence of IgG autoantibodies directed against IgE or the α -chain of the high-affinity IgE receptor (Fc ϵ RI α) expressed on basophils and mast cells, leading to the hypothesis that at least some forms of CIU could be sustained by an autoimmune process.

Objectives: The aim of this study was to investigate the relationship between the presence of anti-IgE or anti-Fc ϵ RI antibodies and the ability to induce wheal-and-flare responses in CIU sera selected for the capacity to give a positive skin test response.

Methods: Fifteen patients with CIU and a positive skin test response to autologous serum were injected intradermally with native serum and with serum heated at 56°C for 30 minutes and then adsorbed on Sepharose-protein G to obtain IgG depletion. Serum levels of anti-IgE and anti-Fc ϵ RI α antibodies were measured by ELISA by using purified IgE and recombinant RI α -soluble double-fusion protein RI α -human serum albumin-RI α , respectively. The histamine-releasing activity of sera was tested by using ELISA with whole human blood from a healthy donor.

Results: All patients had positive cutaneous responses to native serum injection. Anti-Fc ϵ RI α antibodies were present in 14 of 15 native sera, only two of which were able to induce in vitro basophil degranulation. On the contrary, detectable amounts of anti-IgE antibodies were not found in any serum. IgG depletion by protein G resulted in complete (10/14 samples) or considerable (4/14 samples) removal of anti-Fc ϵ RI α antibodies. The two sera endowed with functional activity lost their capacity to trigger histamine release from basophils after heating and protein G adsorption. Nonetheless, heat-decomplemented/

IgG-depleted sera elicited wheal-and-flare reactions comparable with those observed with untreated sera.

Conclusions: These results strongly suggest that skin reactivity to autologous serum could be due to as yet unidentified non-Ig reactants present in the sera of patients with CIU. (*J Allergy Clin Immunol* 2000;106:567-72.)

Key words: Chronic idiopathic urticaria, IgE receptor, autoantibodies, histamine release, cutaneous response

Chronic idiopathic urticaria (CIU) is a form of long-lasting urticaria (>6 weeks) the cause and pathogenesis of which are still poorly understood.¹ In the past few years, it was observed that intradermal injection of autologous serum elicits immediate wheal-and-flare responses in a variable portion of patients with active CIU.² The ability of serum to induce a cutaneous reaction is associated with active disease because it decreases or disappears during remission phases.² These data suggested the presence of histamine-releasing factors (HRFs) able to cause cutaneous mast cell degranulation in sera from patients with CIU. At present, the nature of these HRFs is controversial.

Several authors described the finding of IgG autoantibodies directed to IgE, the α -chain of the high-affinity IgE receptor (Fc ϵ RI α), or both in sera from patients with CIU.³⁻⁷ Subsequent works demonstrated that in some cases these autoantibodies were able to induce histamine release from basophils of healthy donors and from isolated mast cells; a role for activation of the complement pathway was also proposed.^{3,6,8-11} It was therefore hypothesized that skin reactivity to autologous serum could be due to the presence in sera of autoantibodies able to activate the cutaneous mast cells. However, although only a few studies investigated the relationship between cutaneous reactivity to autologous serum and the presence of circulating anti-IgE or anti-Fc ϵ RI α autoantibodies in the same cohort of patients,^{3,6,8} it is well established that there is a discrepancy between the numbers of patients with CIU who have a positive skin test response^{2,3,6,8} and those in whom functional anti-IgE or anti-Fc ϵ RI α can be demonstrated.^{3,6-10} This observation suggests that at least in some patients with CIU, the cutaneous response to autologous serum may be due to non-Ig reactants.

We recently observed that heparinized plasma did not induce a cutaneous response in patients with CIU com-

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Abbreviations used

CIU:	Chronic idiopathic urticaria
FcεRIα:	α-Chain of the high-affinity IgE receptor
gahIgG:	Goat anti-human IgG
HRA:	Histamine-releasing activity
HRF:	Histamine-releasing factor
HRP:	Horseradish peroxidase
HSA:	Human serum albumin
RIα-DFP:	Double-fusion protein RIα-HSA-RIα

pared with autologous serum. Moreover, addition of heparin to sera or adsorption on Sepharose-coupled heparin before testing was associated with loss of cutaneous activity, probably by a direct effect of heparin on the HRFs present in sera.¹²

We therefore hypothesized that HRFs of a different nature could be present in different patients with CIU or even in the same patient. To verify this hypothesis, in a group of patients with CIU and positive skin test responses to autologous serum, we investigated the relationship between the presence of anti-IgE or anti-FcεRIα antibodies, their functional activity, and the ability of sera to cause a positive wheal-and-flare response in both native and heat-decomplemented/IgG-depleted sera. Our results disclosed the presence in almost all native sera of anti-FcεRIα antibodies, only a minority of which were endowed with histamine-releasing activity (HRA) on human basophils. Heat treatment/IgG depletion was associated with abrogation of the *in vitro* functional activity, but the sera retained their ability to induce wheal-and-flare responses. These data strongly suggest that skin reactivity to autologous serum could be sustained by as yet unidentified non-Ig factors.

METHODS

Study population

Fifteen patients with severe CIU and a positive skin test response to autologous serum (14 women and 1 man; age range, 19-58 years) were selected from our CIU patient cohort, which was composed of 132 individuals, 72 of whom had positive reactions to intradermal injection of autologous serum. The duration of urticaria ranged from 6 to 48 months, and all patients were under treatment with cetirizine associated in some cases with steroids. At the time of the study, none of the patients had been given cetirizine during the last 48 hours before testing, and steroid therapy was withdrawn at least 7 days before. All sera were collected and injected during the active phase of the disease.

Moreover, 10 healthy volunteers from the medical staff were tested as normal control subjects for skin reactivity to autologous serum or to heat-decomplemented/IgG-depleted serum.

All patients gave their informed consent to this study. The study was approved by the institutional review committee for human experimentation.

Sera preparation

Serum was obtained from venous blood clotted for 30 minutes at room temperature in silicone-coated Vacutainer tubes (Becton-Dickinson, Marlan, France) and centrifuged at 350g for 15 minutes. Sera of both patients with CIU and normal control subjects were stored at -20°C until use.

Immunoaffinity depletion of IgG

All sera were heated for 30 minutes at 56°C before protein G adsorption. IgG was depleted from serum samples by incubating 1-mL serum aliquots with an equal volume of Sepharose 4B-coupled protein G (Pharmacia-LKB, Uppsala, Sweden). After 30 minutes of incubation at room temperature on a rotating platform, the serum was recovered by centrifugation and stored at -20°C until use. Residual IgG contents in protein G-adsorbed sera were measured by RIA¹³ and were found to be less than 10 µg/mL (not shown). As a control, saline was incubated with Sepharose-protein G and treated as above to exclude the release of skin irritants from Sepharose-protein G.

Anti-FcεRIα antibodies

Sera were tested for the presence of anti-FcεRIα antibodies by ELISA. The extracellular domain of the human FcεRIα chain fused to human serum albumin (HSA) to form the double-fusion protein RIα-HSA-RIα (RIα-DFP), expressed in mammalian CHO cells, was obtained from Novartis (Basel, Switzerland). One microgram of RIα-DFP was immobilized in Costar RIA Strip Plate-8 microtiter plate wells (Costar, Cambridge, Mass) and incubated for 2 hours at 37°C with 100 µL of serum samples diluted 1:50 with PBS/0.05% Tween-20/2% FCS (Sigma, St Louis, Mo). Bound Ig was detected by horseradish peroxidase (HRP)-coupled goat anti-human IgG (gahIgG-HRP; BioRad Laboratories, Hercules, Calif) with the HRP Substrate Kit (BioRad) for development of the color reaction. The plates were read at 405 nm, and OD values were corrected for non-specific binding to HSA-coated wells. Crossreactivity of gahIgG-HRP with human IgE was ruled out by solution-binding assays: human IgE and RIα-DFP were incubated at equimolar amounts (4°C overnight). Formed complexes were added to microtiter plate wells coated with the monoclonal anti-hIgE antibody Le27 (Ce4-specific); captured complexes could be detected with gahIgE-HRP or gaHSA-HRP but not with gahIgG-HRP. The amount of anti-FcεRIα antibodies was calculated by using polyclonal human IgG (Sigma) for comparison and then expressed as IgG concentrations (in nanograms per milliliter).

Anti-IgE antibodies

Determination of the anti-IgE antibody contents in sera was performed an ELISA. Briefly, 1 µg of human IgE (SUS-11, kindly provided by Novartis) was immobilized in Costar RIA Strip Plate-8 microtiter plate wells and incubated for 2 hours at 37°C with 100 µL of serum samples diluted 1:50 with PBS/0.05% Tween-20/2% FCS. Bound Ig was detected by HRP-coupled goat anti-human IgG (Fab specific, Sigma) with the HRP Substrate Kit for development of the color reaction.

Histamine-release assay

Serum samples (1:50 final dilution) were tested for histamine release on human whole blood from a single, highly responsive, healthy donor with low IgE levels (<10 U/mL) by using an enzyme immunoassay (Immunotech International, Marseille, France), according to the manufacturer's instructions.

Skin testing

Fifty-microliter aliquots of native or heat-decomplemented/Sepharose-protein G-adsorbed serum were injected intradermally into the volar forearm. As a control, the patients were also injected with 50 µL of saline or saline incubated with Sepharose-protein G. Skin reactivity was assessed by using a standard histamine prick test (histamine hydrochloride 1%; Bayer, Milan, Italy). All the injection sites were scored 30 minutes after inoculation. The largest diameter (D1) of the wheal-and-flare reaction and the diameter perpendicular

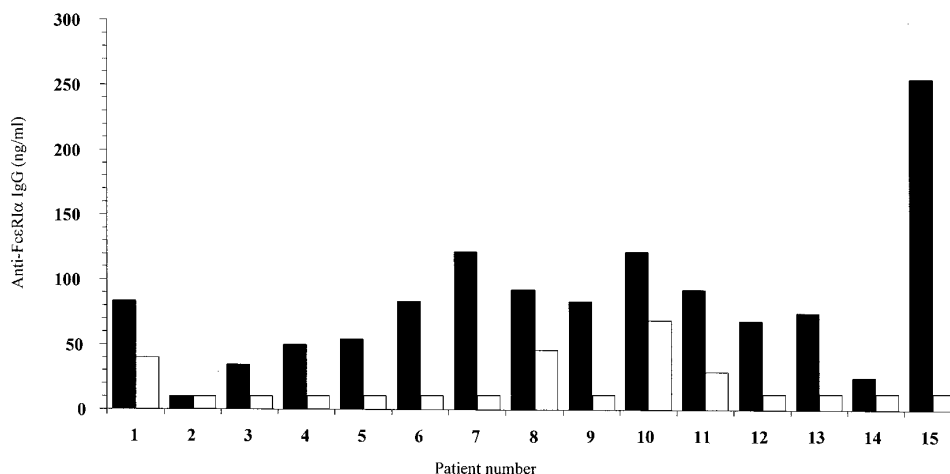


FIG 1. Anti-FcεRIα auto antibody levels in native (black bars) and IgG-depleted (open bars) sera from 15 CIU patients. IgG was depleted from serum samples by Sepharose-protein G adsorption, as described in the "Methods" section. Native and IgG-depleted sera were tested for the presence of anti-FcεRIα antibodies by ELISA. RIα-DFP was coated on plate wells, and sera were added at a 1:50 dilution. Bound IgG was detected with peroxidase-conjugated goat anti-human IgG, and OD values were corrected for nonspecific binding to HSA-coated wells. Anti-FcεRIα IgG concentrations were calculated by using human IgG as a standard in a parallel ELISA with coated anti-human IgG. Results are means of measurements in duplicate.

to this (D2) were recorded according to the following formula: $D1 + D2/2$. Reactions showing a mean wheal-and-flare diameter of less than 0.5 cm were scored as negative. All the skin tests were performed with stored sera (-20°C). The freezing and thawing procedures did not modify the cutaneous reactivity of native or heat-decomplemented/IgG-depleted sera from both patients with CIU and healthy control subjects (not shown).

Statistical analysis

Data were managed by Statgraphics. The Spearman rank correlation test and the Wilcoxon matched pair test were used where appropriate.

RESULTS

In all patients the prick test with histamine elicited positive results, whereas the injection of plain saline always elicited negative results. When autologous serum was injected, significant wheal-and-flare responses were observed in all 15 patients studied (Table I).

None of the native sera showed detectable amounts of anti-IgE autoantibodies (data not shown); on the contrary, it was possible to demonstrate the presence of variable amounts of anti-FcεRIα antibodies in 14 of 15 sera, ranging from 25 to 255 ng/mL (Fig 1). To rule out interference of serum IgE binding to FcεRIα, sera were also tested at 1:200 dilutions without significant differences in the anti-FcεRIα IgG pattern. Furthermore, preincubation of selected serum samples with an excess of the mAb BSW17, which is known to inhibit IgE/FcεRIα binding and to displace receptor-bound IgE,¹⁴ had no effect on the anti-FcεRIα IgG readout (data not shown).

No correlation was observed between the diameter of the wheal-and-flare reaction in individual patients and the amount of anti-FcεRIα antibodies present in serum ($r = -0.26$, $P = .33$ according to the Spearman rank test). When

sera were tested for the ability to trigger histamine release from human blood basophils, only two samples induced significant histamine release (Fig 2, sera 7 and 15).

To verify whether complement inactivation and IgG removal were associated with a change in cutaneous activity, the sera were heated for 30 minutes at 56°C and then absorbed on Sepharose-protein G. As shown in Fig 1, protein G adsorption resulted in a total or considerable (samples 1, 8, 10, and 11) removal of anti-FcεRIα autoantibodies. Accordingly, the two sera with HRA lost their ability to trigger histamine release from human basophils after complement inactivation and IgG depletion (Fig 2).

Nevertheless, injection of treated sera induced wheal-and-flare responses in all patients comparable with those observed with native sera ($\bar{O} 2.46 \pm 0.57$ cm vs 2.42 ± 0.56 cm; $t = 21.5$, $P = .17$ according to the Wilcoxon test). On the other hand, the injection of Sepharose-protein G-incubated saline did not evoke any cutaneous reaction, thus showing a lack of nonspecific skin irritants in Sepharose-protein G-incubated samples (Table I). The lack of nonspecific irritants in the treated sera was confirmed by testing the cutaneous reactivity to both native and heat-decomplemented/IgG-depleted autologous sera in healthy donors; these tests always elicited negative results (not shown).

DISCUSSION

In this study we extend the knowledge about the presence of anti-FcεRIα IgG antibodies in the sera from patients with CIU and a positive skin test response to autologous serum. Moreover, we show, for the first time, that heat-decomplemented/IgG-depleted sera retain the ability to induce wheal-and-flare reactions.

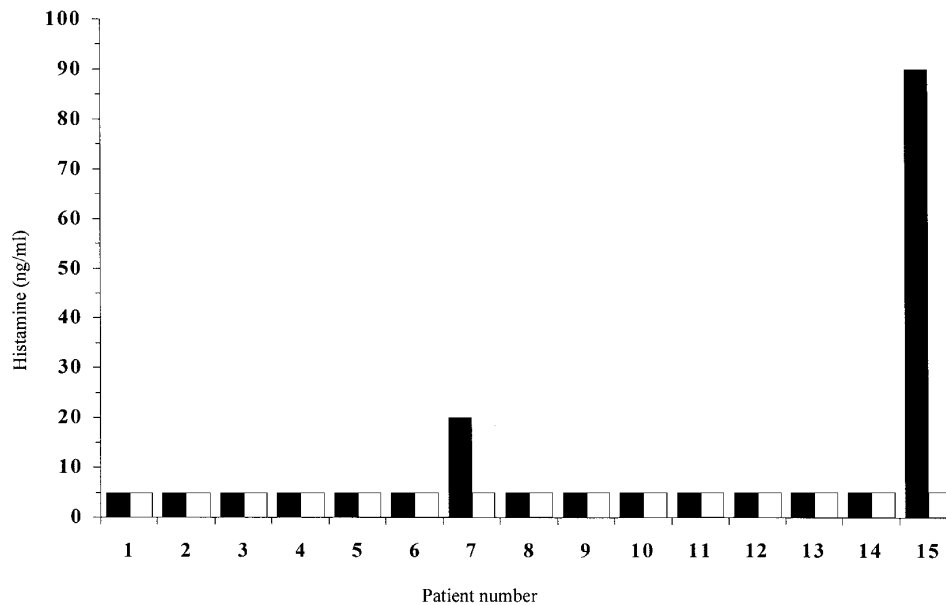


FIG 2. Histamine release from homologous basophils induced by native (black bars) and heat-depleted/IgG-depleted (open bars) sera from 15 CIU patients. Anti-FcεRIα autoantibodies were detected in all sera except in sample 2, ranging in concentration from 25 to 255 ng/mL. Residual amounts of anti-FcεRIα IgG were still present in serum samples 1, 8, 10, and 11 after IgG depletion by Sepharose-protein G. Serum dilutions (1:50) were tested for histamine release by using fresh human blood from a highly responsive healthy donor by Immunotech histamine ELISA, according to the supplier's instructions. Histamine values represent whole blood levels (in nanograms per milliliter). Mean values of measurements made in duplicate are shown.

TABLE I. Skin reactivity to native and heat-depleted/IgG-depleted sera in 15 patients with chronic idiopathic urticaria*

Patient No.	Native serum	Decomplemented/ IgG-depleted serum†	Histamine	Saline	Sepharose-protein G-incubated saline
1	0.7/3.0‡	0.7/3.1	1.0/1.2	Negative§	Negative
2	0.8/2.5	0.6/2.4	0.6/1.0	Negative	Negative
3	1.2/2.6	1.1/2.6	0.4/1.0	Negative	Negative
4	0.7/2.5	0.7/2.3	0.5/1.2	Negative	Negative
5	1.0/2.2	1.0/2.0	0.6/1.0	Negative	Negative
6	0.8/2.6	0.7/2.6	0.7/1.1	Negative	Negative
7	1.3/2.2	1.3/2.0	0.8/1.3	Negative	Negative
8	1.3/3.0	1.3/2.8	0.6/1.1	Negative	Negative
9	0.9/3.3	1.0/3.1	0.8/1.3	Negative	Negative
10	1.0/1.6	1.0/1.4	0.6/0.9	Negative	Negative
11	0.8/1.8	0.9/1.7	0.5/1.4	Negative	Negative
12	1.1/1.6	1.0/1.5	0.6/1.1	Negative	Negative
13	2.2/3.5	2.0/3.2	0.5/1.2	Negative	Negative
14	1.4/2.2	1.4/2.4	0.7/1.1	Negative	Negative
15	1.4/3.4	1.4/3.0	0.8/1.7	Negative	Negative

*Patients with CIU were injected intradermally with native serum, heat-depleted/IgG-depleted serum, saline, and Sepharose-protein G-incubated saline according to the protocol detailed in the "Methods" section. Skin reactivity was assessed by histamine prick test.

†IgG depletion was achieved by incubation of 1 mL of heat-depleted (30 minutes at 56°C) serum with an equal volume of Sepharose-protein G for 30 minutes at room temperature.

‡Mean wheal-flare diameters are given in centimeters and calculated as described in the "Methods" section.

§Mean diameter of less than 0.5 cm.

The finding of anti-IgE and anti-FcεRIα autoantibodies in sera of patients with CIU has been repeatedly reported. Anti-IgE antibodies, isolated³⁻⁵ or in association with anti-FcεRIα autoantibodies,^{6-8,15} have been detected in a variable percentage of patients with CIU by

several authors, with an incidence ranging from 5% to 69%. In the case series reported here, we did not detect anti-IgE antibodies, but our results could simply reflect the small number of patients investigated.

On the other hand, anti-FcεRIα autoantibodies were

found in almost all the sera examined. Our figures are higher than those reported by others^{6,7-10,16} but comparable with those recently reported by Horn et al.¹⁷ These differences may be due to several nonmutually exclusive explanations, including the selection of patients with CIU, the methods used to detect anti-FcεRIα antibodies, and the means of evaluating positive and negative results. As far as the first point is concerned, we selected patients with positive skin test responses to autologous serum, in which the prevalence of anti-FcεRIα antibodies could be particularly high. The detection method also entails critical issues. Although the basophil histamine release assay may be considered the method of choice to identify anti-FcεRIα antibodies endowed with functional activity,^{6,8,16} this approach may not be appropriate when the purpose is to define the actual prevalence of these autoantibodies in a population. On the other hand, one could also argue against results obtained by immunoblotting techniques. In fact, when the α subunit of the Fcε receptor is blotted, its conformational structure may be modified, with eventual loss of reactivity of antibodies directed against conformational epitopes. Indeed, Ferrer et al¹⁰ found a substantial number of patients who had negative test results by immunoblot but had positive basophil histamine responses. This apparent discrepancy could also be due to the working dilution used by these authors to test their sera, assuming that an arbitrary cut-off dilution to discriminate between negative and positive sera may be misleading. In our opinion a quantitative descriptive approach allowing direct comparison of the values obtained in healthy subjects and individuals with CIU would be desirable.

In this regard quantitative ELISA assays with a recombinant soluble FcεRIα have been reported,^{9,17} which exploit the antigen in its native conformation and therefore should be able to detect all anti-FcεRIα antibodies present in the sera. Using an ELISA method with FcεRIα proteins produced in CHO mammalian cells, Horn et al¹⁷ found circulating anti-FcεRIα IgG in all 68 patients with urticaria and in sera from 8 healthy control subjects. These figures are higher than those reported by Fiebiger et al,⁹ who used ELISA with recombinant FcεRIα obtained in insect cells, which differs from CHO proteins in terms of biologic activity.¹⁷ In addition, Fiebiger et al⁹ assumed cut-off values for normal sera that were arbitrarily chosen to define background levels. In our study we used a method very similar to that reported by Horn et al¹⁷ and chose not to use background levels, and therefore the lower prevalence of anti-FcεRIα IgG reported by others may be mainly due to the antigen and the method used for Ig detection and data expression. In any case the real prevalence and physiologic significance of anti-FcεRIα antibodies in normal and CIU sera are still an open question, which may be resolved by careful comparison of data obtained with different functional and biochemical methods in a large case series of both healthy subjects and individuals with CIU.

In our study, although all patients had a positive skin test response, the anti-FcεRIα antibody titer varied great-

ly, and no correlation was recorded between skin responsiveness and anti-Fcε receptor antibody titer. Nonetheless, only two sera with a high titer of anti-FcεRIα antibodies were able to induce histamine release from basophils. These findings raise several considerations concerning the functional activity of these antibodies and their relationship with the skin response to autologous serum. The possibility that the in vitro HRA depends on the amounts of anti-FcεRIα autoantibodies is unlikely because sera with comparable titers of anti-FcεRIα IgG showed a different behavior with respect to basophil degranulation (Figs 1 and 2). On the other hand, the anti-FcεRIα antibodies could differ in their affinity for a common ligand, recognize different epitopes on Fcε receptor, or differ in the ability to activate the complement pathway, as recently suggested.^{9,11}

In our study the two sera endowed with functional activity lost their ability to trigger histamine release from basophils after heat inactivation and protein G adsorption. By contrast, all the treated sera retained the capacity to induce a wheal-and-flare response, thus suggesting that skin reactivity to autologous serum could depend on as yet unidentified non-Ig reactants present in sera of patients with CIU. This idea is supported by our previous finding that heparin addition to CIU sera or adsorption on solid-phase heparin before skin testing was associated with loss of cutaneous activity.¹²

Once we admit that skin reactivity to autologous serum could be due to non-Ig factors, several hypotheses may be advanced. The possibility that the putative HRFs act on mast cells rather than on circulating basophils seems unlikely. In fact, although basophils and mastocytes show many functional differences,¹⁸ Ferrer et al¹⁰ recently found an excellent correlation between histamine release from basophils and cutaneous mast cells stimulated in vitro with sera from patients with CIU. On the other hand, non-Ig HRFs could cause histamine release from skin mastocytes in vivo, although in vitro they are not able to degranulate basophils and mast cells. In this regard one could also advance the provocative hypothesis that the putative HRF could not act on basophils or mastocytes but directly exert their activity on the cutaneous microvasculature bypassing these cells.

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