Inhibitory effect of heparin on skin reactivity to autologous serum in chronic idiopathic urticaria

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Background: Most patients with chronic idiopathic urticaria (CIU) show cutaneous reactivity to intradermal injection of autologous serum. In some cases this reactivity is associated with the presence of autoantibodies directed against IgE or IgE receptors expressed on mast cells, whereas in others no autoimmune mechanisms can be documented.
Objectives: The aims of this study were to compare the cutaneous reactivity to serum and plasma samples in a series of patients with active CIU and to address the mechanisms of the inhibitory effect exerted by heparin on the cutaneous responsiveness to the histamine-releasing factors (HRFs) present in CIU serum.
Methods: Fourteen patients with CIU were injected intradermally with autologous serum, plasma (anticoagulated by either heparin or EDTA), or serum samples to which heparin had been added. The effects of heparin injection on cutaneous responsiveness to allergens was tested in 5 atopic patients. Moreover, in a set of experiments sera were also adsorbed with Sepharose-conjugated heparin.
Results: All the patients had positive cutaneous reactions to autologous serum injection. When heparinized plasma was injected, negative reactions were observed in 12 of 14 patients, and a sizable reduction in the wheal-and-flare reactions was recorded in the remaining 2. Compared with results obtained with serum, no substantial change was observed in 6 of 8 patients injected with EDTA-anticoagulated plasma. When heparin was added to serum, abrogation of skin reactivity was seen; nonetheless, no change in the cutaneous response to allergens was associated with locally administered heparin in 5 atopic patients with no history of CIU. Finally, adsorption of CIU sera with solid-phase heparin abrogated the ability to induce cutaneous reactions in 5 of 7 patients, whereas in the remaining 2 a sizable reduction was observed.
Conclusions: These data indicate that heparin is able to profoundly inhibit the cutaneous response to HRFs present in the sera of patients with CIU. Although the precise level of action of this heparin-mediated effect is unclear from present data, preliminary evidence seems to indicate that heparin could directly interfere with HRFs present in CIU sera. (J Allergy Clin Immunol 1999;103:1143-7.)

Key words: *Chronic idiopathic urticaria, heparin, histamine, cutaneous response*

Chronic idiopathic urticaria (CIU) is a common form of chronic urticaria in which no precise causal factors can be identified.[1] Several factors, including nonsteroidal anti-inflammatory drugs, foods, and additives, are able to induce or worsen this disease, but in contrast to what happens in urticarial forms with defined etiology, their withdrawal usually leads to little or no improvement.[1] In the past few years, it has been observed that in some patients with clinically active CIU intradermal injection of autologous serum elicits immediate wheal-and-flare responses[2]; during remission phases, however, the ability of autologous serum to induce such a reaction decreases or disappears, whereas intradermal administration of active disease serum still induces a wheal-and-flare reaction.[2] These data suggested the presence of histamine-releasing factors (HRFs) able to trigger cutaneous mast cell activation and degranulation in the sera of patients with CIU. These factors seem to be unique to CIU because skin tests performed in healthy subjects or patients with physical urticaria, as well as patients with respiratory allergy or aspirin intolerance, consistently show negative reactions (U. Fagiolo, unpublished data). Subsequent work[3-7] demonstrated the presence of anti-IgE and/or anti-FcεRI autoantibodies in sera from most patients with CIU. In view of this observation, it was suggested that at least some CIU forms could be sustained by an autoimmune process targeted on basophils and mast cells.[8] The functional activity of these autoantibodies is very important, however; only in a minority of cases were these antibodies able to cause in vitro homologous basophil degranulation,[7-8] whereas in most cases they were not capable of triggering basophil degranulation.

The relevance of these IgG autoantibodies to the natural history of CIU is unclear, and these antibodies cannot be considered responsible for all the cutaneous responses to autologous serum in patients with CIU. On the one hand, in a recent study[9] in patients with CIU and positive skin test responses to autologous serum, the

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Supported in part by grants from Ministero del' Università e della Ricerca Scientifica e Tecnologica (60%).
Received for publication Apr 15, 1998; revised Nov 9, 1998; accepted for publication Jan 11, 1999.
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0091-6749/99 $8.00 + 0 **1/1/97160**

Abbreviations used
CIU: Chronic idiopathic urticaria
HRF: Histamine-releasing factor

presence of anti-IgE autoantibodies in circulation was found in about 80% of the subjects but also in about 70% of patients with atopic dermatitis and in about 25% of healthy control subjects. On the other hand, anti-FcεRI autoantibodies were found in about 30% of patients with CIU, as well as in a sizable proportion of patients with dermatomyositis and pemphigo vulgaris[9]; in these latter patients, however, these antibodies did not seem to possess a functional activity in terms of histamine-releasing activity.

To our surprise, we recently observed that some patients with positive skin reactions to autologous serum displayed negative results when heparinized plasma collected at the same time was tested. To clarify this observation, we compared the cutaneous reactivity to serum and plasma samples in a series of patients with clinically active CIU and positive results to intradermal injection of autologous serum. Our data show that cutaneous reactivity to HRFs present in CIU serum is strongly inhibited by heparin, possibly through binding of HRF by heparin itself.

METHODS

Study population

Informed consent for this study was obtained from 14 adult patients with active CIU and positive skin test responses to autologous serum injection. These patients (8 males and 6 females) were selected from a larger CIU patient cohort comprising 98 individuals, 43 of whom had positive reactions (see below) to intradermal injection of autologous serum. All the patients were undergoing treatment with cetirizine associated in some cases with steroids. At the time of this study, none of the patients had received cetirizine during the last 48 hours before testing, and steroid treatment was withdrawn at least 7 days before. In addition, 5 atopic individuals from the medical staff were used as control subjects; 3 had RAST-confirmed positive skin test responses to house dust mites and 2 to grass pollen extracts. Moreover, 8 healthy volunteers were also used as normal control subjects. This study was approved by the institutional review committee for human experimentation.

Serum and plasma preparation

Serum and plasma were obtained from venous blood samples in fasting patients. For serum preparation, blood was clotted at room temperature for 30 minutes in silicone-coated Vacutainer tubes (Becton-Dickinson, Marlan, France) followed by centrifugation. Plasma was obtained from blood anticoagulated with 2 different sodium heparin concentrations (Roche, Basel, Switzerland; 50 and 200 IU/mL) or from EDTA-anticoagulated blood (final EDTA concentration, 6 μmol/L). The tubes were centrifuged at 550*g* for 10 minutes, and cell-free supernatant was collected. In a set of experiments, 0.8 mL serum aliquots were combined with 0.2 mL of isotonic saline containing heparin (1 IU/μL final concentration). As a control, serum was combined with the same quantity of plain saline.

In a set of experiments, serum aliquots (1.0 mL) were incubated in plastic tubes with 1.0 mL of Heparin Sepharose CL-6B (Pharmacia–LKB, Uppsala, Sweden) slurry gel at room temperature, according to the manufacturer's instructions. After 1 hour of incubation under continuous rotation, the tubes were spun, and serum was recovered. As a control, serum aliquots were also incubated with Sepharose CL 6B (Pharmacia-LKB) under the same conditions.

IgG levels in sera before and after solid-phase heparin adsorption were measured by RIA as described elsewhere.[10]

Experimental protocol

For skin testing, serum or plasma samples were used immediately after preparation or after storage at –20°C. The patients with CIU were injected intradermally on the volar surface of the forearms with 50 μL of each of the following: (1) undiluted serum; (2) heparin-anticoagulated plasma (50 or 200 IU/mL); (3) EDTA-anticoagulated plasma (6 μmol/L); (4) serum combined with heparin (1 IU/μL); (5) serum combined with plain saline (0.8 mL + 0.2 mL, respectively); and (6) serum adsorbed with Heparin Sepharose. As controls, the patients were also injected with (1) plain saline; (2) saline containing heparin (50 or 200 IU/mL) or EDTA (6 μmol/L); and (3) Sepharose-adsorbed serum. Skin reactivity was evaluated in all patients by a standard histamine prick test (Histamine hydrochloride 1%; Bayer, Milan, Italy).

To assess the possible influence of heparin on skin reactivity, 5 atopic individuals from the medical staff with no history of CIU were injected intradermally with 20 μL of *Dermatophagoides pteronyssinus* or *Lolium perenne* extract (Lofarma Allergeni, Milan, Italy; 0.04% wt/vol) diluted 1:5 as above with either saline or heparin (1000 or 200 IU/mL final concentration).

All the injection sites were scored 30 minutes after inoculation. The largest diameter (D_1) of the wheal and flare reactions and the diameters perpendicular to these (D_2) were recorded, and results were expressed according to the following formula: $D_1 + D_2/2$. Reactions showing a mean wheal/flare diameter of less than 0.5 cm were scored as negative.

Although all the patients were tested for skin reactivity to whole serum, histamine, and saline, not all the tests could be performed in all patients.

RESULTS

In all patients the skin test with histamine elicited positive results, whereas the injection of plain saline always elicited negative results (Table I). When autologous serum was injected, positive results were observed in all 14 patients studied (Table I); repeated testing on different occasions in the same patients always gave consistent results, with only minor modifications in the degree of the cutaneous response (not shown). In healthy donors testing with autologous serum always elicited negative results (data not shown). When heparin-anticoagulated plasma (200 IU/mL) was injected, the skin test results were negative in 12 of 14 patients (Table I), whereas a sizable reduction in the wheal-and-flare reactions was recorded in the remaining 2 patients. When plasma anticoagulated with 50 IU of heparin was used, a negative reaction was observed in 5 of 7 patients tested, whereas in the other 2 a significant decrease in the reaction was recorded (Table I).

To verify whether the differential behavior of serum and heparin-anticoagulated plasma samples could be due to the nature of the injected material, we also tested some

TABLE I. Skin reactivity to autologous serum or plasma in patients with CIU*

Patient no.	Serum	Heparinized plasma		EDTA plasma	Saline	Histamine
		200 IU/mL	50 IU/mL			
1	0.7/1.8†	Neg‡	ND	ND	Neg	0.5/1.4
2	0.8/1.4	Neg	ND	ND	Neg	0.6/0.9
3	2.1/2.5	Neg	Neg	Neg	Neg	1.0/1.4
4	1.4/3.5	Neg	Neg	1.1/3.0	Neg	0.8/1.3
5	1.0/4.0	0.5/1.0	Neg	ND	Neg	0.5/1.4
6	1.0/3.0	Neg	0.6/1.2	1.0/2.5	Neg	0.5/1.2
7	1.4/2.2	0.6/0.9	Neg	1.1/2.3	Neg	1.0/1.3
8	3.5/4.5	Neg	1.0/1.0	2.6/3.8	Neg	0.8/1.7
9	0.9/1.3	Neg	Neg	ND	Neg	0.5/0.9
10	1.0/1.3	Neg	ND	1.0/1.3	Neg	0.6/1.0
11	1.2/1.4	Neg	ND	1.1/1.4	Neg	0.9/1.6
12	0.9/1.6	Neg	ND	ND	Neg	0.5/0.9
13	1.0/1.8	Neg	ND	ND	Neg	0.7/1.0
14	1.3/2.6	Neg	ND	Neg	Neg	0.6/1.2

Neg, Negative; *ND,* not done.
*Patients with CIU were injected intradermally with serum or plasma samples according to the protocol detailed in the Methods section.
†Mean wheal/flare diameter in centimeters calculated as described in the Methods section.
‡Mean diameter, <0.5 cm.

TABLE II. Effect of heparin on skin reactivity to allergens in atopic individuals*

Subjects†	Allergen	Allergen + heparin (1000 IU/mL)	Allergen + heparin (200 IU/mL)
A	1.5/4.0‡	1.5/4.0	1.3/4.1
B	3.0/5.0	2.8/4.7	2.8/4.7
C	2.2/2.8	2.5/3.4	2.5/3.2
D	1.7/3.2	1.6/3.4	1.6/3.2
E	2.4/4.2	2.2/4.0	2.4/4.0

*Five atopic individuals without a history of CIU were injected intradermally with either allergenic extract or allergenic extract containing the indicated concentration of heparin.
†Subjects A, B, and C were allergic to house dust mites, and subjects D and E were allergic to grass pollen.
‡Mean wheal/flare diameter calculated as described in the Methods section.

TABLE III. Effect of heparin addition and solid-phase heparin adsorption on skin reactivity to autologous serum*

Patient no.	Serum†	Heparin-containing serum	Heparin Sepharose-adsorbed serum
1	0.7/1.8	Neg	Neg
4	1.4/3.5	Neg	Neg
6	1.0/3.0	Neg	ND
7	1.4/3.2	Neg	0.6/1.2
8	3.5/4.5	Neg	Neg
12	0.9/1.6	ND	Neg
13	1.0/1.8	ND	Neg
14	1.3/2.6	Neg	0.8/1.8

ND, Not done.
*Patients with CIU were injected intradermally with autologous serum, with serum to which heparin (1 μ/mL) was added, and with Heparin Sepharose-adsorbed serum. The results were expressed as detailed in the Methods section.
†Serum was combined with plain saline (0.8 mL + 0.2 mL, respectively) to compensate for the dilution caused by heparin addition.

of our patients with EDTA-anticoagulated plasma. No cutaneous reactivity to EDTA-containing saline could be observed in any of the patients tested (not shown). Compared with results obtained with serum, no substantial change in skin responses were observed in 6 of 8 patients injected with EDTA plasma (Table I). On the other hand, in 2 patients who showed a positive reaction to the injection of autologous serum, the inoculation of EDTA-anticoagulated plasma did not give any measurable response (patients 3 and 14 in Table I). These data seemed to indicate that the effect of heparin-anticoagulated plasma and serum was not apparently due to the different composition of serum and plasma. In addition, the maintenance of positive results with EDTA-anticoagulated plasma in most individuals also argued against the possibility that the factor or factors able to trigger mast cell degranulation could be generated during the clotting process.

The substantial maintenance of skin reactivity to EDTA-anticoagulated plasma seemed to suggest an inhibitory effect exerted by heparin on skin reactivity to the factor or factors contained in autologous serum. To verify whether the heparin-associated decrease in skin reactivity to autologous serum could be the result of a general ability of heparin to down-modulate mastocyte activation,[11-13] we tested the effect of heparin addition to allergenic extract in 5 atopic subjects. As shown in Table II, no change in response to allergens was associated with 2 different doses of heparin, thus indicating that the differential activity of autologous plasma, compared with that of serum, was likely not a result of a downregulating effect of heparin on cutaneous mastocyte responsiveness.

Because these data seemed to point to a direct effect of heparin on HRFs present in CIU sera, we addressed the

effect of heparin addition to sera. As shown in Table III, when we injected serum combined with heparin, abrogation of skin reactivity was observed in all cases. In addition, when sera were adsorbed with Heparin-Sepharose, abrogation of the ability to induce cutaneous responses was observed in 5 of 7 cases, whereas in the remaining 2 a significant reduction in the response was seen. No change in cutaneous reactivity to Sepharose-adsorbed control sera was observed. In addition, IgG measurement in 4 sera before and after heparin adsorption showed no change in IgG levels (9.8 ± 0.3 mg/mL vs 9.5 ± 0.4 mg/mL, respectively), thus rendering it unlikely that anti-IgE or anti-FcεRI autoantibodies could be nonspecifically adsorbed on the serum-heparin matrix (not shown). Thus these data strongly suggest that heparin may directly interfere with the putative HRFs present in CIU serum.

DISCUSSION

In this article we show for the first time that heparin is able to profoundly inhibit or completely block the cutaneous response to the HRFs present in sera from patients with CIU. Even though we did not perform a dose-response curve, the results obtained with plasma heparinized with 2 different heparin concentrations suggested that this inhibitory effect could be related to the heparin concentration. This phenomenon did not seem to be linked to factors unique to serum because cutaneous tests performed with EDTA-anticoagulated samples generally did not show significant changes compared with the cutaneous response to the serum counterpart. On the whole, these data strongly point to the ability of heparin to modulate the cutaneous response to HRFs present in serum from most patients with CIU.

The possible level of action of heparin in this context is a central point. It is well known that heparin, besides its anticoagulant activity, is also endowed with a series of biologic activities because of its ability to interact with many plasma proteins or cell surface components.[14] Thus heparin could exert its inhibitory activity either on the cutaneous response to HRFs present in CIU serum by a blockade of these factors, by interfering with mast cell function, or by both mechanisms. Heparin is known to modulate mast cell function by blocking inositol triphosphate binding to its receptor[15] or through the inhibition of some mast cell activators.[16-19] It is unclear whether such an effect is exerted through a specific competition at the receptor site[18] or through a more general mechanism, probably involving a change in the cell surface electrical properties.[20] As shown by other workers,[15] inositol triphosphate receptor blockade requires heparin internalization, and this process is not accomplished before 1 hour after incubation with heparin. Because in our study the inhibitory effect of heparin on the cutaneous response is immediate, we consider it unlikely that heparin effect could be mediated by an interference with second messenger intracellular pathways. In addition, the finding that intradermal administration of up to 1000 IU/mL of heparin was not able to downregulate mast cell responsiveness to allergen-mediated stimuli (Table II) strongly buttresses this idea. Although this latter observation is apparently in contrast with previous evidence,[11-13] this could depend on several variables that include type and doses of heparin used, route of administration, and type of readout used.

The ability of solid-phase heparin to downregulate the cutaneous reactivity of CIU sera in most cases (Table III) strongly points to an intrinsic ability of heparin to bind HRFs in these sera. It is known that heparin, because of its strong negative charge, is able to bind several positively charged molecules and eventually modify their functional activity. This effect is very rapid and has been demonstrated for a variety of molecules.[14,17] The nature of the HRFs present in the serum of patients with CIU is presently obscure, and probably several categories of HRF exist in different patients or even within a same patient. On the basis of our data, we suggest that heparin may be able to bind some of them, thus interfering with their activity. During the past few years, the presence of IgG autoantibodies directed against mast cell–bound IgE or FcεRI in serum of a variable proportion of patients with CIU was reported.[3-7,9,21] Only a minority of CIU sera are able to cause in vitro degranulation of homologous basophils, however. Thus it is likely that in the majority of CIU sera the cutaneous response to autologous serum could be caused by nonimmunoglobulin molecules. Indeed, preliminary experiments by testing the effect of Protein G–adsorbed CIU sera (U. Fagiolo, unpublished results) showed no substantial effect of IgG depletion in our patients. On the other hand, no effects of heparin on antigen-antibody interactions would be theoretically expected. Thus the inhibitory effect of heparin observed here is likely not exerted on HRFs belonging to IgG autoantibodies. Nonetheless, anti-IgE or anti-FcεRI antibodies may play a significant role in some cases. Indeed, in our case series heparin was not able to completely abrogate the effect of autologous sera in some cases (Table I) nor did heparin adsorption always remove histamine-releasing activity (Table III).

In any case, whatever the mechanism or mechanisms of action of heparin in modulating skin reactivity of patients with CIU to autologous serum, our results constitute a solid background for a new approach to understanding CIU and a possible breakthrough in its management. It will be of paramount importance to verify whether the apparent favorable effect of heparin in the model studied here could translate into beneficial effects in a clinical setting. In this regard a limited phase I trial with low heparin doses in our patients with CIU seems to show promising clinical results (U. Fagiolo, manuscript in preparation).

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