

Chronic idiopathic urticaria: infiltrating cells and related cytokines in autologous serum-induced wheals

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Abstract

The term chronic autoimmune urticaria (CAIU) is used for chronic urticaria in subjects who present a whealing response to the intradermal injection of autologous serum, suggesting the presence of pathogenic antibody activities.

In this study, we examined 28 chronic urticaria subjects with positive autologous serum skin test (ASST), all of whom presented autologous serum-induced lesions at different evolutive stages. Punch biopsies were taken from lesional skin of six subjects at 10', eight subjects at 30', six subjects at 60', and four subjects each at 24 and 48 h. Immunological studies focussed on infiltrating cell immunophenotype and related cytokines, chemokines and chemokine receptors, adhesion molecules. Immunohistochemical staining was performed to measure expression of CD3, CD4, CD8, tryptase, eosinophil cationic protein, myeloperoxidase, basophil granular protein, IL-4, IL-5, IL-8, CCR3 and CXCR3, ICAM-1, VCAM and ELAM. Control staining was done on unaffected skin from the patients and normal skin from four healthy donors.

The main infiltrating population was represented by neutrophils, seen focally in both unaffected skin ($P = 0.001$) and healthy controls ($P = 0.003$). IFN- γ and IL-5 were expressed focally in autologous wheals. Significant staining for IL-4 was seen at 30'. CCR3 and CXCR3 were expressed less in autologous wheals than in uninvolved skin ($P < 0.0001$; $P = 0.002$). Cellular adhesion molecules (CAMs) reached their highest expression at 30' and 60' in induced lesions, and they showed strong expression also in unaffected skin (ICAM-1: $P < 0.0001$).

Our data show that the immunoinflammatory features of ASST-induced wheals involve a prevalent role of lymphocytes (with a mixed Th1/Th2 response), with strong neutrophil infiltration and activity and involvement of the chemokine pathway. We interpreted the finding of inflammatory cells and mediator up-regulation in uninvolved CIU skin as a sign of prolonged and widespread "urticarial status".

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Introduction

Chronic urticaria (CU) is currently defined as the daily or almost daily occurrence of wheals for at least 6 weeks, and the term "idiopathic" urticaria (CIU) is used for CU cases in which neither signs of vasculitis nor causative drugs, foods, and/or physical factors can be individuated [1,2].

Previous studies have clarified that chronic idiopathic urticaria (CIU) includes a heterogeneous subset (up to 46% of cases) [2] that presents a previously unidentified serum factor, later discovered to be an antibody that most probably plays an important role in the pathogenesis of CIU. This antibody belongs to the IgG isotype and it reacts against the α -chain of the high affinity IgE receptor (Fc ϵ RI) of basophils and mast cells, or alternatively against IgE itself [3]. Functional properties of antibodies are evidenced in vivo by the intradermal injection of autologous serum

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(autologous serum skin test, ASST) [4], that is able to induce a wheal-and-flare response in patients with CIU; further evidence is seen *in vitro* with the release of histamine from basophils and mast cells elicited by addition of CIU patient serum [5–7].

Since CIU has been hypothesized to have an autoimmune pathogenesis, the term chronic autoimmune urticaria (CAIU) has been proposed for CIU patients with positive ASST and *in vitro* assays. These subjects frequently present an increased incidence of autoimmune disorders (e.g., thyroiditis) and more severe urticarial clinical features than normal CU patients [8].

The literature includes several reports that contribute to better understanding the pathogenesis and effector mechanisms of CIU, focussed in turn on the different cellular populations that infiltrate urticarial lesions. Comprehensive review of the molecular immunopathology of CIU seems to indicate early activation of mast cells [9–11], followed by a lymphocyte and granulocyte cell-mediated hypersensitivity reaction with a Th0 (or, alternatively, a mixed Th1/Th2) cytokine profile [12–19].

However, the real advances in this field came with studies on the temporal evolution and changes of the wheal infiltrating cells. In particular, Grattan et al. [20] in 1990 performed the first serial biopsies of autologous serum-induced wheals in CIU patients. Then, confirming mast cell degranulation by electron microscopy in early wheals, he demonstrated that, like T lymphocytes, perivascular neutrophils and eosinophils showed a tendency to increase from 30 min to 2 h. At 24 h, CD4+ T cells significantly outnumbered CD8+ cells; at 48 h, the neutrophils were clearing, but the eosinophils and lymphocytes persisted.

Also Sabroe et al. [2] sought to determine the timing of the inflammatory infiltrate in the wheals of CIU patients and to detect differences between patients with and without antibodies. These investigators found that eosinophil, neutrophil, and lymphocyte infiltration was more extensive in both “recent” and “established” wheals (less than 4 h and more than 12 h, respectively) than in uninvolved skin of CIU subjects with positive ASST. CIU subjects without antibodies had greater numbers of eosinophils in established wheals than those with antibodies, but the two groups presented no other significant difference, suggesting that eosinophil activation may occur later or be more persistent in urticaria subjects without antibodies [2].

Similarly, in a previous study [21], we tried to find more specific information on the function of the inflammatory response in spontaneous wheals of CAIU subjects. We found similar tryptase staining in both CAIU subjects and healthy controls. Moreover, the lack of any significant differences between lesional and unaffected skin of CAIU subjects in the expression of T lymphocytes, adhesion molecules (ICAM-1 and VCAM-1), and chemokines (C-Chemokine Receptor 3, CCR3) seemed to indicate widespread immunological activation, involving not only lesional tissues, but possibly the entire skin immune system

[21]. These findings recapitulate Natbony’s previously published conclusions [22].

In the present study, we examined autologous serum-induced lesions at different evolutive stages to find further information about the pathological mechanisms of CAIU. We carried out thorough investigations of immunophenotype of infiltrating cells, related cytokines, chemokines and chemokine receptors, and adhesion molecules.

Methods

Patients

The study comprised twenty eight patients (15 M, 13 F; age range 23–68; median age 42,08 years) diagnosed with chronic urticaria. All subjects had a disease duration of more than 3 months, with recurrent whealing at least twice a week. All the subjects presented a positive autologous serum skin test (ASST), as defined by Grattan et al. [23] and positive *in vitro* serum-evoked histamine release assays (HRA). HRA was performed on basophils of healthy donors as previously described [24]. Antihistamines were discontinued 1 week before skin testing in all cases and none of the subjects was taking other drugs. The exclusion criteria were negative response to ASST, predominant physical urticaria, allergic urticaria (IgE-mediated), urticaria-vasculitis, C₁-esterase inhibitor deficiency, drug or alcohol abuse, pregnancy, and lactation. Four age- and sex-matched healthy subjects were recruited as controls. Baseline investigations included search for circulating immunocomplexes (CIC) and organ and non-organ specific antibodies to evidence any autoimmune stigmata in our patients.

Biopsies

Six of the 28 patients with CIU and positive ASST were subjected to 4 mm punch biopsies taken from an autologous serum-induced wheal 10 min after development, the established baseline time. Bioptic specimens were taken from eight subjects at 30 min, from six at 60 min, and from four each at 24 and 48 h. Additional biopsies were taken from apparently uninvolved skin of four of the above subjects before the ASST was performed (*t* = 0). Finally, four control specimens were obtained from the healthy donors.

The biopsies were performed under local ring anesthesia (without adrenaline) and covered in cryoprotectant (Tissue-Tek, Miles INC, USA) before storage at –80°C.

Immunohistochemistry

Tissue specimens were cut into 5-μm sections that were then stained with hematoxylin–eosin for routine immunohistochemical examination.

The various types and titers of monoclonal antibodies (Mo Abs) used included MoAbs against tryptase at 1:50

(Dako Ltd., High Wycombe, UK) to detect mast cells. For the detection of human T lymphocytes, we used CD3 at 1:20 pan-specific for T cells, CD4 at 1:50 specific for T-helper cells, CD8 at 1:20 specific for cytotoxic/suppressor T cells (Dako). Neutrophils stained with Mo Abs against myeloperoxidase (MPO) at 1:100 (Dako). Eosinophils were labeled with anti-EG1 at 1:150 and anti-EG2 at 1:300 (Pharmacia and Upjohn Diagnostics Ab, Uppsala, Sweden) that bind to the epitope of eosinophilic cation protein (ECP) in the non-secreted and secreted forms. BB1 at 1:10, a novel Mo Ab (the kind gift of Dr A.F. Walls, University of Southampton, United Kingdom) was used to find human basophil granular protein.

We studied the cytokines in tissue specimens using Mo Abs anti-IL4 at 1:20, anti-IL-5 at 1:50, anti-IL8 at 1:100, anti-IFN- γ at 1:100 (R&D Systems, Europe Ltd. Abingdon, UK). The Mo Abs for integrins used were ICAM-1 at 1:50 (Immunotech, Marseille, France), V-CAM at 1:50 (R&D Systems), ELAM at 1:100 (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). We also studied two chemokine receptors using antibodies against CCR3 at 1:50 and anti-CXCR3 at 1:50 (R&D Systems).

Frozen sections were air-dried and fixed in acetone (5 min) before staining. Immunolabeling was performed using rabbit anti-mouse bridging antibodies conjugated with alkaline phosphatase (1:10 dilution, 30 min) (Dako) followed by incubation with murine alkaline phosphatase anti-alkaline phosphatase (APAAP) complexes (1:30 dilution, 30 min) (Dako). Two independent observers did “blind” assessments of the histological sections. For quantitative analysis, we used the mean of the stained cells counted in three adjacent microscopic fields (250 $\times$) of each section.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD) of the mean. The first step consisted in comparing the mean of stained cells in autologous wheal sections and then calculating statistical differences between the mean numbers observed at different times. Later, we compared results from lesional, uninvolved and healthy skin. Statistical significance ($P < 0.05$) was assessed by Student's t test.

Results

Immunological profile

Circulating anti-thyroid autoantibodies were detected in 15 CIU subjects (7 had anti-thyreoglobulin and 10 anti-peroxidase autoantibodies). Eight subjects had anti-nuclear autoantibodies with a titer of 1:40. Two CIU subjects had anti-gastric parietal cell antibodies, one anti-pancreatic islet, none had CIC, and 7 had anti-*Helicobacter pylori* antibodies.

Table 1
Time evolution of infiltrating process in autologous wheal, uninvolved skin of CIU patients and healthy control skin (mean $\pm$ SD)

	MoAb	Autologous wheal				Uninvolved skin				Healthy skin		$P^{(7)}$			
		10' (baseline) $n = 6$	30' $n = 8$	$P^{(1)}$	60' $n = 6$	$P^{(2)}$	24 h $n = 4$	$P^{(3)}$	48 h $n = 4$	$P^{(4)}$	Uninvolved skin		Healthy skin		
											$n = 4$		$P^{(5)}$	$n = 4$	$P^{(6)}$
Lymphocytes	CD3	26.7 ± 14.1	28.5 ± 17	0.8	21.1 ± 9.7	0.4	20 ± 10.5	0.4	26.6 ± 16.8	0.9	30.7 ± 11.3	0.6	4.0 ± 2.0	<0.0001	
	CD4	22.6 ± 10.4	26.2 ± 8.6	0.5	26.7 ± 12.7	0.5	30.6 ± 9.2	0.2	30.3 ± 4.9	0.2	44.9 ± 13	<0.0001	6.3 ± 1.3	<0.0001	
	CD8	8.7 ± 5.4	9.1 ± 7.6	0.9	9.0 ± 7.7	0.9	7.0 ± 2.0	0.5	12.3 ± 5.8	0.3	20.8 ± 9.2	0.0002	4.6 ± 0.9	0.01	
Mast cells	Try	21.7 ± 11.3	18.4 ± 7.3	0.5	20.6 ± 9.0	0.8	18.5 ± 12	0.6	19 ± 1.3	0.6	23.7 ± 6.0	0.6	22.9 ± 0.9	0.7	
Neutrophils	MPO	24.3 ± 6.4	49.1 ± 7.2	0.02	84.9 ± 31	0.0001	75.6 ± 2.0	0.0001	49 ± 2.6	0.0001	13.7 ± 8.9	0.001	17.6 ± 1.7	0.003	
	EG1	7.8 ± 4.7	13.1 ± 7.0	0.1	16.8 ± 11.1	0.09	8.0 ± 0.1	0.9	8.0 ± 0.1	0.9	3.3 ± 3.2	0.4	2.0 ± 0.1	0.003	
	EG2	7.5 ± 4.9	16.0 ± 9.0	0.07	17.3 ± 6.4	0.08	19.3 ± 5.0	0.006	28 ± 2.8	0.0001	3.3 ± 3.5	0.1	2.0 ± 0.1	0.001	
Basophils	BB1	4.3 ± 2.7	11.0 ± 3.2	0.0002	6.6 ± 2.2	0.06	4.0 ± 1.7	0.8	3.2 ± 1.2	0.8	4.6 ± 2.9	0.8	1.6 ± 1.1	0.007	

n : number of investigated patients.

$P^{(1)}$: comparison between 10' and 30'.

$P^{(2)}$: comparison between 10' and 60'.

$P^{(3)}$: comparison between 10' and 24 h.

$P^{(4)}$: comparison between 10' and 48 h.

$P^{(5)}$: comparison between autologous wheals at 10' and uninvolved skin.

$P^{(6)}$: comparison between autologous wheals at 10' and healthy control skin.

$P^{(7)}$: comparison between unaffected skin and healthy control skin.

Histologic examination

All biopsies from affected skin showed interstitial dermal edema, dilated venules with endothelial swelling and a perivascular and interstitial mixed-cell infiltrate in agreement with the original diagnosis of urticaria. We did not observe any signs of vasculitis-like endothelial degeneration, vessel necrosis, leukocytoclasia, fibrin deposition or erythrocyte extravasation.

Cellular infiltrate

In autologous wheals, lymphocytes (CD3+, CD4+, CD8+) were located mainly in the perivascular upper- and mid-dermis. No significant variations in mean numbers were evidenced at different time checks (Table 1, Fig. 1). The CD4/CD8 ratio was about 2:1 at all times.

CIU subjects with positive ASST showed more numerous CD4- and CD8-positive cells in apparently uninvolved skin than in lesional skin. Induced lesions and unaffected skin showed significant differences in CD4 cells up to 60' and CD8 cells up to 24 h.

On the other hand, skin from healthy donors showed lower T lymphocyte counts (Table 1). Statistical difference was always significant for CD4, and only at 48 h for CD8.

In skin biopsies from autologous wheals, tryptase-positive (+) mast cells were detected in the upper- and mid-dermis with a prevalent perivascular and periadnexal pattern. The intensity of staining did not change remarkably in autologous serum-induced wheals at subsequent times of observation (Table 1). Mast cell mean counts were similar to those of unaffected skin of the same patients and normal control specimens (Table 1). Statistical analyses did not evidence any significant differences within a group at different times or between uninvolved patient skin and healthy controls.

In autologous wheals, MPO+ neutrophil granulocytes, located in the upper perivascular dermis, increased progressively, representing at 60' the major cellular population infiltrating autologous wheals (Fig. 1). The mean of MPO+ cells at 60' significantly outnumbered those at both 10' and 48 h (Table 1). Neutrophil infiltration was significantly lower in both unaffected CIU skin and specimens from healthy controls than in autologous wheals (Table 1).

In lesional skin, eosinophils were located mainly in perivascular dermal sites. Eosinophil functional activity was studied by immunohistochemical markers EG1 and EG2 that detect the intra- and extracellular isoform of ECP, respectively. We observed a progressive increase in EG1+ cells from 10' to 60' followed by decreases at 24 and 48 h, but this trend was not statistically significant (Table 1). EG1+ cells were poorly represented in uninvolved skin of urticaria subjects as well as in healthy donor specimens (Table 1).

EG2 expression increased up to 48 h, but this increase was significant only after 24 h. EG2 staining was weak in

samples of both uninvolved skin from CIU subjects and healthy controls.

In autologous wheals, basophils were located predominantly in intra- and perivascular sites in the superficial dermis and showed prevalently intracellular staining. A statistically significant increase for BB1 was evident at 30' (Fig. 1), followed by a trend to slow decrease. Interestingly, we detected relevant amount of these cells also in unaffected skin of CIU subjects, but they were poorly represented in the skin of healthy controls.

Cytokines, chemokines, and chemokine receptors

In autologous wheals, IFN- γ -positive cells showed focal dermal staining with constant mean counts at subsequent observations (Table 2). Staining in wheal specimens at 24 h was significantly different from that observed in uninvolved skin of urticaria subjects. Skin specimens from healthy controls did not show any IFN- γ expression (Table 2).

IL-4 staining, possibly attributable to activated T lymphocytes, mast cells, and basophils, increased in lesional skin within the first 30' (Fig. 2), when it showed the highest expression, and then it started to decrease from the peak. However, this trend was not statistically significant (Table 2). Skin of healthy controls was negative for IL-4 expression, whereas uninvolved skin specimens from CIU subjects showed a low mean value that differed significantly from that of the autologous wheal at 10' (Table 2).

In autologous wheals, focal perivascular staining for IL-5 was detectable in the dermis from the time of 30' (Fig. 2) and it started decreasing in the first hour thereafter (Table 2). In uninvolved skin, IL-5 was assessed at a mean value of 3.6 ± 4.0 , significantly different from the 60' count in lesional skin (Table 2). No reactivity for IL-5 protein was found in skin of healthy controls.

IL-8, a monocyte-, neutrophil-, eosinophil-, and mast cell-related chemokine, was moderately expressed in epidermis and dermis with constant intensity in autologous wheals (Table 2). There was moderate IL-8 staining in uninvolved skin of affected subjects, but none in the skin sections of healthy controls. (Table 2).

Chemokine receptor expression of lesional skin at different times (particularly CXCR3) was very similar to that of healthy control skin, except at 30' when CXCR3 showed a higher cell count in lesional skin. However, no significant difference was found between the 10' and 30' values (Table 2), and when compared to uninvolved CIU skin, the difference was significant only at 10'. Otherwise, CCR3 staining was slighter than that in CXCR3 at the different times, but without significant variation (Table 2). Uninvolved skin showed a mean CCR3 expression of 14.1 ± 6.6 significantly different from that of the autologous wheal at 10'. No significant differences were observed between lesional skin and that of healthy controls.

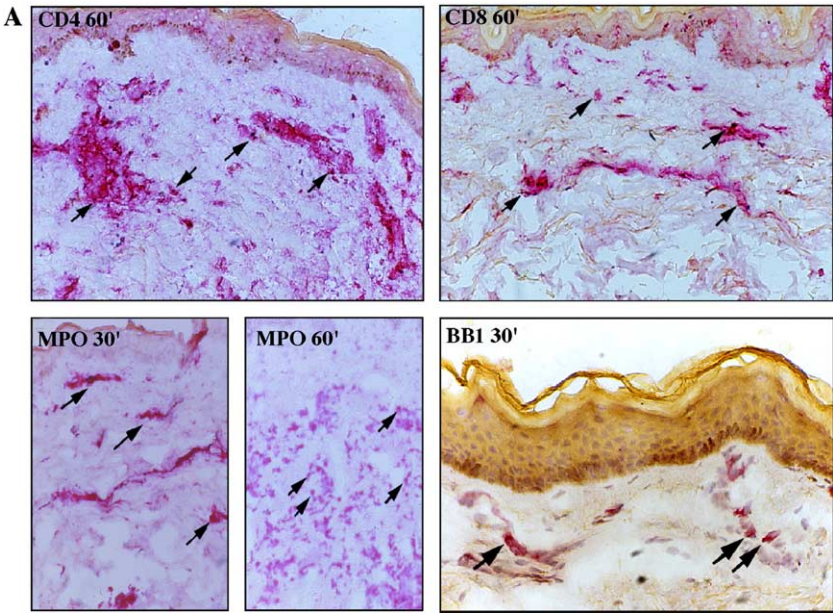


Fig. 1.

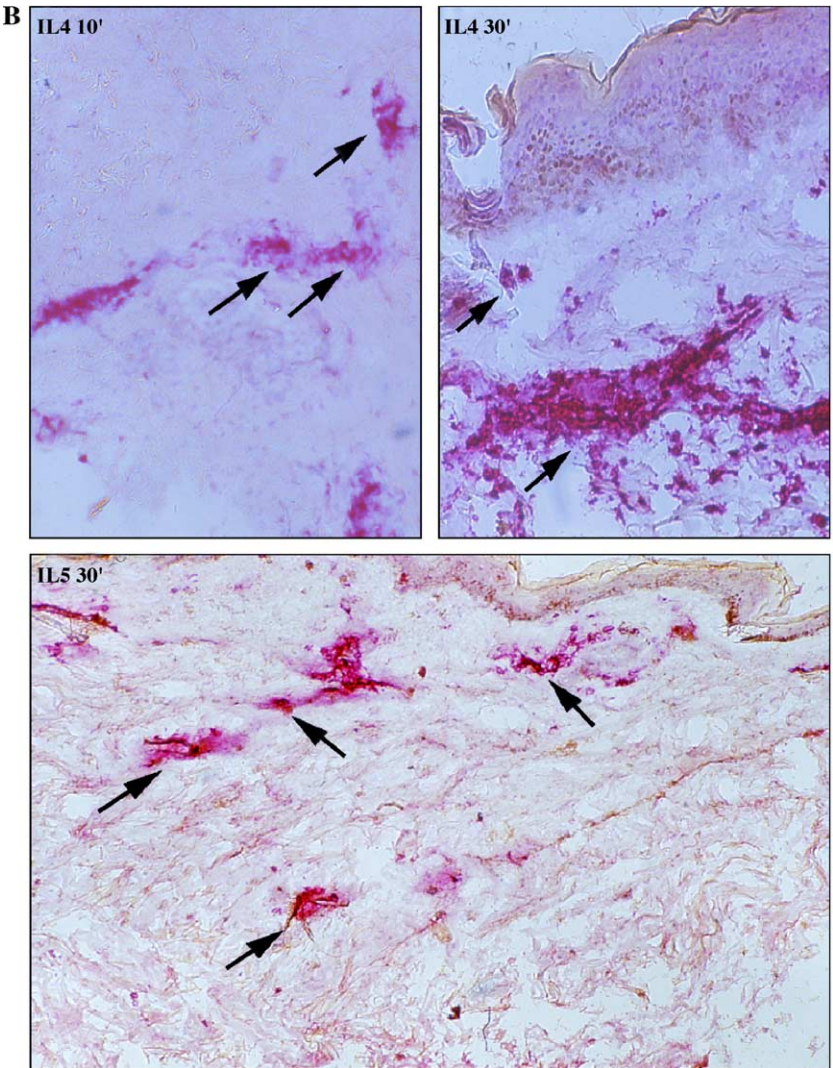


Fig. 2.

Cellular adhesion molecules (CAMs)

In autologous wheals, ELAM expression was slightly but not significantly increased at 60' (Table 2).

In uninvolved CIU skin ELAM expression was assessed at a mean of 6.7 ± 4.6 , a value not significantly different from that of autologous wheals.

The highest expression of ICAM-1 was reached at 30' but overall there was no significant difference in expression between autologous wheals and skin of healthy controls (Table 2). VCAM showed the highest expression at 48 h with statistically relevant increase compared to the 10' value (Table 2). These CAMs were intensely expressed in uninvolved skin of urticaria subjects (Table 2).

Discussion

In this study, ASST-induced wheals of CIU subjects were sequentially biopsied at different times after their appearance and specimens were subjected to investigation of immunophenotype of infiltrating cells and related molecules. Our data describe evolutive stages of the wheal-and-flare skin process and are compared to findings from both uninvolved skin of the same urticaria subjects and skin specimens from healthy donors. The aim of the study was to glean information about the pathogenesis and effector mechanisms of CIU, considered a model of dynamic inflammation.

We note that ASST alone is not sufficient to define CIU as an autoimmune disease. However, ASST has often been chosen as an experimental model (2,20) because it has an effect very similar to that of the physiological stimulus that causes whealing in urticaria. Moreover, it is performed in vivo and in the very serum-donor subjects affected with CIU, which allows one to avoid the many biases involved in in vitro procedures.

One must also consider that in the large population of skin test-positive subjects, different subsets can be distinguished on the basis of whealing persistence. In fact, in our series, only a minority of CIU subjects had long-lasting lesions (up to 24 and 48 h). We can suppose that several individual factors influence the duration of “idiopathic” urticaria wheals, for instance, the nature and functional properties of the “serum factor” contained in CIU sera (anti-FcεRI antibodies, anti-IgE antibodies, other histamine-releasing activities with different molecular characteristics). Other variables that may influence wheal duration are basophil releasability that varies largely from one subject [25] to another, and/or basophil concentration in lesional tissue.

We detected in our specimens early participation of T lymphocytes, already present at 30' and persisting until 48 h, which emphasizes their role in inflammatory response. As mentioned above, this finding reproduces that of Grattan, who also found a progressive increase in numbers of T cells after the first determination (30') up to 24 h. Since T cell numbers were significantly higher in uninvolved than in affected skin of CAIU subjects, one might suggest that a “latent” skin inflammation [26] extends to the entire surface of the skin and mucosae, lowering the reactive threshold to triggering stimuli. This “urticarial status” [25–28] could also explain the extreme spreading characteristic of CIU wheals. The principal difference between our investigation and that of Grattan regards the CD4/CD8 ratio, that was about 2:1 in our series, whereas in Grattan's study CD8 cells outnumbered CD4 cells at all times, except at 24 h.

In regard to mast cells, we did not observe significantly different numbers of these cells in autologous wheals, unaffected skin of the same subjects and healthy controls, contrary to the findings of increases in mast cell in CIU lesions reported in the literature [11,27]. Moreover, in our specimens, mast cell counts were constant at different times in autologous wheals, deduced from the lack of significant fluctuations in tryptase staining from 30' to 48 h. This observation, however, is not incompatible with the predominant role of tryptase+ mast cells and related mediators in hive formation, but merely suggests that we should consider their activation to occur very early, as previously proposed [11,12]. We might speculate that involvement of mast cells, in particular their activation and degranulation, is predominant at the very onset of the induced lesion (when they would prime the immediate phase of inflammation), and that afterwards, they play a less important role as revealed by the lack of substantial differences in their numbers at different times.

MPO staining, performed to quantify neutrophils, closely resembled the findings reported in the previous literature [20]. Neutrophils represented the main component of the cellular infiltrate, the most diffuse dermal neutrophilia appearing at 60', with lower detectable staining at the other times. The hypothesis that neutrophil granulocytes might play an important role in the development of wheals, through the release of inflammatory mediators, is supported by the very weak MPO expression observed in both uninvolved skin of CIU subjects and healthy control skin. These cells, recruited only in those sites where a lymphocytic activation is already started, might leave blood vessels and accumulate exclusively in whealing skin.

The reasons for the high chemoattraction of neutrophils in CIU lesional skin remain to be clarified, and may entail

Fig. 1. CD4-positive T lymphocytes with mainly perivascular distribution in the upper and mid dermis of autologous serum-induced wheal at 60'; CD8 expression in lesional skin at 60'; MPO staining at 30' in a biopsy from autologous wheal; MPO staining at 60' in a biopsy from autologous wheal; BB1-specific staining in lesional skin (autologous wheal, 30').

Fig. 2. IL-4 expression in lesional skin (autologous wheal, 10'); IL-4 expression in lesional skin (autologous wheal, 30'); Focal perivascular staining for IL-5 detectable in the dermis at 30' in autologous wheal.

Table 2
Timing of cytokine, chemokine and chemokine receptor, CAM expression in autologous wheal, uninvolved skin of CIU patients and healthy control skin (mean $\pm$ SD)

MoAb	Autologous wheal										Uninvolved skin		Healthy skin	$P^{(7)}$	
											$P^{(5)}$	$P^{(6)}$			
	10' n = 6	30' n = 8	$P^{(1)}$	60' n = 6	$P^{(2)}$	24 h n = 4	$P^{(3)}$	48 h n = 4	$P^{(4)}$	n = 4	N = 4				
Cytokines	IFN- γ	1.7 $\pm$ 1.3	2.7 $\pm$ 3.4	0.5	2.0 $\pm$ 1.4	0.7	3.6 $\pm$ 0.5	0.02	2.0 $\pm$ 1.2	0.7	1.3 $\pm$ 1.5	0.5	negative*	0.002	0.002
	IL-4	15.9 $\pm$ 7.8	25.9 $\pm$ 14.4	0.1	9.4 $\pm$ 5.3	0.1	12.0 $\pm$ 14.1	0.5	10.0 $\pm$ 4.1	0.2	5.0 $\pm$ 2.0	0.02	negative*	<0.0001	<0.0001
	IL-5	negative*	10.0 $\pm$ 7.8	0.0006	9.9 $\pm$ 2.9	<0.0001	4.3 $\pm$ 1.1	<0.0001	4.5 $\pm$ 0.7	0.0004	3.6 $\pm$ 4.0	0.004	negative*	0.8	0.004
	IL-8	8.7 $\pm$ 3.9	12.0 $\pm$ 8.6	0.4	10.1 $\pm$ 4.0	0.5	6.6 $\pm$ 7.0	0.8	6.0 $\pm$ 3.0	0.2	12.2 $\pm$ 8.6	0.06	negative*	0.0004	0.0002
Chemokines	CXCR3	26.0 $\pm$ 14.3	33.0 $\pm$ 16.7	0.4	20.0 $\pm$ 7.6	0.3	14.0 $\pm$ 3.4	0.1	15.6 $\pm$ 3.0	0.1	47.5 $\pm$ 15.0	0.002	20.0 $\pm$ 10.1	0.1	0.0004
	CCR3	5.3 $\pm$ 3.0	8.8 $\pm$ 9.1	0.3	6.5 $\pm$ 2.8	0.4	6.7 $\pm$ 5.7	0.6	4.3 $\pm$ 1.1	0.5	14.1 $\pm$ 6.6	<0.0001	5.2 $\pm$ 2.8	0.4	0.03
CAMs	ICAM-1	16.3 $\pm$ 8.4	27.8 $\pm$ 10.3	0.06	22.5 $\pm$ 6.6	0.1	14.6 $\pm$ 8.6	0.7	23.3 $\pm$ 1.5	0.1	33.5 $\pm$ 9.8	<0.0001	21.1 $\pm$ 9.3	0.1	0.004
	VCAM	9.3 $\pm$ 2.8	11.2 $\pm$ 3.7	0.3	8.0 $\pm$ 3.4	0.4	10.6 $\pm$ 2.5	0.4	13.0 $\pm$ 0.1	0.03	16.7 $\pm$ 6.6	0.001	6.8 $\pm$ 4.8	0.1	0.004
	ELAM	6.5 $\pm$ 4.9	5.9 $\pm$ 4.0	0.8	8.5 $\pm$ 5.0	0.5	5.0 $\pm$ 4.3	0.6	3.6 $\pm$ 1.1	0.2	6.7 $\pm$ 4.6	0.5	4.9 $\pm$ 1.5	0.7	0.4

n: number of investigated patients.

Negative*: for statistical purpose, a conventional mean value of 0.1 was chosen.

$P^{(1)}$: comparison between 10' and 30'.

$P^{(2)}$: comparison between 10' and 60'.

$P^{(3)}$: comparison between 10' and 24 h.

$P^{(4)}$: comparison between 10' and 48 h.

$P^{(5)}$: comparison between autologous wheal at 10' and unaffected skin.

$P^{(6)}$: comparison between autologous wheal at 10' and healthy control skin.

$P^{(7)}$: comparison between unaffected skin and healthy control skin.

simultaneous involvement of various mechanisms. Some of these mechanisms involve IL-8 that is important in chemotaxis, as well as in inducing shape change and degranulation of cells [28]. However, it seems unlikely that it is the only chemotactic molecule, especially since we found a similar expression of this chemokine also in unaffected CIU skin, where neutrophils are significantly less numerous. Thus, some other granulocyte chemoattractant and/or activating system might be sustained by other cytokines, such as IL-4 (that acts by enhancing the respiratory burst and phagocytic properties [29]), mast cell-derived TNF- α and GM-CSF [30], complement fragment C5a [31], and by CXCR3.

An effector role in the development of wheals can also be suggested for eosinophils, shown in lesional skin by EG1 staining; this was remarkably similar to the neutrophil trend in both ASST-induced lesions and hive-spared skin. Eosinophil chemotaxis to lesional sites would be followed by secretion of eosinophil cationic protein (ECP), confirmed by the progressive increase of EG2.

We have also demonstrated, using the well-defined phenotype marker BB1, that basophils are present, indeed, in autologous wheal biopsies at all times. Since basophils express their Fc ϵ RI, it seems likely anti-Fc ϵ RI autoantibodies can directly interact with these cells to activate them in situ. Moreover, the persistent staining for BB1 at 48 h might explain the prolongation of wheals characterizing the "autoimmune" subset of CIU patients.

The cellular infiltration pattern has already been investigated by other authors, and the novelty of the present study consists in the immunohistochemical detection of cytokines and chemokines. To date, few studies [12,32,33] have been carried out to define the cytokine profile of the sera and/or tissues of CIU patients. The only available data consist in the serum increase of TNF- α and IL-10 in CIU subjects compared to healthy controls, despite similar IL-4 levels in the two groups, suggesting that an IgE-dependent mechanism might not be primarily involved in the etiopathogenesis of CIU.

IL-4 plays a role in cutaneous mast cell degranulation and regulates the synthesis of IgE [34], and IL-5 is reported to exercise specific chemotactic activity on eosinophils and can induce the full pathway of eosinophil differentiation [35]. Moreover, as shown by Ying et al. [12], in addition to the prominent role of IL-4 and IL-5, also IFN- γ is detected in skin biopsies from CIU subjects (although not in allergen-induced late-phase reactions). This finding suggests that there is a balance between Th1 and Th2 cytokines in urticarial inflammation, with early involvement of Th2-like molecules (possibly derived from T helper lymphocytes, basophils and mast cells) and later participation of Th1 activity, demonstrated by the significant increase of IFN- γ at 24 h.

We investigated CXCR3 and CCR3 expression as prototypes of α and β -chemokine families.

Activated Th lymphocytes are the cellular source of these molecules that in turn exert a chemoattractant function on

the very leukocytes. Alpha-chemokines (CXC-) promote neutrophil attraction towards tissues, whereas β -chemokines (CC-) recall monocytes, lymphocytes, basophils, and eosinophils with variable selectivity [36,37]. Both CXCR3 and CCR3 showed a peak at 30' in autologous wheals, followed by a progressive decrease, suggesting that in early whealing such recruiting mechanisms might work to attract neutrophils, eosinophils, and basophils. CXCR3 receptor is involved in classical Th1 responses, while CCR3 is primarily (although not exclusively) associated with Th2 pattern. These findings further confirm the combined lymphocyte response. Moreover, the overlapping trend of IL-4 and CCR3 in autologous wheals would further support the proposed pathogenic role of basophils.

In addition to chemokines, other key players in tissue localization of leukocytes are represented by cellular adhesion molecules (CAMs). CAMs are cell surface proteins that are important as mediators of close cell–cell and cell–matrix interaction. They possess unique specificity that allows them to interact selectively with ligands that may be other adhesion molecules or matrix proteins [35].

Cassano et al. [27] have recently detected increased expression of ELAM and VCAM in unaffected skin of CIU patients compared to that of healthy controls. This finding indicates that non-lesional skin presents signs of latent inflammation, in agreement with previous [21,38] and present studies showing high levels of adhesion molecules in the uninvolved skin of patients with chronic urticaria.

However, in addition to CAMs, also T cells, eosinophils, basophils, and chemokine receptors were often overexpressed in unaffected CIU skin compared to controls and lesional skin specimens. The observed up-regulation of inflammatory cells, soluble mediators and adhesion molecules in uninvolved CIU skin could reinforce the hypothesis of minimal persistent inflammation in the apparently unaffected tissues. We suppose that, analogously to what has been demonstrated in pathologic atopy-related conditions like asthma and atopic dermatitis [39,40], in CIU, the repeated whealing episodes would modify the cutaneous microenvironment and the communication signals between the skin and the immune system. This, in turn, would lead to an inflammatory cell influx towards the entire skin surface to support chronic cycles of injury and inflammation.

To conclude, our data all indicate the importance of neutrophil and basophil infiltration and activity in ASST-induced wheals. However, the quantitatively limited eosinophilic infiltration suggests that these cells have less influence on autologous wheal than spontaneous lesions [21]. Despite CAM expression in uninvolved and possibly lesional CIU skin, it seems worth remarking that ASST-induced wheals do not present higher levels of ELAM than unaffected CIU skin does: this finding seems to indicate the possible existence in CAIU of other “serum factors” whose main function is neutrophil recruitment.

Altogether, our data on ASST-induced lesions evidence a specific kinetics of cellular infiltration, mostly sustained by T helper lymphocytes and neutrophils, with a mixed Th1/Th2 response. While there is no significant difference in the numbers of mast cells found in autologous wheals and healthy skin at any of the time points evaluated, we noticed that increased numbers of basophils are present at baseline (10') in wheals compared to healthy skin, with additional recruitment at 30'.

Finally, as reported previously [12], even uninvolved skin of CIU subjects presents an apparently latent inflammatory state characterized by a lymphocytic and granulocytic cellular infiltrate.

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