

CELLULAR INFILTRATE AND RELATED CYTOKINES, CHEMOKINES, CHEMOKINE RECEPTORS AND ADHESION MOLECULES IN CHRONIC AUTOIMMUNE URTICARIA: COMPARISON BETWEEN SPONTANEOUS AND AUTOLOGOUS SERUM SKIN TEST INDUCED WHEEL

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Received December 5, 2005 – Accepted April 6, 2006

In recent years, the demonstration of circulating functional autoantibodies against the high affinity IgE receptor or against IgE themselves in about one-third of patients with chronic idiopathic urticaria has suggested that an autoimmune mechanism might be involved in the pathogenesis of the disease [the so-called chronic autoimmune urticaria (CAIU)]. In this study we compare findings from serum-induced and spontaneous wheals with regard to immunohistochemical markers of disease activity and discuss whether autologous serum skin test (ASST) may be regarded as an *in vivo* experimental model of the physiological stimulus causing urticaria skin condition, or if it does not reproduce the whole of the mechanisms operating in the development of spontaneous wheals. By means of immunohistochemical technique, we analyzed specimens from just-developed spontaneous wheals and from serum-evoked wheals of six CAIU patients, to glean information on cellular infiltrate and related cytokines, chemokines, chemokine receptors and adhesion molecules. According to the results of our study, spontaneous urticaria lesions seem to be sustained by a characteristic inflammation pattern where neutrophils, adhesion molecules, IL-8 and chemokine receptors play a main role in flogosis triggering and, consequently, disease development. On the contrary, ASST-induced wheals seem to imply different activities mainly represented by basophil granulocytes and related molecules, i.e. IL-4. Although it represents an efficacious diagnostic instrument to screen CAIU patients, ASST is not an exhaustive model of the many immunopathogenic pathways operating in the development of urticaria lesions, especially with regard to systemic activation networks.

Chronic urticaria (CU) is a common skin disorder, characterized by the recurrence of an itchy, eruptive wheal-and-flare response of at least six weeks duration. When no physical, allergic, infectious or drug-related causes can be identified, CU is called "idiopathic" (CIU) (1).

In the past few years, the demonstration of circulating functional autoantibodies against the

high affinity IgE receptor (FcεRI), or against IgE themselves, in about one-third of patients with CIU has suggested that an autoimmune mechanism might be involved in the pathogenesis of the disease (2-4). The positive response to the intradermal injection of autologous serum (autologous serum skin test, ASST) (4) and the serum-induced histamine release assay (HRA) from basophils are actually useful in

Key words: chronic autoimmune urticaria, cytokines, cellular infiltrate, adhesion molecules, autologous serum-induced wheal

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0394-6320 (2006)

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screening patients for the presence of circulating autoantibodies and in defining a specific subset of CIU, the so-called "chronic autoimmune urticaria" (CAIU). Commonly, an increased incidence of autoimmune disorders (i.e. Hashimoto thyroiditis, rheumatoid arthritis, vitiligo) and more severe cutaneous clinical features (pruritus, spread of wheals) have been documented in patients with CAIU (4).

Several consistent contributions in better understanding the mechanisms of cutaneous CIU lesion development have been supplied by previous literature. Many authors have focused on different cellular populations infiltrating urticarial lesions, in turn confirming the primary effector role of mast cells (5-6) and the participation of lymphocytes (7-8), neutrophils (9) and eosinophils (10).

Moreover, a real advance in this topic and an innovative approach, consisting in following the time evolution of the wheal infiltrating cells, have been given since 1990 by Grattan, who first performed diachronic biopsies of autologous serum-induced wheals in patients with CIU (11).

Over the past few years, our work group, identifying CAIU subjects by the routine use of ASST and HRA, has investigated the skin immunopathology of spontaneous wheals as regards infiltrating cells and related cytokines, chemokines, chemokine receptors and adhesion molecules (12). Later, analogous immunohistochemical studies were performed on skin specimens from serum-induced wheals of the same patients (13) who were biopsied at different times from the appearance of the lesions, in order to describe evolutive stages of the wheal-and-flare skin process. In this way, we gleaned information on the pathogenesis and effector mechanisms of CIU, according to the novel concept of urticarial inflammation as a dynamic process.

With a view to completing the analysis of our data, in this novel study we compare findings from serum-induced and spontaneous wheals in regard to immunohistochemical markers of disease activity. Possible differences and/or similarities between the two kinds of wheals are highlighted, to finally discuss whether ASST may be regarded as an *in vivo* experimental model of the physiological stimulus causing urticaria skin condition or if, conversely, it does not reproduce the whole of the mechanisms

operating in the development of spontaneous wheals although representing an efficacious diagnostic instrument to screen CAIU patients.

MATERIALS AND METHODS

Patients

Six patients (4 F; 2 M, age range 23-68; median age 42.08 years), diagnosed as suffering from CIU, were selected. All the subjects had shown recurrent whealing at least twice a week. They had also had a positive autologous serum skin test (ASST) according to Grattan (14) and positive histamine release *in vitro* assay (15).

Antihistamines were discontinued one week before skin testing. None of the patients was taking NSAIDs, corticosteroids or immunosuppressants, either topically or systemically. Three out of six subjects had been on short courses of oral corticosteroids, but a 40-day wash-out period was granted in all cases. A negative response to ASST, predominantly physical urticaria, allergic urticaria (IgE-mediated), urticaria-vasculitis, C₁-esterase inhibitor deficiency, drug or alcohol abuse, pregnancy and lactation were considered as exclusion criteria. The patients did not suffer from any infectious or inflammatory systemic disorder. As control, four healthy subjects similarly aged and sex-matched were recruited. None of the control subjects was taking any drug.

In order to confirm the correlation of a positive ASST with an autoimmune diagnosis in our patients, baseline investigations included the detection of circulating immunocomplexes (CIC), autoantibodies (antinuclear, anti-gastric parietal cells, anti-pancreatic islet cells, anti-mitochondria, anti-thyroglobulin, anti-peroxidase) and anti-*Helicobacter Pylori* antibodies (Table I). Baseline investigations were also performed in control subjects (Table I).

Biopsies

Wheal duration was assessed, inviting the hospitalized patients to call for a member of the medical team at the onset of an acute urticaria episode. The development of lesions was followed by the operator, who also took the biopsies from spontaneous wheals at designated times and then performed the ASST at three to six hours from the beginning of the observation. Mean duration of spontaneous wheals was also registered. This way, in all CIU patients a 4 mm punch biopsy was taken from a spontaneous wheal at about 10' from its appearance, and at both 10' and 30' from the development of serum-evoked lesions.

Four additional control specimens were collected from healthy donors.

Biopsies were performed under local ring anaesthesia (without adrenaline) and covered in cryoprotectant

Table I. Baseline investigations performed in selected patients and controls.

Histamine-release assay	Positive in all patients	
Circulating immunocomplexes	-	Negative in controls
Antinuclear Abs	Present in 2 patients	
Anti-gastric parietal cells Abs	Present in 1 patient	
Anti-pancreatic islet cells Abs	-	
Anti-mitochondria Abs	-	
Anti-thyroglobulin Abs	Present in 3 patients	
Anti-peroxidase Abs	Present in 2 patients	
Anti-Helicobacter Pylori Abs	Present in 1 patient	
Others:	Anti-smooth muscle Abs in 1 patient Anti-TSH receptor Abs in 1 patient	

Table II. Comparison between spontaneous and autologous wheals at 10' and 30'.

	Spontaneous wheal 10' (n=6)	Autologous wheal 10' (n=6)	p	Autologous wheal 30' (n=6)	p	Healthy skin (n=4)
CD3	34.8±15.2	26.7±14.1	Ns	29.8±16.0	Ns	4.3±1.9
CD4	27.2±10.6	22.6±10.4	Ns	28.4±7.9	Ns	6.7±1.0
CD8	10.8±5.7	8.7±5.4	Ns	7.5±7.0	Ns	4.2±1.2
TRY	22.3±7.6	21.7±11.3	Ns	20.1±6.0	Ns	22.6±3.3
EG1	20.9±7.6	7.8±4.7	<0.0001	12.7±5.5	0.004	2.1±0.3
EG2	25.9±5.5	7.5±4.9	<0.0001	12.0±9.1	<0.0001	2.0±0.1
MPO	84.8±40.1	24.3±6.4	<0.0001	43.7±37.5	0.001	14.3±2.3
BB1	5.77±0.83	4.3±2.7	Ns	12.3±2.4	0.001	1.5±1.0
IFN γ	3.0±2.0	1.7±1.3	Ns	0.4±0.2	<0.0001	Neg.
IL4	19.7±11.0	15.9±7.8	Ns	27.2±14.4	0.02	Neg.
IL5	36.6±17.7	Neg.	Nd	5.1±3.8	<0.0001	Neg.
IL8	15.9±11.9	8.7±3.9	0.01	12.8±6.5	Ns	Neg.
CCR3	11.9±3.1	5.3±3.0	<0.0001	8.1±9.9	Ns	5.6±2.5
CXCR3	48.6±11.2	26.0±14.3	<0.0001	32.7±15.2	0.002	18.2±9.8
ICAM	44.1±7.0	16.3±8.4	<0.0001	23.4±16.1	<0.0001	21.5±12.1
VCAM	17.1±3.5	9.3±2.8	<0.0001	12.3±3.3	0.002	7.3±4.0
ELAM	18.5±7.2	6.5±4.9	<0.0001	7.5±5.1	<0.0001	5.3±0.9

Ns = not significant; Neg = negative; Nd = not determinable

(Tissue-Tek, Miles INC, USA) before storing in a -80°C refrigerator.

Immunohistochemistry

Tissue specimens were cut into 5 μ m sections which were stained for routine ematoxylin-eosin examination and for immunohistochemistry.

Monoclonal antibodies (Mo Abs) against tryptase at 1:50 (Dako Ltd, High Wycombe, UK) were employed for mast cell detection. Mo Abs for human T lymphocytes included the following: CD3 at 1:20 pan-specific for T cells, CD4 at 1:50 specific for T-helper, CD8 at 1:20 specific for cytotoxic/suppressor T cells (Dako). Neutrophils were stained with Mo Abs against

myeloperoxidase (MPO) at 1:100 (Dako). Eosinophils were labelled by anti-EG1 at 1:150 and anti-EG2 at 1:300 monoclonal antibodies (Pharmacia & Upjohn Diagnostics Ab, Uppsala, Sweden) that bind to eosinophil cationic protein (ECP) and to ECP and eosinophil-derived neurotoxin, respectively. BB1 at 1:10, a novel Mo Ab (the kind gift of Dr A.F. Walls, University of Southampton, UK) was used to find human basophil granular protein.

We studied the related 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). Mo Abs for integrins included the following: 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 chemokines using antibodies against CCR3 at 1:50 and anti-CXCR3 at 1:50 (R & D Systems).

Before staining, frozen sections were air-dried and fixed in acetone (5 min). Immunolabelling 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

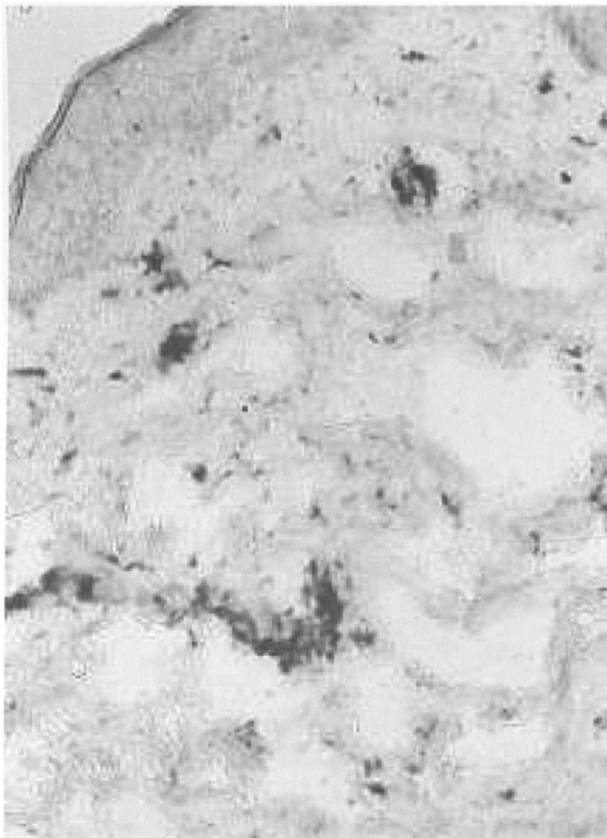


Fig. 1. (APAAP) Perivascular MPO expression at 30' in the upper dermis from a serum-induced wheal.

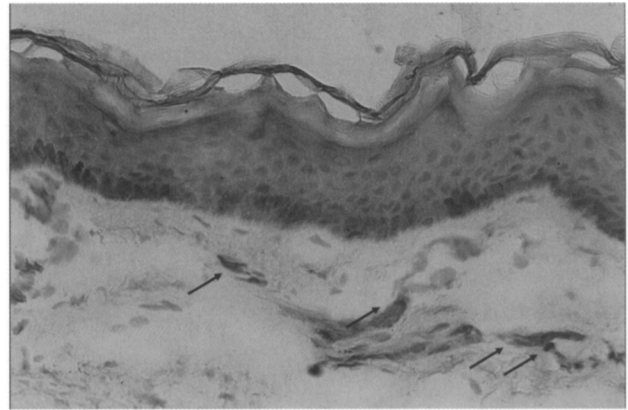


Fig. 2. (APAAP) Mainly extracellular BB1 expression in induced lesions at 30'.

dilution, 30 min) (Dako).

Assessment of histological sections was undertaken "blind" by two independent observers. For quantitative analysis, stained cells were counted and added in three adjacent microscopic (250X) fields of each section.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD). The first step consisted in comparing the mean of stained cells between spontaneous and serum-induced wheal sections, the latter evaluated at 10' and 30'. Later, we compared results from CIU biopsies to healthy skin. Statistical significance ($p < 0.05$) was assessed by Student's *t* test.

RESULTS

Duration of wheals

All CAIU subjects displayed long-lasting lesions. In fact, both spontaneous and serum-induced wheals were singularly present at 6 hours from the appearance in all patients. In two cases most of the spontaneous lesions and induced wheals were still evident after 24 and 48 hours respectively.

Immunohistochemistry

Results of our study are summarized in Table II.

Cellular infiltrate

In both spontaneous and serum-evoked wheals, T lymphocytes were localized in the upper- and mid-dermis, with prevalent perivascular distribution. CD4/CD8 ratio was about 3:1 in all groups. CD3 and CD4 cells slightly increased in induced wheal from 10' to 30', while CD8-positive lymphocytes

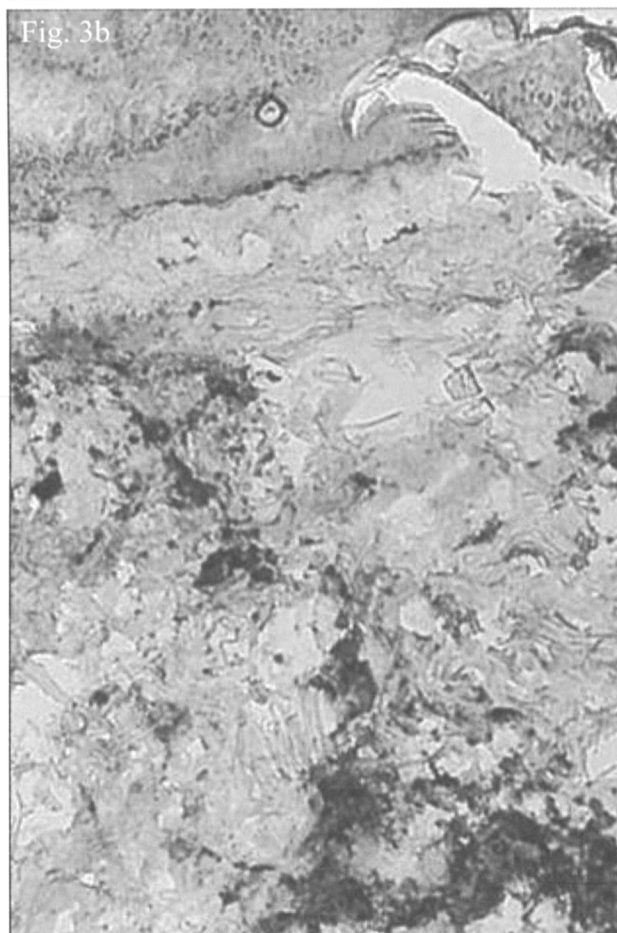
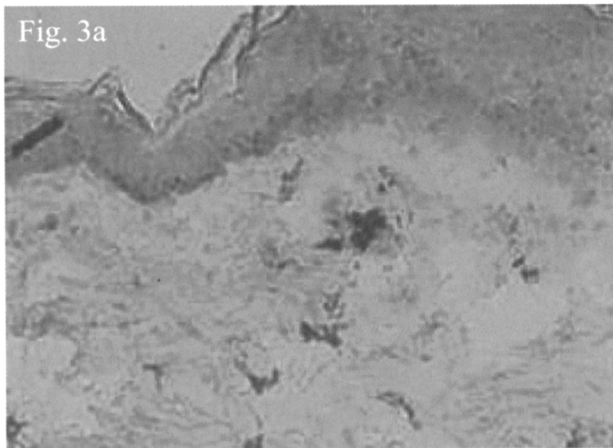


Fig. 3a and Fig. 3b. (APAAP) Increasing expression of IL-4 positive cells in autologous serum-induced lesions from 10' to 30'. Stained cells are distributed perivascularly in the upper dermis.

showed a constant mean count at both time checks. No significant difference could be noticed between spontaneous and serum-induced lesions.

Tryptase-positive mast cells were detected in the upper and lower dermis with a perivascular and

periadnexal distribution. Their mean was similar in both spontaneous and autologous serum-induced wheals, with lack of statistical significance.

A significant eosinophil infiltration was revealed in spontaneous lesions, as assessed by the immunohistochemical detection of EG1 and EG2. In serum-induced wheals, a progressive staining increase from 10' to 30' was observed for both MoAbs, but mean counts remained consistently lower than in spontaneous wheals ($p < 0.0001$; $p = 0.004$).

A similar trend was appreciated for MPO-positive neutrophil granulocytes. A tendency to increase up to 30' was clearly present in induced wheals (Fig. 1), but the mean counts remained significantly lower than in spontaneous lesions. Neutrophils were located in the upper perivascular dermis and represented the major infiltrating population in spontaneous wheals.

In spontaneous lesions, BB1 expression showed a main dermal extracellular pattern, while superficial dermal perivascular localization was seen in serum-induced lesions. There was a significant difference in induced wheals only at 30' ($p = 0.001$) (Fig. 2).

Cytokines, chemokines and chemokine receptors

In spontaneous wheals, a focal expression for IFN- γ was present on infiltrating cells of the perivascular sites of the dermis. A similar expression was also noticed in evoked lesions at 10', but this cytokine was rapidly clearing from ASST-induced hive, with the difference becoming significant at 30' ($p < 0.0001$) as compared to spontaneous wheals.

IL-4 positive cells in ASST-induced lesions outnumbered at 30' the mean count in spontaneous ones, with statistical significance ($p = 0.02$). At 10' by contrast, IL-4 expression was nearly overlapping in the two groups (Fig. 3a and 3b). Stained cells, possibly identifiable in activated T lymphocytes, mast cells and basophils, were distributed perivascularly in the upper dermis. Significantly higher expression in spontaneous wheals as compared to evoked wheals was detected for IL-5. At 10' in induced lesions cytokine expression was negative, and only a focal expression was reached at 30' (Fig. 4) with $p < 0.0001$ when compared to spontaneous lesions. The chemokine IL-8 was also present more in spontaneous than in induced lesions, with significant difference at 10' ($p = 0.01$).



Fig. 4. (APAAP) Focal expression for IL-5 at 30' in induced lesions.

Chemokine receptor CCR3 was identified in the superficial perivascular areas in both spontaneous and induced wheals, while CXCR3 expression was detected in the epidermis and in both perivascular and vascular dermal areas. Overall, their expression was significantly higher in spontaneous lesions.

Cellular adhesion molecules (CAMs)

In both groups ICAM expression was intense and diffuse in the vascular and perivascular dermal sites, while VCAM and ELAM showed a slight-to-moderate expression on endothelial cells and around vessels. All molecules showed a similar trend to increase from 10' to 30' in ASST-induced wheals, but mean counts of stained cells remained constantly lower than in spontaneous lesions of the same patients ($p < 0.0001$; $p = 0.002$).

DISCUSSION

In this study we aim at comparing the immunohistochemical features of just-developed spontaneous wheals of six chronic urticaria patients and serum-induced lesions of the same subjects biopsied at 10' and 30', with a view to acquiring further information on the pathogenesis and effector mechanisms of CIU.

Regarding T lymphocytes, results from our data show that there is no significant difference between spontaneous and ASST-induced lesions, in both lymphocyte total rate and cell subpopulations. These findings suggest a main role for CD4+ lymphocytes in spontaneous and ASST-induced wheal at any time they were analyzed, since T-cells produce a wide spectrum of mediators involved in inflammatory cell recruitment, mast-cell degranulation and eosinophils chemotaxis (16).

A similar behaviour in both kinds of wheals was observed for mast-cells. In fact, tryptase staining did not show any statistical difference between spontaneous and ASST-induced lesions, leading to the hypothesis that mast cell recruiting pathways are similarly operative both *in vivo* and in the experimental serum-evoked wheal. Mast cells have indeed an effector role in skin lesion development, based on the release of stored and newly synthesized mediators. (17)

The lack of statistically significant difference in lymphocytes and mast cells counts between spontaneous and serum-induced lesions at any time could be explained by the existence of a minimal persistent inflammation involving the entire skin surface of CIU patients that, for the action of any triggering agent, could reach a limit threshold of clinical appearance, as previously proposed by Cassano and co-workers. (18)

With regard to eosinophils, EG1 and EG2 staining showed a preponderant presence of these cells in spontaneous wheals, with significant difference at both 10' and 30' as compared to ASST-induced ones. In serum-evoked lesions, the slow increase of eosinophils from 10' to 30', that could be justified by the lack of IL-5 at 10', might suggest a predominant role for eosinophils in lesion persistence rather than development, as previously described for allergic asthma and atopic dermatitis in which eosinophil

granulocytes play a significant role in disease chronicization. (19)

Basophil infiltration was revealed in both spontaneous and serum-induced lesions by BB1, a basophil granule-specific monoclonal autoantibody that recognizes a protein of 124 kDa and does not cross-react with mast cells, eosinophils, neutrophils, lymphocytes or macrophages (20). BB1 staining showed a predominantly extracellular pattern in spontaneous wheals and mixed intra- and extra-cellular in those ASST-induced. This finding seems to suggest earlier degranulation in naturally-occurring lesions (10'), as opposed to a slower accumulation of basophils in serum-evoked wheals, according to which BB1+ granulocyte mean counts at 30' were higher than in spontaneous lesions, and they continued growing (60' and 24 h, data not shown) (13). These data are in accordance with those observed in bronchial asthma, in which basophils are implied in late-phase response (21). Basophils granulocytes, possessing high affinity IgE- receptors (FcεRI) that are cross-linked upon engagement of receptor bound IgE with corresponding antigens (22), may play a direct role in the pathogenic mechanism of CAIU.

Neutrophils were significantly more represented in spontaneous than in induced wheals at both 10' and 30'. It has been previously demonstrated, in fact, that neutrophil infiltration of autologous serum-induced hives was similar to that of spontaneous lesions only at a 60' time point (12-13) (data not shown).

Overall, MPO expression trend was similar to IL-8 that is implied in neutrophil extravasation and homing in inflammatory tissues (23).

IL-5 staining showed a major expression in spontaneous lesions when compared to serum-evoked wheals at 30', closely reproducing eosinophil immunohistochemical findings. Since IL-5 can induce differentiation and chemotaxis on eosinophils (24), it can be presumed that eosinophils and the related cytokine IL-5 are involved in the development and maintenance of the inflammatory process inborn in spontaneous wheals.

IFN-γ, although more represented in urticaria spontaneous lesions, showed low levels in both wheals underlining the presence of a mixed Th1/Th2 pattern (25), even with a Th2 predominance, in urticarial inflammation.

IL-4 positive cells in serum-evoked lesions

outnumbered at 30' the mean count in spontaneous ones, with a pattern similar to that shown by basophils, which are the main cellular source for rapid IL-4 generation (26). This finding might suggest the existence, in ASST-induced wheal, of a more Th2-polarized (but mixed, anyway) immunological bearing, if compared to spontaneous urticaria lesion behaviour.

Regarding the investigated adhesion molecules (ICAM-1, VCAM and ELAM) and chemokine receptors CCR3 and CXCR3, our entire findings have shown a significantly major expression in spontaneous lesions in comparison to induced wheals at both determinations. This trend closely reproduces that observed for neutrophils.

The overall evidence emerging from the present study suggests a balance of different inflammatory systems implied in spontaneous and induced CIU lesions. In particular, it could be concluded that spontaneous urticaria lesions seem to be sustained by a characteristic inflammation pattern where neutrophils, adhesion molecules, IL-8 and chemokine receptors play a main and rapid role in flogosis triggering and, consequently, disease development. On the contrary, ASST-induced wheals seem to imply different activities (basophils granulocytes and IL-4) with only a later involvement of neutrophil granulocytes. The reasons for this behaviour have not yet been clarified but, presumably, the wheal induction by intradermal injection of serum is able to provoke a peculiar condition of local inflammation regulated by a concentration gradient in the closer microenvironment surrounding the injection site. Spontaneous CAIU lesions, by contrast, appear to depend on the triggering of inflammation mechanisms that are already present in a "latent urticarial status" (18).

It is presumable that in CIU, the whole of the skin surface is involved in a persistent inflammation, mainly due to mast cell minimal activation, analogously to what was demonstrated in pathologic atopy-related conditions (19). The recurrent whealing episodes would modify the links and the communication signals between the skin and the immune system. The inflammatory cell influx towards the entire skin surface could progressively reach a limit threshold of clinical manifestation due to any urticariogen stimulus.

To sum up, from these data it may be concluded

that ASST, although representing a valid diagnostic tool to screen CAIU patients, is not an exhaustive model of the many immunopathogenic pathways operating in the development of urticaria lesions, especially in regard to systemic activation networks.

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