

Upregulation of TNF- α and IL-3 expression in lesional and uninvolved skin in different types of urticaria

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Background: Although mast cells are known to secrete a broad spectrum of proinflammatory and immunomodulatory cytokines, the role of these molecules in mast cell-dependent cutaneous inflammation is not clear.

Objective: We decided to study biopsy specimens from lesional and nonlesional skin of patients with acute, chronic recurrent, delayed pressure, and cold urticaria; from fleeting wheals of prick test reactions to allergens; and from normal skin of non-allergic subjects.

Methods: Cryostat sections were stained by immunohistochemistry with antibodies against IL-3, IL-8, TNF- α , and mast cell-specific tryptase. In serial sections with tryptase and each cytokine, reactivity of mast cells was studied as well.

Results: Compared with normal skin and prick test reactions, immunoreactivity for TNF- α and IL-3 was significantly increased on endothelial and perivascular cells of the upper dermis in all urticaria lesions. In nonlesional skin comparable upregulation was noted on endothelial cells and for TNF- α on perivascular cells of patients with delayed pressure urticaria. In addition, TNF- α was expressed throughout the epidermis in lesional and nonlesional skin of patients with all types of urticaria, but not in normal control subjects. Sequential biopsy specimens from patients with cold urticaria showed upregulation of TNF- α and IL-3 on endothelial cells 30 minutes after elicitation of lesions with an ice cube. In contrast to these findings, epidermal immunoreactivity, as well as endothelial and perivascular cell expression of IL-8, were only slightly altered in urticaria compared with normal skin. In sequentially stained sections, few tryptase-positive mast cells reacted to TNF- α , few reacted to IL-3 in pressure urticaria only, and practically none stained for IL-8.

Conclusion: These findings suggest that the cytokines studied here are involved in the pathology of urticaria, possibly by inducing subthreshold inflammation in endothelial cells of uninvolved skin. (*J Allergy Clin Immunol* 1999;103:307-14.)

Key words: Urticaria, IL-3, TNF, IL-8, cytokines, mast cells, endothelial cells, keratinocytes

Urticaria is one of the most common skin diseases, and mast cells are generally viewed as key elicitors of its typical wheal-inducing pathology because these cells can be stimulated to rapidly secrete vasoactive and chemotactic mediators, thus inducing edema and, in more persistent lesions, leukocytic infiltrates involving neutrophils, eosinophils, and monocytes.^{1,2} Reasons for the induction, maintenance, and spontaneous resolution of the disease are largely unknown, however.

In recent years, human mast cells have been shown to express and secrete a broad spectrum of proinflammatory and immunomodulatory cytokines at the mRNA and protein level,³ but the role of these molecules in urticaria is largely unexplored. In the past, increased levels of IL-6 have been measured in blisters raised over pressure urticaria lesions,⁴ and we have demonstrated an increased expression of migration inhibitory factor in several types of urticaria.⁵ More recently, increased serum levels of TNF- α were measured in a patient with exercise-induced urticarial vasculitis,⁶ and we have observed increased lesional expression of IL-3 and TNF- α in neutrophilic urticaria.⁷ In other types of allergic diseases, research has focused on the expression of cytokines in mast cells, and IL-4, IL-5, IL-6, IL-8, GM-CSF, and TNF- α have indeed been demonstrated to a variable extent in mast cells of the bronchial, nasal, and conjunctival mucosa in normal and allergic subjects and furthermore in cutaneous allergic reactions at the protein and mRNA level.⁸⁻¹³

In this study we have decided to examine 3 cytokines with regard to their possible role in urticaria because they are released abundantly from cultured human mast cells after in vitro stimulation³ and because they might be of potential relevance to the pathology of urticaria due to the following specific properties.

TNF- α is a 17-kd nonglycosylated protein that can be produced by practically all common resident skin cells, as well as by infiltrating leukocytes.¹⁴ The molecule can induce mediator release from skin mast cells,¹⁵ activate and upregulate endothelial adhesion molecules,¹⁶ and contribute in a major way to leukocytic infiltration in IgE-dependent late-phase cutaneous reactions.¹⁷

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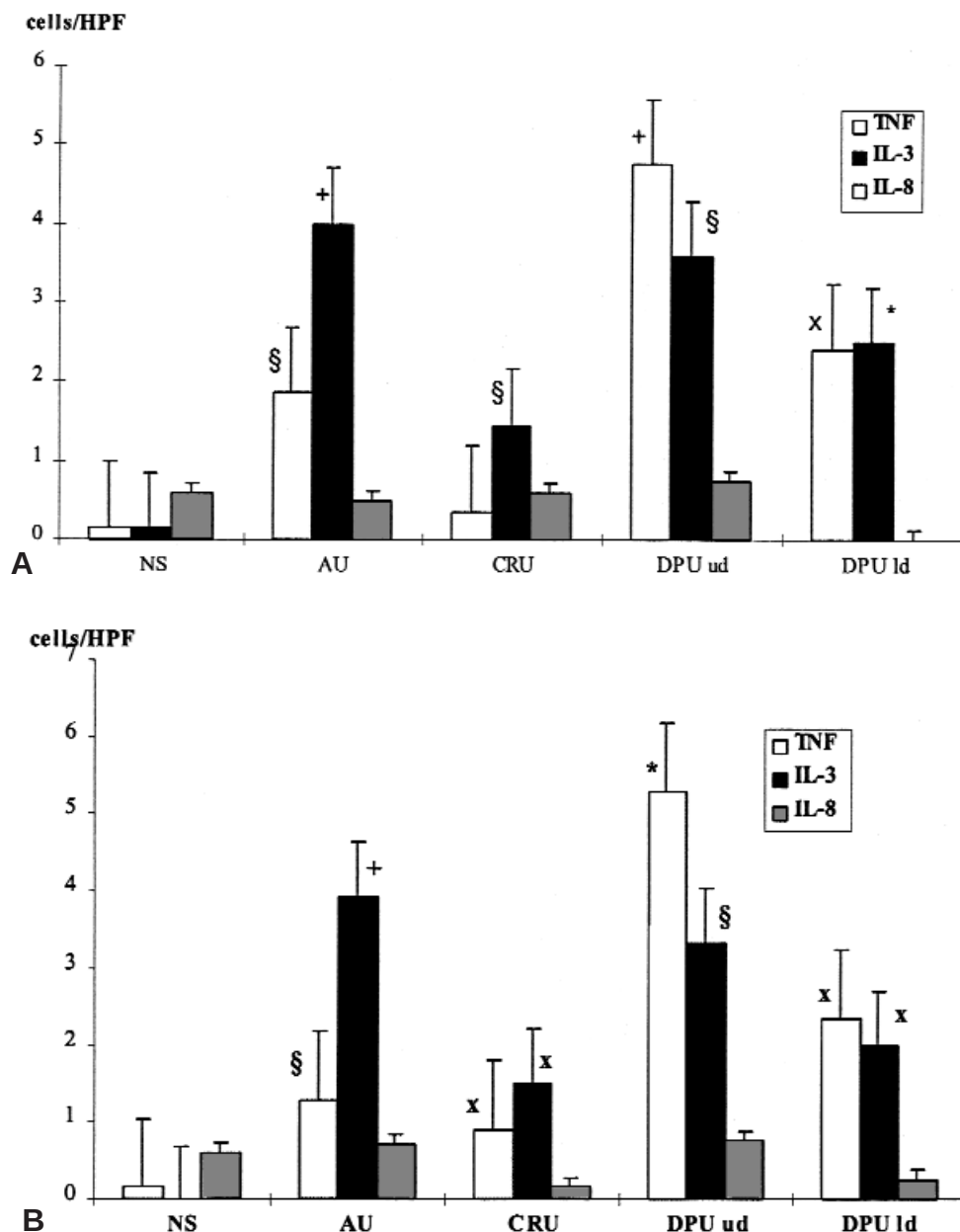


FIG 1. Immunoreactivity of endothelial cells for TNF- α , IL-3, and IL-8 in normal skin and in different types of urticaria. Immunoreactivity is shown separately for lesional (**A**) upper dermis (*ud*) and nonlesional skin (**B**) of the same patients. For DPU, findings in the lower dermis (*ld*) are shown as well. Data are expressed as means $\pm$ SD of numbers of cells per microscopic high-power field (HPF). $\times P < .05$, $\S P < .01$, $+P < .001$, $*P < .0001$ (all compared with normal skin [NS]).

Abbreviations used

AU: Acute urticaria
CRU: Chronic recurrent urticaria
DPU: Delayed pressure urticaria
ICAM: Intercellular adhesion molecule

IL-8, an 8-kd chemokine that can also be produced by and stored intracellularly in a broad range of skin resi-

dent cells and infiltrating leukocytes in the skin, represents one of the most potent neutrophil chemoattractants and activators of secretion.^{18,19} IL-8 also exerts in vitro chemotactic activity on human mast cells²⁰ and on activated eosinophils,^{21,22} and it enhances histamine-induced wheal response and neutrophil infiltration after injection into human skin.²³ Finally, it is released from human skin and nasal polyps after antigen challenge.^{24,25}

IL-3 is a 15-kd pleiotropic hematopoietic growth fac-

TABLE I. Endothelial cytokine immunoreactivity in prick test lesions and contralateral normal skin and in skin before and after elicitation of cold urticaria with an ice cube

	TNF- α	IL-3
Prick test subjects (n = 7)		
Normal skin	0.83 $\pm$ 1.54	2.40 $\pm$ 2.37
Prick test sites at 15 min	1.72 $\pm$ 3.44	1.16 $\pm$ 0.66
Cold urticaria (n = 1)		
Normal skin	1.01 $\pm$ 0.98	5.31 $\pm$ 1.93
Lesional skin at 30 min	4.36 $\pm$ 0.84*	13.00 $\pm$ 2.68*

In the patient with cold urticaria, no increased reactivity was noted in biopsy specimens taken 5 and 15 minutes after elicitation of response.

*Data are expressed as means $\pm$ SD; * P < .0001 compared with normal skin.

tor that is secreted by T lymphocytes, monocytes, granulocytes, and mast cells.^{3,26-28} It enhances eosinophil mediator release and migration and prevents apoptosis of these cells,²⁹⁻³¹ and it induces chronic endothelial expression of P-selectin,³² an adhesion molecule that is markedly upregulated in serum and tissue sections of patients with dermatographic and chronic urticaria.³³

The present data show that these 3 selected mast cell cytokines are indeed expressed and that TNF- α and IL-3 are upregulated in lesional and nonlesional skin of patients with different types of urticaria compared with normal control subjects, with only low-level immunoreactivity of the cytokines in mast cells.

METHODS

Biopsy material

Biopsy specimens were taken after informed consent from 9 patients with acute urticaria (AU), 10 patients with chronic recurrent urticaria (CRU), 6 patients with delayed pressure urticaria (DPU), 7 nonatopic patients with positive prick test responses to mite antigen, and 1 patient with cold urticaria. Only specimens from patients consenting to have biopsies from both lesional and corresponding contralateral uninvolved skin were included. Normal skin, obtained from 10 subjects during cosmetic surgical procedures, was used as a control. Subjects treated with H1-type antihistamines within the last 3 days (4 weeks for astemizole) or with systemic corticosteroids within the last 3 weeks before biopsy were excluded.

Biopsy specimens were taken by elliptical excision with a scalpel or with a 4 to 6 mm diameter punch from recently evolved wheals. In patients with acute and chronic urticaria, the estimated age of the spontaneously developed lesions ranged from 2 to 6 hours. DPU lesions were elicited by application of 900 and 1800 g static pressure for 10 and 20 minutes on the upper back, and biopsies were performed at 2 hours after their appearance. Prick test sites underwent biopsy 15 minutes after allergen application, and cold urticaria lesions underwent biopsy at 5, 15, and 30 minutes after elicitation with an ice cube. Before biopsy, sites were anesthetized by circumferential injection of 1% lidocaine without vasoconstrictive additives.

Staining procedures

Immunohistochemistry was performed on sections from snap-frozen specimens, as previously described,³⁴ by using the alkaline phosphatase-antialkaline phosphatase technique and the following antibodies at the dilutions indicated: anti-human IL-3 monoclonal

TABLE II. Immunohistochemical expression of TNF- α and IL-8 in the epidermis of biopsy specimens from patients with urticaria and normal donors

	TNF- α		IL-8	
	Lesional	Nonlesional	Lesional	Nonlesional
AU (n = 9)	3.9 $\pm$ 2.1†	3.6 $\pm$ 1.8‡	6.5 $\pm$ 2.9	5.4 $\pm$ 2.0*
CRU (n = 10)	3.7 $\pm$ 1.8‡	2.9 $\pm$ 1.9†	8.1 $\pm$ 2.0	6.8 $\pm$ 3.3
DPU (n = 6)	4.7 $\pm$ 1.4‡	4.8 $\pm$ 1.4‡	6.7 $\pm$ 2.9	6.6 $\pm$ 2.1
NS (n = 10)	—	0.6 $\pm$ 0.8	—	7.8 $\pm$ 1.6

Data are expressed as means $\pm$ SD of positive cells/high-power field.

NS, Normal skin.

* P < .05 compared with normal skin.

† P < .01 compared with normal skin.

‡ P < .001 compared with normal skin.

IgG₁ antibody (1:80); anti-human TNF- α rabbit polyclonal antibody (IgG and IgM, 1:6000; both from Genzyme, Ismaning, Germany); anti-human IL-8 monoclonal IgG₁ antibody 52E8 (1:5);³⁵ monoclonal anti-tryptase AA1 IgG₁ antibody (1:50) (kindly provided by A. Walls, Southampton, UK); and anti-desmin (1:200) and UCHL1 (1:50) antibodies as negative and positive controls, respectively (both from Dako, Hamburg, Germany).

Evaluation of specimens

All specimens were coded by the technician and evaluated in a blinded fashion by at least 2 independent observers who counted positive cells in different cellular compartments of the skin. Data are expressed as positive cells/high-power microscopic field at 400 $\times$ magnification. For identification of cytokines in mast cells, sequential sections stained for tryptase and the respective cytokine were studied, with 50 to 100 mast cells being evaluated in each specimen.

Statistical analysis was performed by using Student's *t* test for unpaired samples, paired Student's *t* test for the comparison of lesional versus nonlesional skin in the same patient, and Wilcoxon rank test for evaluating a possible correlation between mast cell numbers and cytokine reactivity in different specimens.

RESULTS

Compared with specimens from nonallergic normal subjects, there was an overall increase of immunoreactivity toward IL-3 and TNF- α in all types of urticaria in lesional skin, with a slightly lower increase of expression in nonlesional skin, as illustrated for endothelial cells in Figs 1, A and B and 2, A. No changes were observed in prick test wheals of nonatopic subjects after 15 minutes compared with normal skin (Table I). IL-8 immunoreactivity was, on the other hand, expressed in the epidermis and dermis of normal subjects and patients with urticaria, with decreased expression in certain subtypes of urticaria (Figs 1, 3, and 4 and Table II). Expression of the cytokines studied differed markedly in cutaneous cell types and compartments, as detailed below.

Endothelial immunoreactivity

In upper dermal vessels there was a marked upregulation of endothelial reactivity toward TNF- α and IL-3 in lesions of all types of urticaria studied, as summarized in Fig 1, A and illustrated for endothelial IL-3

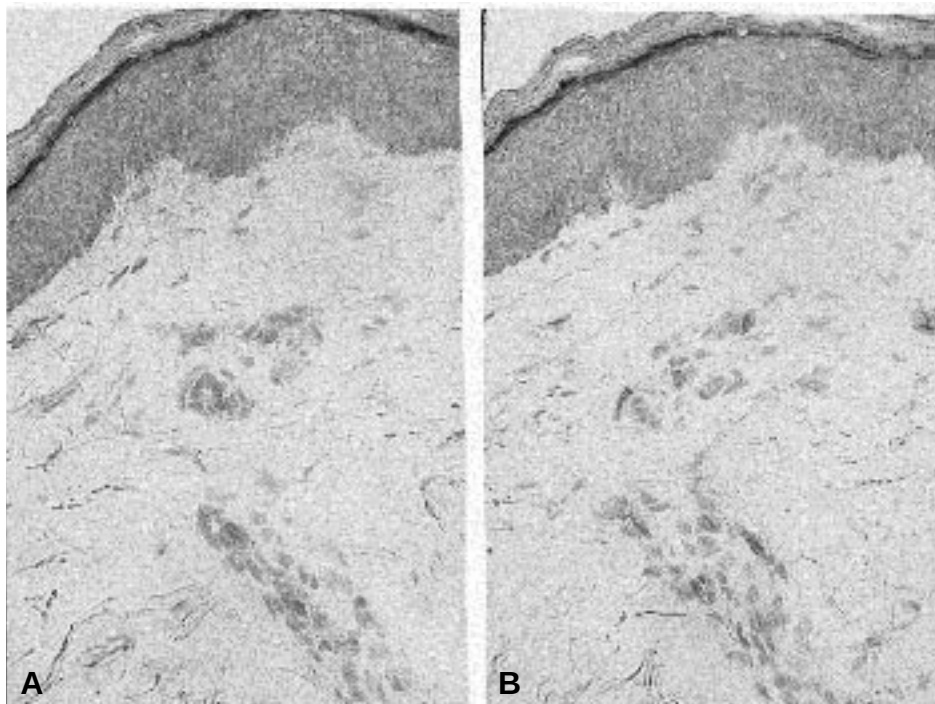


FIG 2. IL-3 immunoreactivity in an AU lesion (**A**) compared with mast cell tryptase (**B**). Note the distinct staining patterns of the 2 antibodies, with IL-3 reacting primarily with endothelial cells, and mast cell-specific tryptase reacting with cells in a perivascular distribution (original magnification $\times 400$).

TABLE III. Percentage of tryptase-positive mast cells exhibiting cytokine immunoreactivity in normal skin and in skin biopsy specimens from patients with different types of urticaria

	TNF- α	IL-3	IL-8
AU (n = 9)	0.05	0.00	0.00
CRU (n = 10)	0.06	0.01	0.00
DPU (n = 6)	0.05	0.06	0.01
NS (n = 10)	0.00	0.00	0.00

Data were calculated from 500 to 1200 mast cells evaluated in sequential sections of each biopsy specimen.

expression in AU lesions (Fig 2, A). Unexpectedly, endothelial cells in biopsy specimens of nonlesional skin in the same patients were only slightly less reactive than those of lesional skin (Fig 1, B), with no significant differences between lesional versus nonlesional skin.

Changes were most pronounced in DPU lesions, with significant upregulation of immunoreactivity also in the lower dermis (Fig 1, A and B), whereas in other types of urticaria, reactivity in the lower dermis was scanty (not shown). In CRU lesions upper dermal endothelial reactivity was least pronounced (Fig 1, A and B). In the one patient with cold urticaria, baseline endothelial reactivity in the upper dermis remained unchanged in lesional specimens taken 5 and 15 minutes after cold provocation compared with nonchallenged skin, but in lesions taken 30 minutes after elicitation, a marked and statistically

significant upregulation was found for both TNF- α and IL-3 (Table I).

Immunoreactivity for IL-8 was only weak in endothelial cells of upper dermal vessels, and no significant changes were noted in prick test, cold urticaria (not shown), or lesional and nonlesional urticaria biopsy specimens compared with control skin (Fig 1, A and B).

Perivascular and interstitial cell immunoreactivity

A moderate level of immunoreactivity toward TNF- α and IL-3 was noted in perivascular cells of skin from normal donors, with distinctly higher levels for IL-8 (Fig 3). Upregulation of immunoreactivity in these cells was seen for TNF- α and IL-3 in the lesional upper dermis of patients with AU and DPU, whereas changes in CRU and in the lower dermis of all types of urticaria failed to reach significance compared with normal skin. Significant decrease of IL-8 immunoreactivity was noted, however, in all urticaria lesions (Fig 3). In nonlesional skin statistically significant upregulation of perivascular immunoreactivity, compared with that of normal skin, was noted only for TNF- α in upper dermal DPU biopsy specimens ($P = .006$) (not shown).

Separate evaluation of cells scattered in the dermal connective tissue without association to vessels or appendages yielded similar, but less striking, results (not shown). Statistically significant increases were

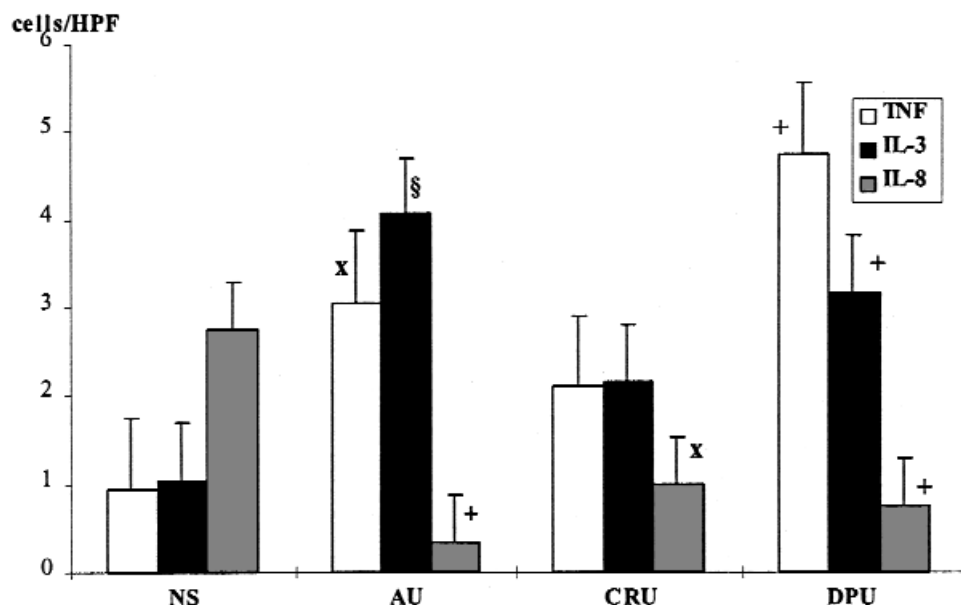


FIG 3. TNF- α , IL-3, and IL-8 immunoreactivity of perivascular cells in upper dermis biopsy specimens from the skin of normal donors and from the lesional skin of patients with urticaria. Data are expressed as means $\pm$ SD of numbers of cells per high-power microscopic field (HPF). * $P < .05$, § $P < .01$, + $P < .001$ (all compared with normal skin [NS]).

noted only for TNF- α in AU lesions ($P = .001$) and in the nonlesional upper dermis in DPU lesions ($P = .009$).

Epidermal immunoreactivity

Of the 3 cytokines studied, IL-3 was not expressed at all in the normal epidermis. In urticaria only a distinct staining of the basement membrane zone was noted (Fig 2, A), which was not observed in normal skin. TNF- α was expressed only in a few scattered epidermal cells in normal skin, but it was markedly upregulated in lesional and uninvolved skin of urticaria (Table II and Fig 4, B). In contrast, IL-8 was markedly expressed in the suprabasal region of the epidermis of all specimens and also in normal skin, with only a minor decrease of reactivity in acute urticaria (Table II and Figure 4, A). Immunoreactivity for both IL-8 and TNF- α was also detected in the stratum corneum and in epithelial appendages, such as hair follicles and distal sweat ducts, in urticaria specimens, particularly in DPU specimens (not shown).

Cytokine expression and mast cells

On statistical analysis of a possible correlation between cytokine-positive dermal perivascular and interstitial cells and mast cell numbers (Wilcoxon rank test), significant positive correlations were observed for TNF- α in AU and DPU specimens (always $P < .05$), as well as for IL-3 in AU, CRU, and DPU specimens (always $P < .05$). A negative correlation was found for IL-8 in AU ($P = .017$), CRU ($P = .009$), and DPU specimens ($P < .025$).

Because the distribution pattern of tryptase-positive mast cells in urticaria lesions was clearly distinct from that of cytokine-positive cells, as shown for AU and IL-3

(Figs 2, A and B), detailed analysis of a possible coexpression of mast cell tryptase and cytokines was done in sequential sections stained for tryptase, followed by 1 of the 3 cytokine antibodies. As shown in Table III, only a few TNF- α -positive mast cells were detected in urticaria specimens, IL-3 reactivity was noted only in mast cells of DPU specimens, and IL-8 staining of mast cells was negligible throughout, with no differences of cytokine reactivity in any sections between lesional and uninvolved skin (not shown).

DISCUSSION

The present analyses of cytokine immunoreactivity in the skin of patients with urticaria have yielded a number of novel findings. Specifically, the data indicate a central role for endothelial cells in urticaria pathology, whereas the contribution of mast cell-derived cytokines plays probably only a minor role. The demonstration of TNF- α upregulation in keratinocytes also suggests a potential involvement of the epidermis in urticaria, and most interestingly, cytokine changes seem not to be restricted to lesional skin. The quantitative expression of IL-3 and TNF- α in different types of urticaria is furthermore in line with data on the intensity of the inflammatory cell infiltrate on the lesions, which is most pronounced in AU and DPU, with lower expression in CRU where counter-regulatory processes may negatively influence the extent of the chronic disease process.³⁶

A key involvement of the endothelium in urticaria has already been suggested in previous studies in which endothelial GMP-140 (P-selectin), and subsequently intracellular adhesion molecule (ICAM)-1 and endothe-

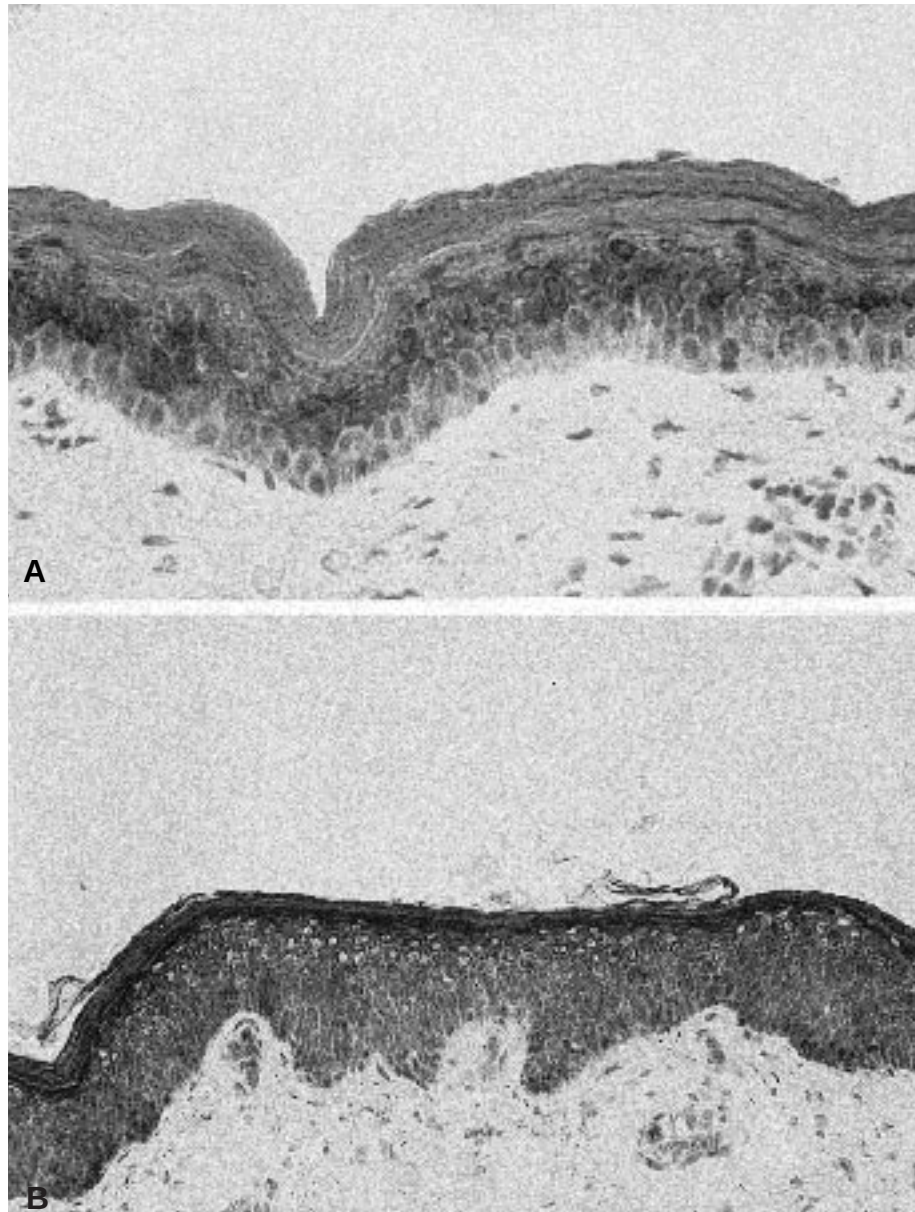


FIG 4. Immunoreactivity for IL-8 (**A**) and TNF- α (**B**) in sections from patients with acute urticaria (original magnification $\times 400$).

lial leukocyte adhesion molecule-1, were shown to be upregulated in cold and cholinergic urticaria;³⁷ MHC class II molecules in several types of urticaria;³⁸ P-selectin, E-selectin, ICAM-1, and vascular cell adhesion molecule-1 in dermographic urticaria;³³ E-selectin in DPU;³⁹ and E-selectin and ICAM-1 in urticarial vasculitis.⁶ A mast cell dependence of this endothelial adhesion molecule upregulation is supported by animal studies with histamine perfusion and by the induction of P-selectin-dependent leukocyte rolling after mast cell activation.^{40,41} Because IL-3 and TNF- α can also initiate endothelial adhesion molecule expression,^{16,32} it is possible that these cytokines can enhance endothelial activa-

tion and even boost their own expression on these cells in an autocrine fashion because they are already expressed in uninvolved skin as well.

The low numbers of cytokine-positive mast cells in urticaria agree with recent observations in allergic conjunctivitis,⁴² but they differ from studies dealing predominantly with mucosal mast cells in different types of allergic reactions.^{9,11-13} A possible explanation for this divergence of findings may be related to cytokine expression in mast cell subtypes because IL-5 and IL-6 upregulation has been shown to be restricted to tryptase-positive, chymase-negative mast cells.¹¹ These cells are rare in normal human skin, although they increase during

diverse inflammatory processes.⁴³ Taken together, the present findings show that mast cells contribute to only a minor extent to TNF- α and IL-3 expression in urticaria. The analysis of a correlation between tryptase-positive mast cell numbers and perivascular and interstitial dermal cytokine reactivity suggests, however, that both parameters depend in a similar way on the associated urticarial inflammation.

On the basis of clinical observations and histologic examinations,³⁶ the pathology of wheal reactions is currently thought to be limited to the dermis, particularly the dermal vasculature with its surrounding leukocytic infiltration. An involvement of the epidermis in urticaria, as shown here for TNF- α expression, has so far only been reported recently for TNF- α and IL-6 by *in situ* hybridization and immunostaining in acute urticaria.⁴⁴ The underlying mechanisms might involve stimulation of keratinocytes by means of the low-affinity IgE receptor (CD23),⁴⁵ but because IgE-dependent mechanisms are only rarely involved in urticaria,¹ other stimuli might be operative as well.

A particularly fascinating finding in this study is the significant upregulation of cytokine expression in nonlesional skin. Abnormal responsiveness and increased histamine have been reported before in nonlesional skin of patients with chronic urticaria,⁴⁶ as has increased expression of vascular adhesion molecules,^{33,37} MHC class II molecules,³⁸ β_2 -integrins,²⁸ and mast cell numbers.³⁶ Increased expression of eosinophil major basic protein has been reported in DPU only.⁴⁷ These changes might be induced by a generalized subthreshold stimulation of the skin by circulating allergens, by increased serum cytokines like TNF- α ,⁶ or by shed receptors like P-selectin.³³ The upregulation of inflammatory molecules in the nonlesional skin of patients with urticaria may represent a low-level priming of the cutaneous inflammatory and immunologic response apparatus, allowing for the maintenance of the disease even when a disease-eliciting factor is not apparent, as holds for most types of urticaria.

In view of the findings with IL-3 and TNF- α , the lack of upregulation of IL-8 seems puzzling on first sight because IL-8 is abundantly secreted by stimulated human mast cells *in vitro*.^{3,24} However, the data fit with data reported for normal skin and inflammatory diseases in which a high epithelial IL-8 expression was observed independently of the source of the mAb.^{35,42} Currently, it is thought that the mAb for IL-8 used in this study detects a nonfunctional, intracellularly stored form of the molecule, which is released extracellularly in its active form.⁴⁸ A decreased IL-8 reactivity in inflammatory dermatoses³⁵ and in urticaria, as observed here in epidermal and perivascular cells (Table II and Fig 3), is in line with this concept. Taken together, the involvement of IL-8 in urticaria remains to be further elucidated.

If one views the present findings together with recent data from the literature, the pathophysiologic events in urticaria might be summarized as follows: Mast cells release a number of preformed and rapidly generated vasoactive mediators, which induce vasodilatation,

sludging of the capillary blood flow, and rolling of leukocytes along the endothelium. Upregulation and activation of adhesion molecules on endothelial cells and of their counterreceptors on leukocytes results in subsequent adhesion of leukocytes to vessel walls and their transmigration into lesional tissue where they can further amplify the local wheal reaction by producing further vasocative lipid mediators and leukocyte-activating, chemotactic cytokines. Low-level initiation of endothelial activation in the entire integument by means of circulating cytokines and other mediators, soluble receptor molecules, or allergens might facilitate development of wheals even on minor stimulation. The specific nature of these circulating factors and the contribution of diverse cell types other than mast cells and endothelial cells in urticaria will need to be further clarified.

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