

T_H1/T_H2 cytokines and inflammatory cells in skin biopsy specimens from patients with chronic idiopathic urticaria: Comparison with the allergen-induced late-phase cutaneous reaction

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Background: Previous studies have demonstrated infiltration of eosinophils, neutrophils, monocytes, and T cells in the skin of patients with chronic idiopathic urticaria (CIU), suggesting a possible T_H2-type cytokine pathology analogous to the allergen-induced skin late-phase reaction (LPR).

Objective: We sought to compare skin biopsy specimens from patients with CIU and the allergen-induced skin LPR for mRNA⁺ cells for IL-4, IL-5, and IFN- γ and inflammatory cell infiltration.

Methods: Skin biopsy specimens were obtained from 13 patients with CIU (6 had positive results for Fc ϵ RI autoantibodies), 6 nonatopic control subjects, and 6 atopic subjects (before and after cutaneous allergen challenge). Cryostat sections were processed for immunohistochemistry and in situ hybridization by using the ³⁵S-riboprobe.

Results: There were significant increases in the numbers of intradermal CD3⁺ ($P = .007$), CD4⁺ ($P = .004$), CD8⁺ ($P = .012$), and CD25⁺ ($P = .018$) T cells, as well as eosinophils ($P = .02$), neutrophils ($P = .01$), basophils ($P = .004$), and macrophages ($P = .0014$) in patients with CIU compared with numbers in nonatopic control subjects. There were lower numbers of tryptase-positive mast cells ($P = .048$). In the epidermis of patients with CIU, but not in that of normal subjects or in allergen-challenged biopsy specimens, there were increased numbers of CD3⁺ T cells ($P = .039$). The profile of inflammatory cell infiltration in the allergen-induced skin LPR was similar to that in patients with CIU. In patients with CIU there was a T_H0 cytokine profile, with significant increases in IL-4 ($P = .0029$), IL-5 ($P = .0025$), and IFN- γ ($P = .037$) mRNA⁺ cells. As expected, in the skin LPR there was an increase in IL-4 ($P = .0082$) and IL-5 ($P = .0051$), but not IFN- γ , mRNA⁺ cells.

There were no significant differences in either the numbers of inflammatory cells or the cytokine pattern between patients with and without autoantibody.

Conclusion: The molecular immunopathology of CIU is that of an eosinophil and basophil cell-mediated hypersensitivity reaction. Thus it is similar to the allergic skin LPR but has a T_H0 rather than a T_H2 cytokine profile. (*J Allergy Clin Immunol* 2002;109:694-700.)

Key words: T_H1/T_H2-type cytokines, chronic urticaria, inflammatory cells

The histology of the lesions of chronic idiopathic urticaria (CIU) consists of a cellular infiltrate characterized initially as a nonnecrotizing perivascular infiltrate with mononuclear cells^{1,2} and an early infiltration with variable numbers of neutrophils and eosinophils.³ Therefore the reaction has features in common with the allergen-induced skin late-phase reaction (LPR) in atopic subjects,^{4,5} although basophil infiltration in CIU has not been demonstrated. CD4⁺ T cells are also predominant in CIU lesions, but the T_H subpopulation and the cytokines they secrete have not been characterized. In this study we have further characterized the infiltrating cell types and the profiles of T_H1/T_H2 cytokine expression in patients with CIU by using immunohistochemistry and in situ hybridization. Our findings were compared with those regarding the allergen-induced skin LPR.

Among our study group were a subpopulation of patients with circulating IgG autoantibody reactive with the α subunit of the IgE receptor.⁶⁻⁹ This antibody initiates an urticarial eruption by cross-linking the IgE receptor, thereby activating cutaneous mast cells.^{7,10-12} The reaction is augmented by complement activation⁸ to liberate complement component 5a (C5a).^{13,14} Thus we also ascertained whether this subgroup of patients with CIU have a distinctive molecular immunopathology.

METHODS

Human subjects

Skin biopsy specimens were obtained from patients with CIU ($n = 13$), normal nonatopic subjects ($n = 6$), and atopic individuals ($n = 6$) 24 hours after challenge with either allergen or diluent. Subjects with CIU were selected from patients with recurrent hives of greater than 12 weeks' duration, occurring at least 4 times a week, with individual lesions lasting over 4 hours. Patients with physical-

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Supported by the Wellcome Trust (UK).

Received for publication October 22, 2001; revised January 17, 2002; accepted for publication January 17, 2002.

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0091-6749/2002 \$35.00 + 0 1/85/123236

doi:10.1067/mai.2002.123236

Abbreviations used

C5a: Complement component 5a
CIU: Chronic idiopathic urticaria
LPR: Late-phase reaction

ly induced urticaria or clinical evidence of urticarial vasculitis were excluded. Antihistamine treatment was discontinued for at least 48 hours before the serum samples and skin biopsy specimens were collected, and none of the patients were taking tricyclic antidepressants, corticosteroids, or immunosuppressive drugs at the time of venipuncture or in the recent past. Sera from normal subjects served as a control. Atopic subjects and nonatopic control subjects were recruited as previously described.⁵ Atopy was defined as a history of seasonal or perennial rhinitis, asthma, or both and the presence of a positive skin prick test response to an aeroallergen in the presence of a positive histamine and negative vehicle controls. Patients taking oral medication were not included in this study. All subjects gave informed consent, and the study was approved by the Ethics Committee of the Royal Brompton and Harefield NHS Trust, London, and the Institutional Review Board of the Medical University of South Carolina.

Leukocyte isolation

Fifteen milliliters of whole blood from donors was mixed with 1.5 mL of 0.2 mol/L EDTA 10% and with 3.0 mL of 3% dextran-3% glucose-0.15mol/L NaCl saline solution. It was sedimented for 90 minutes, the supernatant was removed, and the cells were placed into 50-mL conical tubes that were washed 3 times with Hank's balanced salt solution-human serum albumin (HBS-HSA; Gibco BRL), 4 mmol/L HEPES, and 0.3% human serum albumin. The cells were then resuspended in HBS-HSA containing 2 mmol/L CaCl₂ and 1 mmol/L MgCl₂. This leukocyte concentration was reconstituted to the original concentration in blood.

Histamine release assay

One healthy blood donor whose cells were reactive with anti-IgE antibody and who has been shown to be particularly responsive to chronic urticaria¹⁴ was chosen for the studies reported here. This donor was atopic, with a serum IgE level of 25 IU/mL. Seventy-five microliters of serum from patients with chronic urticaria or buffer was incubated with 75 mL of leukocyte suspension for 40 minutes at 37°C. After incubation, the supernatants were separated by means of centrifugation at 3000 rpm for 5 minutes at 4°C, and histamine release was determined. Two replicate aliquots of cells were boiled to determine the total basophil histamine content. Histamine release was measured by means of enzyme immunoassay (Immunotech International, Marseille, France), according to the manufacturer's instructions. All histamine release experiments were performed in duplicate, and the results were expressed as a percentage of total histamine content. Spontaneous histamine release from cells was less than 8% of total histamine.

Histamine release activities were tested in 13 patients (3 had prominent angioedema as well): 6 had positive results, 1 had equivocal results, and 6 had negative results. The results are shown in Table I.

Study design and processing specimens

In patients with CIU, skin biopsy specimens were taken from individual urticarial lesions that had persisted for over 4 hours, as previously described.^{1,2} Atopic subjects were injected intradermally with 30 BU of a relevant allergen (0.02 mL) into individual sites on the extensor aspect of the forearm. A second site was injected with a similar volume of diluent. Skin biopsy specimens were taken with 1% plain lidocaine as the local anesthetic 24 hours after aller-

TABLE I. Comparison of histamine release activity (cutoff is 15%) between patients with and without autoantibody with CIU

Subject No.	Age (y)	Sex	Clinical diagnosis	Histamine release activity (%)
1	39	F	CIU and AE	18
2	43	M	CIU	(11)
3	38	F	CIU	(8)
4	49	M	CIU	27
5	47	M	CIU and AE	(7)
6	60	F	CIU	100
7	39	M	CIU	(7)
8	37	M	CIU and AE	100
9	51	M	CIU	20
10	31	F	CIU	(12)
11	43	F	CIU	(15)
12	51	F	CIU	(10)
13	18	M	CIU	19

AE, Angioedema.

gen or diluent challenge. Single skin biopsy specimens were taken from healthy, nonatopic control subjects. Tissue biopsy specimens were fixed immediately in 4% paraformaldehyde (BDH Chemicals Ltd, Dagenham, United Kingdom) in 0.1 mol/L PBS, pH 7.4, and washed in 15% PBS-buffered sucrose (Sigma Chemical Co, Poole, United Kingdom), embedded in OCT compound (Raymond Lamb, London, United Kingdom), and then snap-frozen in isopentane (BDH Chemicals) precooled in liquid nitrogen. Cryostat sections (6 µm) were cut from biopsy specimens, mounted on 0.1% poly-L-lysine-coated slides, dried overnight at 37°C, and then stored with silica gel (BDH Chemicals) at -80°C until use.

Histology and immunohistochemistry

The alkaline phosphatase-antialkaline phosphatase technique was used for single staining to enumerate immunoreactive cells,^{4,5} and Congo red staining was used for total eosinophils. Double-immunofluorescence staining was used for identification of CD4⁺/CD25⁺ T cells, as previously described.¹⁵ Monoclonal antibodies were purchased as follows: anti-CD3, anti-CD4, anti-CD8, and anti-CD25 (Becton Dickinson, Cowley, Oxford, United Kingdom); anti-tryptase (Chemicon, Temecula, Calif); anti-neutrophil elastase (Dakopatts, High Wycombe, United Kingdom); and anti-CD68 (Dakopatts). BB1, a novel mAb recognizing human basophil granular protein was a kind gift from Dr A. F. Walls (University of Southampton, United Kingdom). Optimal concentrations of all antibodies used were determined in pilot experiments. Briefly, the sections were incubated with the mAbs against cellular markers for 30 minutes, washed in Tris-buffered saline (50 mmol/L Tris-HCl and 150 mmol/L NaCl), and then incubated with rabbit anti-mouse immunoglobulin (Dakopatts) for 30 minutes. After washing in Tris-buffered saline, the sections were incubated with soluble complexes of alkaline phosphatase and mouse antialkaline phosphatase (alkaline phosphatase-antialkaline phosphatase, Dakopatts) for a further 30 minutes and developed with Fast Red (Sigma) as chromogen for signal visualization. For immunostaining of BB1⁺ basophils, the slides were pretreated with 0.1% saponin (Sigma)-PBS for 30 minutes, incubated with BB1 (1:10) overnight at room temperature, and then processed as described above.⁵

Positive cells stained red after development with Fast Red. Omission or substitution of the primary antibody with an irrelevant antibody of the same species was used as a negative control. The positive cells within the whole sections were counted, and the results were expressed as the total number of positive cells per square mil-

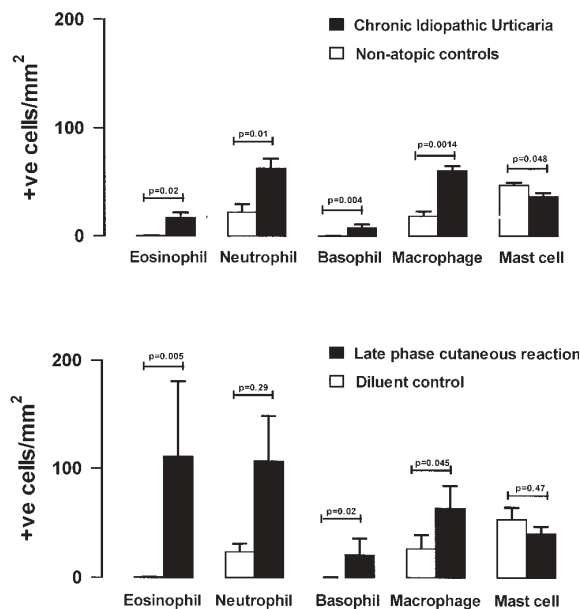


FIG 1. Congo red–positive eosinophils, elastase-positive neutrophils, BB1+ basophils, CD68+ macrophages, and tryptase-positive mast cells in skin biopsy specimens from healthy, nonatopic control subjects (*open columns*, $n = 6$; *top*) and patients with CIU (*filled columns*, $n = 13$; *top*) and diluent challenge sites (*open columns*, $n = 6$; *bottom*) and allergen-induced skin LPR (*filled columns*, $n = 6$; *bottom*). The results are expressed as the numbers of positive cells per square milliliter of biopsy specimen. The differences between patients with CIU and healthy, control subjects or allergen-induced skin LPR sites and diluent-challenged control sites were analyzed by using the Mann-Whitney U test.

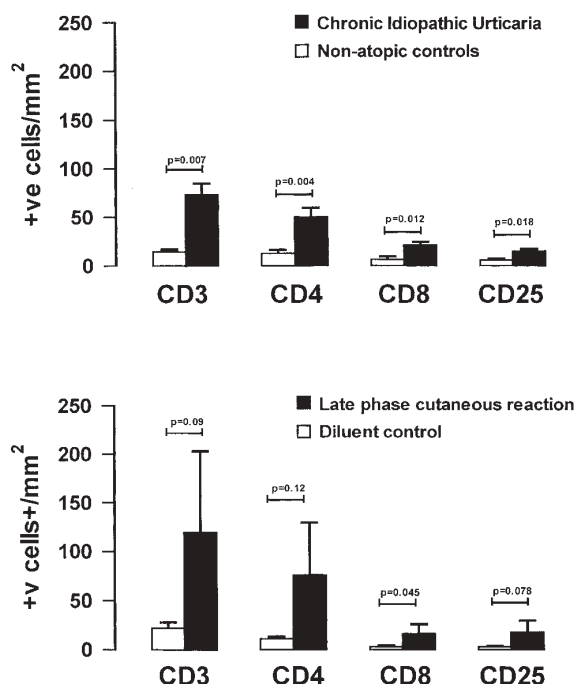


FIG 2. The numbers of CD3+, CD4+, CD8+, and CD25+ T cells in skin biopsy specimens from healthy, nonatopic control subjects (*open columns*, $n = 6$; *top*) and patients with CIU (*filled columns*, $n = 13$; *top*) and diluent challenge sites (*open columns*, $n = 6$; *bottom*) and allergen-induced skin LPR sites (*filled columns*, $n = 6$; *bottom*). The results are expressed as the numbers of positive cells per square milliliter of biopsy specimen. The differences between patients with CIU and healthy, control subjects or allergen-induced skin LPR sites and diluent-challenged control sites were analyzed by means of the Mann-Whitney U test.

limeter of biopsy specimen (each section was about 2.5–3.0 mm²). The coefficient variability of the duplicate counts obtained from all slides was less than 5%. No immunoreactivity was observed in sections stained with omission of the primary antibody or substitution of this antibody with an irrelevant antibody of the same species.

In situ hybridization

All reagents were from Sigma Chemicals, unless otherwise indicated. The cDNA fragments of IL-4, IL-5, and IFN- γ used to generate riboprobes were used in the previous studies.^{16–19} Briefly, riboprobes (antisense or sense) were synthesized in the presence of adenine triphosphate, guanosine triphosphate, cytosine triphosphate, ³⁵S-uridine triphosphate, and appropriate RNA polymerases (T7 or Sp6), respectively.^{16–19}

Slides were defrosted for the experimental procedure. Permeabilization, prehybridization, and hybridization protocols were as described previously.^{16–19} Incubation in N-ethyl maleimide, iodoacetamide, and triethanolamine reduced nonspecific binding of the ³⁵S-uridine triphosphate–labeled probes. In addition, the experiments were performed under very high stringency conditions (hybridization at 50°C and posthybridization washing at 60°C in 0.1 $\times$ standard saline solution citrate). Negative controls used hybridization with the sense probe and pretreatment of slides with RNase A (Promega, Southampton, United Kingdom) before hybridization with the antisense probe. For autoradiography and identification of mRNA+ signals, slides were performed and counted as previously described.^{16–19} The results were expressed as the total number of positive cells per square millimeter of biopsy specimen. The coefficient of variability of the duplicate counts obtained from all slides was less than 5%.

Statistical analysis

Data were analyzed with a statistical package (Minitab Release 7; Minitab Inc, State College, Pa). The Mann-Whitney U test was then used for comparison between 2 groups. For all tests, P values of less than .05 were considered statistically significant.

RESULTS

Immunohistochemistry

In patients with CIU, infiltrating inflammatory cells were located mainly in the dermis and deep dermis. The numbers of Congo red–positive eosinophils and elastase-positive neutrophils were significantly increased in patients with CIU compared with nonatopic control subjects ($P = .02$ and $P = .01$, respectively; Mann-Whitney U test), even though the numbers of cells were slightly less than those in biopsy specimens from allergen-induced skin LPRs (Fig 1). The numbers of BB1+ basophils were significantly greater in patients with CIU than those in healthy subjects ($P = .004$, Fig 1). CD68+ macrophages were also significantly greater in patients with CIU than those in control subjects (Fig 1). In contrast, the numbers of tryptase-positive mast cells in urticaria were slightly but significantly decreased from those in control subjects ($P = .048$, Fig 1). Degranulation of neutrophils, eosino-

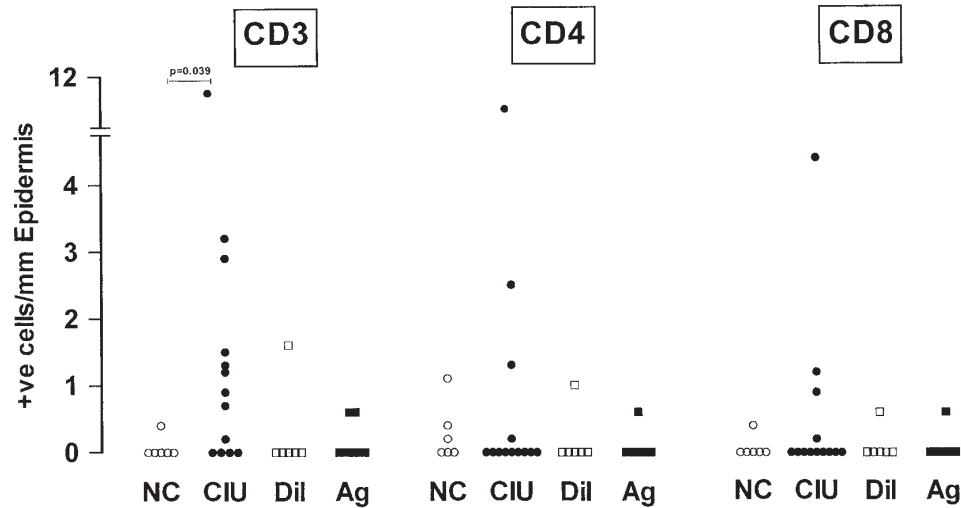


FIG 3. Infiltrating CD3⁺, CD4⁺, and CD8⁺ T cells in epidermis of skin biopsy specimens from healthy, nonatopic control subjects (*open circles*, n = 6), patients with CIU (*filled circles*, n = 13), diluent challenge sites (*open squares*, n = 6), and allergen-induced skin LPR sites (*filled squares*, n = 6). The results are expressed as the numbers of positive cells per milliliter of skin epidermis. The differences between patients with CIU and healthy control subjects or allergen-challenged skin LPR sites and diluent-challenged control sites were analyzed by means of the Mann-Whitney U test. NC, Nonatopic control subject; Dil, diluent-challenged skin sites; Ag, antigen-challenged skin LPR sites.

phils, basophils, and mast cells was also observed in patients with CIU. Compared with control subjects, there were significant increases in the numbers of CD3⁺, CD4⁺, CD8⁺, and CD25⁺ T cells in biopsy specimens from patients with urticaria ($P < .01$, Fig 2). A similar cell infiltration was observed in biopsy specimens taken 24 hours after allergen challenge, as previously reported (Figs 1 and 2).⁵ Double-immunofluorescence staining showed that the majority of CD25⁺ T cells were CD4⁺ T cells. Thus in patients with urticaria, the mean percentage of CD4⁺/CD25⁺ cells was $80.5\% \pm 2.8\%$, and in the allergen-induced LPR, it was $70.7\% \pm 2.8\%$.

Compared with the allergen-induced skin LPR sites and with control subjects, there were significant increases in the numbers of T lymphocytes in the epidermis, which were mainly CD3⁺ T cells (Fig 3). There were no significant differences in the numbers of any cell types between patients with and without autoantibodies (Table II).

In situ hybridization

In patients with CIU compared with healthy control subjects, there were significant increases in the numbers of cells expressing mRNA for IFN- γ ($P = .037$), IL-4 ($P = .0029$), and IL-5 ($P = .0025$, Fig 4). In the LPR sites, compared with diluent-challenged sites, there were greater numbers of cells expressing IL-4 ($P = .0082$) and IL-5 ($P = .0051$) but no significant differences in IFN- γ (Fig 4). No significant differences were observed between patients with and without anti-Fc ϵ RI autoantibodies (Table II).

Figs 5, 6, and 7 show examples of immunohistochemistry for infiltration of inflammatory cells and in situ hybridization for IL-4 mRNA in patients with CIU and in allergen-induced LPR sites.

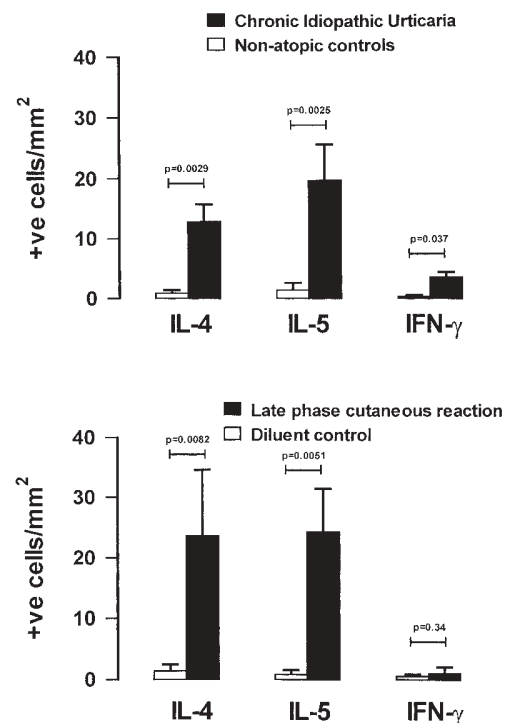


FIG 4. The numbers of cells expressing mRNA for IL-4, IL-5, and IFN- γ in skin biopsy specimens from healthy, nonatopic control subjects (*open columns*, n = 6; *top*) and patients with CIU (*filled columns*, n = 13; *top*) and diluent challenge sites (*open columns*, n = 6; *bottom*) and allergen-induced skin LPR sites (*filled columns*, n = 6; *bottom*). The results are expressed as the numbers of positive cells per square milliliter of biopsy specimen. The differences between patients with CIU and healthy control subjects or allergen-challenged skin LPR sites and diluent-challenged control sites were analyzed by means of the Mann-Whitney U test.

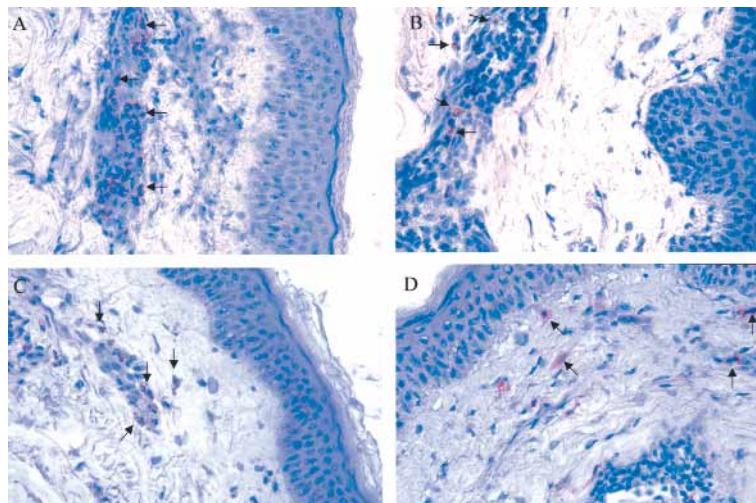


FIG 5. CD3⁺ T cells (**A**), Congo red-positive eosinophils (**B**), elastase-positive neutrophils (**C**), and CD68⁺ macrophages (**D**) in skin biopsy specimens from patients with CIU. Positive cells are stained red (arrows).

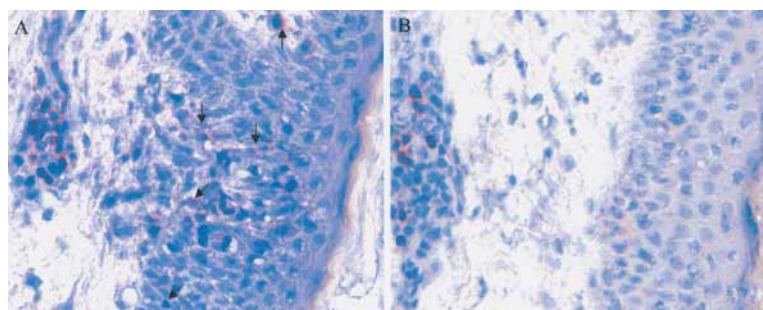


FIG 6. **A**, CD3⁺ T cells (arrows) in epidermis and dermis in patients with CIU. **B**, CD3⁺ T cells in the dermis only in allergen-induced skin LPR sites.

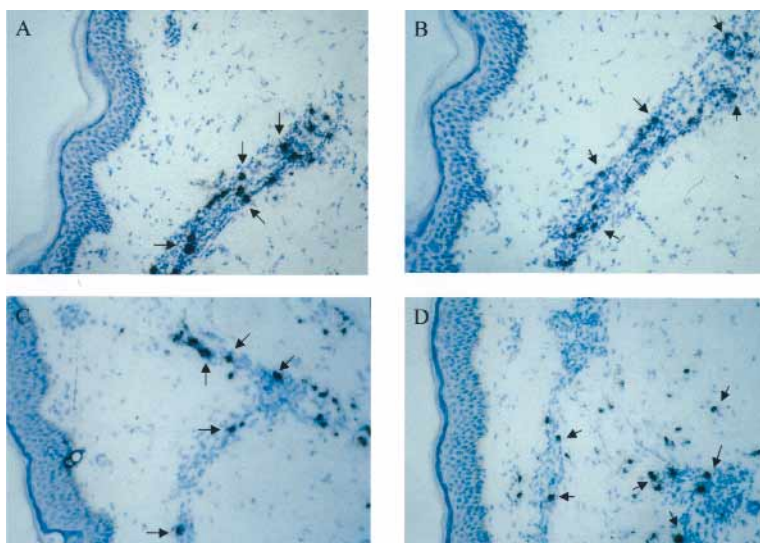


FIG 7. IL-4 and IL-5 mRNA⁺ cells in skin biopsy specimens from allergen-challenged LPR sites and patients with CIU. Positive hybridization with ³⁵S-labeled IL-4 (**A**) and IL-5 (**B**) antisense riboprobes (arrows) from a biopsy specimen taken 24 hours after allergen challenge. Positive hybridization signals for IL-4 (**C**) and IL-5 (**D**, arrows) from a biopsy specimen from patients with CIU.

TABLE II. Comparison of the numbers of inflammatory cells and mRNA⁺ cells in skin urticarial lesions between patients with and without autoantibody

Subjects	Eosinophils	BB1	CD3	CD4	CD8	CD25	Tryptase	CD68	Elastase	IL-4	IL-5	IFN- γ
Auto antibody positive	11.5 (0.0-49.3)	3.4 (0.0-28.1)	40.0 (26.6-94.6)	32.2 (16.2-92.1)	18.8 (13.0-40.5)	10.2 (7.6-21.9)	21.2 (17.4-59.0)	57.4 (33.6-70.5)	62.6 (30.8-101.6)	13.6 (3.3-19.9)	16.9 (4.4-25.5)	3.1 (0.0-7.7)
Auto antibody negative	10.5 (0.0-45.3)	2.5 (0.9-36.8)	77.0 (38.8-164.2)	32.7 (11.8-121.1)	21.2 (1.1-37.3)	11.9 (5.7-37.4)	40.8 (23.1-56.4)	66.6 (31.9-76.6)	47.9 (15.8-121.3)	5.5 (0.7-40.1)	12.3 (1.7-77.4)	2.2 (0.0-9.8)

DISCUSSION

Studies of the pathogenesis of chronic urticaria indicate that a subpopulation of patients have a cutaneous autoimmune disease associated with antibody to the IgE receptor. Therefore the principal mechanism in CIU may be similar to that of urticaria caused by an allergen because cross-linking the IgE receptor would be an initiating stimulus common to both situations. Whereas atopic allergy is a well-defined T_H2-type immunologic response, autoimmune diseases are variable, although many (ie, multiple sclerosis, juvenile diabetes, rheumatoid arthritis, or Hashimoto thyroiditis) are associated with T_H1 lymphocytes.²⁰⁻²⁴ On the other hand, conditions such as systemic lupus erythematosus²⁵ or Graves' disease²⁶ have features of a T_H2 response. The major association of CIU with other autoimmune disorders is with Hashimoto thyroiditis,²⁷⁻²⁹ in which 25% of patients with CIU have antithyroid antibodies, regardless of thyroid functional status.³⁰

The data presented herein suggest that the infiltrating T cells in patients with chronic urticaria had a T_H0 phenotype because there were significant increases in IFN- γ , IL-4, and IL-5 mRNA⁺ cells. Alternatively, it is also possible that there is a mixture of T_H1/T_H2 cell types in the skin of patients with CIU. Allergen-induced LPRs, although resembling the lesion of chronic urticaria histologically, produce IL-4 and IL-5 but not IFN- γ . These differences may reflect the immune responsiveness of the patients, as well as the differences in antigenic stimulation (ie, allergen [LPR] vs α subunit [subpopulation of patients with chronic urticaria] or unknown stimulus [remainder of patients with chronic urticaria]). We have not subdivided patients with chronic urticaria into those who also have atopy (allergic rhinitis, asthma, and atopic dermatitis) and those who do not to determine whether the cellular infiltrate would differ. Another variable and distinction between the lesions of chronic urticaria and those of an allergen-induced LPR site would be an enhancing role for complement in chronic urticaria¹³ with release of C5a,¹² a chemotactic factor for neutrophils, eosinophils, monocytes, and basophils. Perhaps some of the unusual patients with neutrophil-predominant chronic urticaria have a more prominent role for complement. Finally, the timing of the biopsy in relation to the establishment and resolution of the lesion may affect the relative numbers of cells seen in patients with chronic urticaria. The patients with LPRs underwent biopsy at a single time point (ie, 6 hours).

Both LPRs and C5a-mediated chemotactic responses should have basophil recruitment as part of the inflammatory cell infiltrate. Here we demonstrate, by use of the well-defined phenotype marker BB1, that basophils are indeed present in the lesions of chronic urticaria. Thus the antibody to Fc ϵ RI α can also interact with these cells to activate them in situ, whereas basophils remaining within the vasculature appear to be desensitized.³¹

Whether the number of mast cells within the lesion of chronic urticaria is abnormal is not clear. One initial report suggested increased number of cells by means of chloroacetate esterase staining,¹ which was consistent with other data demonstrating a striking increase in a skin histamine levels.³² The latter observation could alternatively represent basophil infiltration or even increased histamine content per cell. Other studies with use of tryptase as a marker did not find increased numbers of mast cells,³³ and in this study we found diminished numbers. Furthermore, increased tryptase levels were observed in suction-blister fluid from patients with chronic urticaria.³⁴ These observations may, in part, reflect degranulation. Chloroacetate esterase stains granular elements in both neutrophils and mast cells, and the staining properties may not correspond to the tryptase distribution within cells.

It is of interest that approximately 60% of patients with chronic urticaria have no evidence of a circulating autoantibody, although their clinical presentation is strikingly similar to those who do, and their skin biopsy specimens are similar as well.³ We also found their cytokine profile to be the same. Because thyroid autoimmunity does not segregate in a strict fashion with antibody to the IgE receptor, perhaps all such patients have an autoimmune basis, but this particular autoantibody is not present.

In summary, we demonstrate that the basic molecular immunopathology of patients with CIU is that of an eosinophil and basophil cell-mediated hypersensitivity reaction with a T_H0 cytokine profile. Whether these changes are the results of ongoing mast cell degranulation is yet to be determined.

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