INVESTIGATIVE REPORT

Cytokine Production of CD4+ and CD8+ Peripheral T Lymphocytes in Patients with Chronic Idiopathic Urticaria

BEATRIX IRINYI[1], MAGDOLNA ALEKSZA[2], PÉTER ANTAL-SZALMÁS[2,3], SÁNDOR SIPKA[2], JÁNOS HUNYADI[1] and ANDREA SZEGEDI[1]

[1]*Department of Dermatology,* [2]*3rd Department of Internal Medicine and* [3]*Department of Clinical Biochemistry and Molecular Pathology, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary*

The aim of this study was to investigate the characteristic cytokine pattern of patients with chronic idiopathic urticaria. Using flow cytometry, we examined the frequency of IL-4, IL-10, IL-13 and IFN-γ producing CD4+ and CD8+ T cells in the peripheral blood mononuclear cells at a single cell level. In patients with chronic idiopathic urticaria, the frequency of IL-10 producing CD4+ and CD8+ T cells was significantly higher than that of control subjects, while the frequency of IFN-γ producing helper and cytotoxic T cells was significantly lower. The proportion of IL-4 producing CD4+ T cells from patients with urticaria was significantly lower. The ratio of IL-4 producing CD8+ T cells and the proportion of IL-13 producing CD4+ and CD8+ T lymphocytes did not show any significant difference between patients and controls. In our study, we could observe neither a dominant Th1 nor a dominant Th2 type cytokine pattern. We found a significant elevation in the intracellular IL-10 level which may be the cause of the down-regulated Th1 and Tc1 and partly Th2 lymphocyte functions. *Key words: chronic urticaria; cytokines; flow cytometry; Th1; Th2.*

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Andrea Szegedi, Department of Dermatology, University of Debrecen Medical and Health Science Center, 98. Nagyerdei str., Debrecen 4012, Hungary. E-mail: aszegedi@jaguar.dote.hu

Urticaria is a common skin reaction characterized by transient, pruritic, slightly erythematous weals is defined as chronic if occuring daily, or almost daily, over the course of at least 6 weeks. The diagnosis of chronic idiopathic urticaria (CIU) can be made by the exclusion of urticarial vasculitis, predominant physical urticarias, known causative foods or drugs and intercurrent febrile illnesses or infectious disease states (1).

Emerging data are providing new evidence that CIU, in at least a subset of patients, is an autoimmune disease (2). Sabroe & Greaves (1) identified three groups of patients with CIU: (*i*) those with functional circulating autoantibodies to FcεRIα or to IgE, (*ii*) those with mast cell-specific histamine-releasing activity with heat stable factors and non-immunoglobulin mediator, and (*iii*) those with a negative autologous serum test and no evident circulating histamine-releasing factors. Sera from patients with CIU containing anti-FcεRIα antibodies can cause weal formation on intradermal injection *in vivo* and histamine release from basophils and mast cells *in vitro* (3, 4). For some cases of CIU, the concept of an autoimmune aetiology is also supported by its association with HLA-DR4 and with autoimmune thyroid diseases, and by the observation that treatment with intravenous immunoglobulin or plasmapheresis may produce clinical remission (5–9).

In both mice and humans, functionally distinct helper T cell subsets, known as Th1 and Th2 cells, are characterized by the patterns of cytokines they produce; a distinction can also be made between cytotoxic (Tc) cells (10, 11). This classification of the cellular immune response is a useful model for explaining both the different types of protection as well as the pathogenic mechanisms of several immunopathological disorders. A prevalent Th1 lymphokine profile is observed in organ-specific autoimmune diseases, whereas Th2 cell responses tend to predominate in systemic sclerosis and in allergic diseases (12, 13).

In this study, our aim was to investigate whether there is a characteristic cytokine pattern in patients suffering from CIU. Using flow cytometry, we examined the frequency of IL-4, IL-10, IL-13 and IFN-γ producing CD4+ and CD8+ T cells in the peripheral blood mononuclear cells (PBMC) at a single cell level.

MATERIALS AND METHODS

Patients and controls

Twenty-two patients (4 men and 18 women, mean age 40 years, range 11–76) with almost daily attacks of urticaria and a mean continuous disease duration of 16.3 months (range 3–48) were investigated. A primary diagnosis of CIU was made when an aetiologic factor accounting for the majority of lesions could not be elicited from history, physical or laboratory evaluations. We excluded patients with lesions lasting for more than 24 h and suspicious for urticaria vasculitis, patients with physical urticarias, with food allergy or those taking drugs that could exacerbate chronic urticaria. None of the patients had an infectious disease. Fourteen (63.3%) patients gave an immediate weal response to an autologous serum skin test and two (9%) of these had anti-thyroid antibodies.

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Antihistamine treatment was stopped 4 days before skin-testing and blood-taking. Control blood samples were also obtained from 22 healthy volunteers (6 men and 16 women, mean age 36 years, range 15–60).

Measurement of immunoglobulin levels

Serum IgG, IgA, IgM, IgE, IgG1, IgG2, IgG3 and IgG4 levels were measured by a BEHRING nephelometer (Dade Behring, Leiderbach, Germany).

Detection of allergen-specific IgE

Food and inhalant allergens were detected by "Allergoset-IgE Food" and "Allergoset-IgE Inhalant" kits (INTEX Diagnostika, Basel).

Detection of lymphocyte subpopulations and activated T cells

Heparinized venous whole blood was stained with anti-human monoclonal antibodies (mAb) – anti-CD3-FITC, anti-CD8-FITC (both from Sigma, St. Louis, MO, USA), anti-CD4-PE, anti-CD19-PerCP, anti-CD69-PE or anti-HLA-DR-RD1 (all from Coulter-Immunotech, Hialeah, FL, USA) – for 30 min at room temperature in darkened conditions. Erythrocytes were lysed and stained cells were fixed using the Coulter QPrep protocol. The fluorescence analysis was performed on a Coulter EPICS XL-4 flow cytometer (Coulter Hialeah, FL, USA) with a 488 nm argon ion laser. Emitted fluorescence was determined by means of 525, 575 and 635 nm bandpass filters. Data of 5000 cells were acquired and analysed by System II software. Gates were set on lymphocytes according to forward and side-scatter properties, and results were expressed as the percentage of surface CD markers in the lymphocyte gate.

Measurement of intracytoplasmic cytokines

Heparinized whole blood (1 ml) was diluted at a 1 for 2 volume rate in RPMI (Sigma). T cells were stimulated with phorbole myristate acetate (Sigma, 25 ng/ml) and ionomycin (Sigma, 1 μg/ml) for 4 h at 37°C in a humidified CO_2 incubator. Blockage of cytokine secretion and intracellular accumulation was achieved by treating with Brefeldin A (Sigma, 10 μg/ml). The CD4+ and CD8+ cells were stained with 10 μl of quantum red labelled anti-human CD4 or anti-human CD8 mAb for 30 min at room temperature in dark. Two millilitres of diluted FACS lysing solution (Becton-Dickinson, Mountainview, CA, USA) was added to each tube for 10 min in order to lyse the erythrocytes and to fix the plasma membrane of leukocytes. Samples were centrifuged at 500 × *g* for 5 min and supernatants were removed. Diluted FACS permeabilizing solution (0.5 ml, Becton-Dickinson) was added to each sample and these were incubated for 10 min. After a washing step, the permeabilized cells were stained with FITC-labelled anti-human IFN-γ and phycoerythrin (PE)-labelled anti-human IL-4 (both from Becton-Dickinson) or with PE-labelled anti-human IL-10 (Caltag) or PE-labelled anti-human IL-13 (Becton-Dickinson) antibodies for 30 min at room temperature in dark. The samples were washed again and fixed in 500 μl of 1% paraformaldehyde (Sigma). Cells were analysed by a Coulter EPICS XL-4 flow cytometer. Analyses gates were set on lymphocytes in accordance with forward and side-scatter and their CD4 or CD8 positivity. Results were expressed as the percentage of cytokine-producing cells in each CD4+ or CD8+ T-cell population.

Statistical analysis

Data were analysed by two-tailed Student's *t*-test for unpaired samples.

RESULTS

Serum immunoglobulin levels

We measured the serum immunoglobulin levels in the blood of patients with CIU by nephelometry. In the case of IgA, IgM and IgG levels we did not see significant deviations when comparing data of the patients and the control group (data not shown). The level of IgE in patients with CIU was elevated, but statistically it was not significant (Table I). Allergen-specific IgE was not detected in the sera of our patients, and the rate of eosinophil granulocytes did not differ significantly compared to the healthy controls (data not shown).

Frequency of CD3+, CD4+, CD8+ and CD19+ cells in PBMC

PBMC from 22 healthy control subjects and 22 patients with CIU were examined for the frequency of CD3+, CD4+, CD8+, and CD19+ T cells by flow cytometry.

The actual lymphocyte subpopulations were separated on the basis of cell surface markers (CD molecules). In the case of CIU patients, the proportion of CD3+, CD4+, CD8+ and CD19+ T and B lymphocytes did not show any significant deviation compared with the control group. We also determined the percentage of activated T cells within the T lymphocyte population. While examining T lymphocytes producing surface HLA-DR molecules, a significantly increased number of HLA-DR+/CD3+ T cells was observed as compared with the control subjects (3.84 ± 2.31% versus 1.55 ± 0.75%; $p < 0.01$). In the case of the other activation marker (CD3+/CD69+), we could not observe any differences compared with the control group (0.92 ± 0.49% versus 0.98 ± 1.2%) (Fig. 1).

Ratio of IL-4, IL-10, IL-13 and IFN-γ producing CD4+ cells in PBMC

We examined the frequency of IL-4, IL-10, IL-13 and IFN-γ producing CD4+ T cells by flow cytometry. The ratio of IL-10 producing CD4+ T cells in the patients with CIU was significantly higher than that of control subjects (6.1 ± 5.4% versus 2.1 ± 1.19%; $p < 0.01$)

Table I. *Serum immunoglobulin levels (mean ± SD) in controls and patients with chronic idiopathic urticaria (n = 22 for both)*

	IgG	IgA	IgM	IgE
Controls (g/l)	11.2 ± 2.3	1.8 ± 0.7	0.9 ± 0.3	96.8 ± 21
Patients (g/l)	10.6 ± 2.4	2.0 ± 0.8	1.3 ± 0.6	146 ± 111

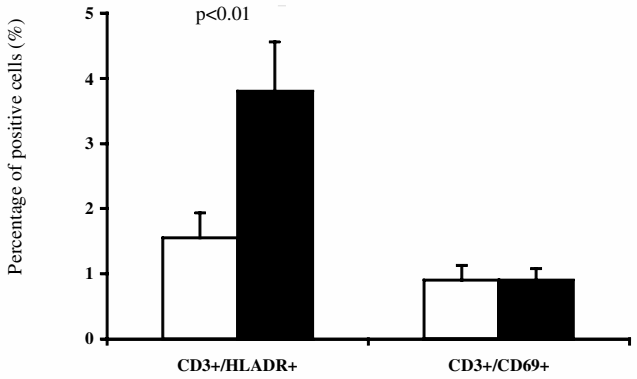

Fig. 1. Detection of activated T cells in patients with chronic idiopathic urticaria (CIU). In order to quantify the rate of activated T cells in patients with CIU, whole blood T lymphocytes of controls (□, $n=22$) and patients with CIU (■, $n=22$) were labelled by a PE-conjugated anti-HLADR or anti-CD69 mAb in combination with a FITC-labelled anti-CD3 mAb. The samples were measured by a Coulter EPICS XL-4 flow cytometer and lymphocytes were identified based on their scatter properties. The rate of CD3+/HLADR+ and CD3+/CD69+ double positive cells expressed on the y-axis as the percentage of positive cells among all lymphocytes.

(Fig. 2B). In contrast, the frequency of IFN-γ producing helper T cells from patients with CIU was significantly lower than that of control subjects (17.0 ± 5.7% versus 22.06 ± 6.25%; $p < 0.002$) (Fig. 2A). The proportion of IL-4 producing CD4+ T cells from patients with CIU was also significantly lower than that of healthy control groups (0.5 ± 0.86% versus 1.12 ± 0.71%; $p < 0.03$) (Fig. 2C). The ratio of IL-13 producing CD4+ lymphocytes was higher in patients than in the control group, but the difference was not remarkable (0.21 ± 0.31% versus 0.14 ± 0.09%) (Fig. 2D).

Ratio of IL-4, IL-10, IL-13 and IFN-γ producing CD8+ cells in PBMC

Cytokine production of the CD8+ T cells was also measured by flow cytometry. The ratio of IL-10 producing CD8+ T cells in patients with CIU was significantly higher than in the case of healthy control subjects (8.63 ± 7.21% versus 1.98 ± 1.47%; $p < 0.001$) (Fig. 2B). The ratio of IFN-γ producing CD8+ T cells in patients with CIU was significantly lower than in healthy control subjects (27.6 ± 12.2% versus 43.4 ± 8.45%; $p = 0.0003$) (Fig. 2A). The frequency of IL-4 producing CD8+ T cells was higher in patients compared with the control group (1.22 ± 4.04% versus 0.59 ± 0.72%) (Fig. 2C), and the ratio of IL-13 producing CD8+ T cells from patients with CIU was also higher than that of healthy controls (Fig. 2D), but neither difference was statistically significant.

Comparison of the autologous serum skin test positive and negative patient groups

In the present study, 14 of the 22 patients gave an immediate weal response in the autologous serum skin test; 8 patients were negative. We made comparisons between the data of the autologous serum skin test positive and negative patient groups and observed no significant difference between the two groups with regard to the immunoglobulin levels, the subpopulations of peripheral lymphocytes, the frequency of activated T cells and the expression of intracellular cytokines (data not shown).

DISCUSSION

The aetiology of chronic urticaria is still obscure. In the vast majority of subjects the causative allergen cannot be identified. The current concept is that CIU is autoimmune in origin, at least in a subpopulation of patients (14). In the present study, we investigated the characteristic cytokine production of T cells in the PBMC of patients suffering from CIU.

First we measured the serum immunoglobulin levels. In the case of the IgG, IgA and IgM levels we did not find any difference, while the median serum IgE level in patients was higher than in the control population, but the difference was not statistically significant. Sabroe et al. (15) detected normal IgE levels in the sera of patients with CIU. They found lower serum IgE levels in those patients who had circulating anti-FcεRIα or anti-IgE antibodies than in patients without autoantibodies. Allergen specific IgE was not found in our patient population.

Barlow et al. (16) investigated T-cell subsets in biopsy specimens of patients with CIU and showed a preponderance of CD4+ cells over CD8+ cells, while Malet et al. (17) detected a significantly decreased CD8+ T-cell count in the peripheral blood of chronic urticaria patients. In our patient population, there was no significant difference in the proportion of the CD3+, CD4+, CD8+ and CD19+ T and B lymphocytes as compared to the control group. While the number of CD3+ T lymphocytes was not elevated, the ratio of activated, HLA-DR expressing CD3+ T lymphocytes was significantly higher.

With regard to the cytokine measurements, we could detect an elevated IL-10 production both in CD4+ and CD8+ T cells. The immune-regulatory cytokine IL-10 is produced by both Th1 and Th2 lymphocytes and inhibits their antigen-specific proliferation and cytokine production (18). IL-10 has the ability to down-regulate several major functions of Th1 cells and macrophages and can control also the Th2-mediated inflammatory processes (19). IL-10 also acts by down-regulating IL-12 production by antigen presenting cells, thereby promoting the differentiation of naive Th0 cells toward a Th2 profile, although the Th2-type response is down-regulated in some situations as well (20, 21). Our results indicate that in consequence of the increased IL-10 production the function of both Th1 and Tc1 lymphocytes is impaired, which results in a decreased

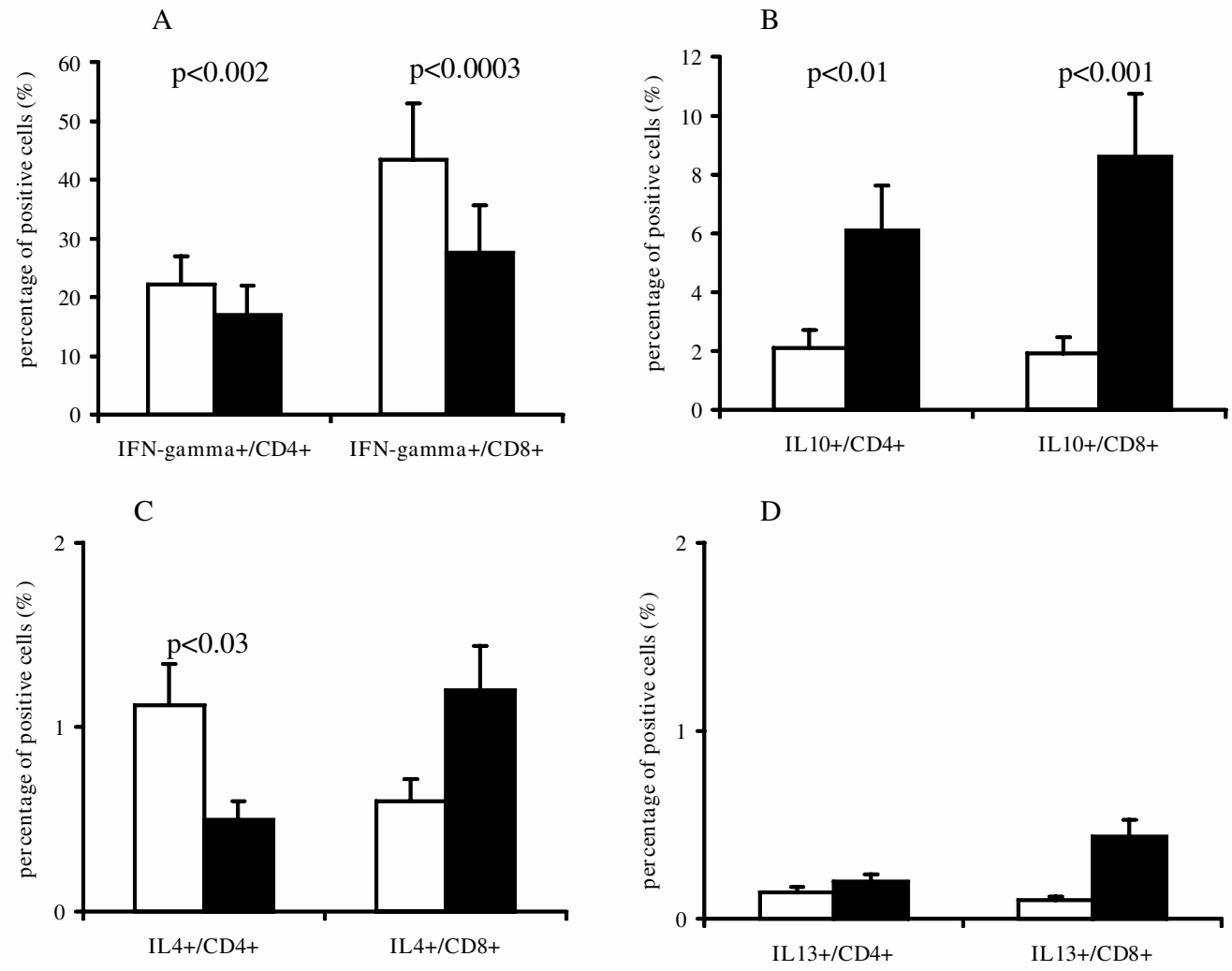

Fig. 2. Intracellular cytokine expression of stimulated T helper and T cytotoxic cells of patients with chronic idiopathic urticaria (CIU) and healthy controls. Whole blood T lymphocytes of healthy controls (□) and patients with CIU (■) were stimulated by phorbol myristate acetate (PMA) and ionomycin in the presence of berefeldin A for 4 h at 37°C. The CD4 and CD8 surface molecules were marked by quantum red-labelled mAbs, then the cells were fixed and permeabilized and the intracellular cytokines IFN-γ (A), IL-4 (C), IL-10 (B) and IL-13 (D) were stained by specific FITC-labelled or PE-labelled mAb. The samples were measured with a Coulter EPICS XL flow cytometer and the rate of cytokine-containing cells among the CD4-positive and CD8-positive T cells was determined. The bars and error bars present the mean ± SD of the result from controls and patients. The differences between the healthy controls and the patient with CIU were calculated by the Student's t-test.

intracellular IFN-γ content. The significant decrease in the intracellular IL-4 level of the CD4 + T lymphocytes can be the result of the inhibitory effect of IL-10 on Th2 cells. On the other hand, IL-10 may not completely inhibit the cytokine production of Th2 and Tc2 lymphocytes because the decreased IFN-γ content could have an opposing influence. Since IFN-γ has the ability to inhibit Th2 cytokine production, an impaired IFN-γ production could be the reason for the increased intracellular IL-4 level in CD8 + cells. According to the literature, the inhibitory activity of IL-10 is not prominent on IL-13 production. This may explain the increased IL-13 production which we observed in both CD4 + and CD8 + T lymphocytes, although these differences were not significant.

Besides the inhibitory effects of IL-10, this cytokine also has immunostimulatory properties, especially on B cells. This effect could contribute to the autoantibody production which is observed in patients with CIU. It has recently become clear that between 27% and 50% of patients with CIU have functional autoantibodies directed against the α-chain of the high affinity IgE receptor (FcεRIα) (22). These antibodies are identified by autologous serum skin testing and confirmed by histamine release studies or immunoblotting. In the present study, 14 of the 22 patients gave an immediate weal response in the autologous serum skin test, whereas 8 patients were negative. However, we did not observe any significant difference between the two groups.

The last decade has been an exciting time for the proponents of the Th1 and Th2 paradigm. Nevertheless, the Th1/Th2 polarization cannot always be demon-

strated. In systemic autoimmune diseases such as in systemic lupus erythematosus and Sjögren's syndrome, both the Th1 and Th2 responses may be pathogenic (13). It is usually accepted that thyroid autoimmunity is mediated by Th1 cells (12), but recent results show a major difference in the balance of Th1 and Th2 cytokines secreted by cultures of thyroid and peripheral blood lymphocytes (23). Our data do not show a characteristic Th1 type or Th2 type cytokine pattern in the PBMC of patients suffering from CIU. From these results we cannot conclude to the cytokine pattern of lesional skin in CIU patients; further investigation is needed to answer this question (24, 25, 26).

Taken together, our study has produced the first results on the cytokine production of PBMC in chronic urticaria. We could observe neither a dominant Th1 nor a dominant Th2 type cytokine pattern and our results were similar with regard to the cytotoxic T cells. We measured a significant elevation in the intracellular IL-10 level and this may be the cause of the down-regulated Th1 and Tc1 and partly Th2 lymphocyte functions.

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