

Immune Profiles of Patients with Chronic Idiopathic Urticaria

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Key Words

Chronic idiopathic urticaria · Immunology · T lymphocytes · Cytokines · Chemokines · Adhesion molecules

Abstract

Background: The immunologic characterization of chronic idiopathic urticaria (CIU) is still incomplete. In particular, it is not known if positivity to the intradermal autologous serum skin test (ASST) identifies an immunologic subset of CIU patients. **Methods:** Nineteen CIU patients and 15 healthy controls were enrolled in the study. A diagnostic flowchart was designed to select CIU patients, who were then analyzed by ASST. Cytokine and chemokine production and the expression of adhesion molecules was measured in patients and controls. **Results:** In CIU patients compared to controls, it was found that (1) TNF- α , IL-10, MIP-1 α and RANTES production was augmented and IL-2 and INF- γ reduced, and (2) CD44, CD11a and CD62L expression on CD4 and CD8 cells was augmented. Additionally, TNF- α and chemokine production was significantly increased in CIU patients with a negative ASST (p-; n = 10) compared to patients with a positive response to the test. **Conclusions:** The presence of an inflammatory process in CIU patients is suggested by the findings that the production of both TNF- α and chemokines as well as the expression of adhesion molecules is increased in these patients.

Similarly to what is seen in rheumatoid arthritis, augmented IL-10 production might be secondary to the attempt to hamper the inflammatory milieu. Immune profiles are particularly altered in CIU p- patients, in whom a more aggressive therapeutic strategy might be considered.

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Introduction

Urticaria is one of the most common cutaneous pathologies. According to American data, 15–25% of the population is affected by urticaria at least once in their lifetime [1], and a chronic evolution is seen in more than 25% of these cases [2]. Chronic urticaria is characterized by the presence of daily clinical manifestations for a period longer than 6 weeks. Chronic urticaria is defined as idiopathic (CIU) in 70–80% of patients, as it is impossible to find a pathogenic agent. These considerations have led to the hypothesis that an autoimmune etiology may be responsible for CIU [3, 4].

Circulating autoantibodies against IgE or against the high-affinity IgE receptor (Fc ϵ RI) can be found in up to 35–45% of patients with CIU. Gruber et al. [4] found that IgE-specific antibodies are increased in up to 10% of individuals affected by CIU. The presence of anti-Fc ϵ RI antibodies was first shown in these patients by Hide et al. [5] and was subsequently confirmed by a number of studies

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[6–8]. These autoantibodies are functional, as demonstrated by both in vivo (induction of a wheal-flare response subsequent to the intradermal injection of autologous serum) and in vitro (basophil and histamine release assays) experimental evidence [9–11]. The pathogenesis of CIU in patients without autoantibodies remains unclear, although a mast cell-specific histamine-releasing factor has been shown to be active in a small proportion of these individuals. Sabroe et al. [12] clearly demonstrated that the severity of CIU was greater in patients with autoantibodies compared to patients without autoantibodies. Thus, autoantibody-positive patients had significantly higher wheal scores off treatment, wheals at more sites, higher itch scores for the most severe episode of itching and a significantly higher number of associated systemic symptoms [12]. Finally, a specific circulating factor that favors the release of histamine from mast cells through a mechanism independent of FcεRI-specific antibodies has been described in a small but somewhat uncertain percentage of patients [13].

The fundamental role of the immune system in the pathogenesis of CIU was evidenced by histologic analyses of cutaneous lesions. Hence, urticaria swellings are characterized by a perivascular infiltrate in the absence of vascular damage, nuclear debris or red cell extravasation [14, 15]. The predominant cell type found in the lesions is the activated T helper lymphocyte that expresses HLA-DR antigen; these cells are arranged perivascularly. Additionally, the lesional skin contains an increased number of mast cells. The kinetics of leukocyte recruitment to the inflammatory site has been studied with selected primary antibody markers of adhesion molecules [16]. In these studies, wheals of patients with pressure urticaria showed increased expression of E-selectin and intracellular adhesion molecule 1 (ICAM-1) on vascular endothelial cells and of vascular cell adhesion molecule 1 on perivascular cells. The importance and the early involvement of these adhesion molecules in the generation of cutaneous swellings was recently confirmed by serological [17] and bioptical [18] analyses. Thus, an augmented concentration of TNF-α, an inflammatory cytokine that induces endothelial cell activation by increasing the expression of adhesion molecules, was described to be present in tissue specimens of urticaria patients.

In this study, we analyzed type 1 (IL-2 and IFN-γ) type 2 (IL-4 and IL-10) and proinflammatory (TNF-α) cytokine generation and chemokine (MIP-1α and RANTES) production as well as the expression of adhesion molecules in peripheral mononuclear cells of CIU patients. The results confirm the presence of an inflammatory pro-

Table 1. Demographic characterization of CIU patients and HCs

Group	n	Sex (M/F)	Age, years
CIU patients	19	7/12	39.15 (23–61)
HCs	15	5/10	38.7 (23–54)

Age is shown as the mean, with the range in parentheses.

cess in CIU. This inflammatory process is particularly severe in CIU patients with a negative response to the autologous serum skin test (ASST).

Subjects and Methods

Patients and Controls

Nineteen subjects with CIU were consecutively enrolled in the study. All patients reported daily symptoms of urticaria lasting for at least 6 weeks. A group of 15 sex- and age-matched healthy control donors (HCs) was also analyzed. Clinical and epidemiological characteristics of patients and controls are shown in table 1a. Diagnosis of CIU was obtained via a diagnostic flowchart (fig. 1). Patients were put on a diet free of dye- and histamine-releasing foods for 4 weeks; if this diet was not effective, patients were examined for hematologic parameters (chemistry panel, complete blood count and sedimentation rate) and underwent parasitologic stool analysis. Patients were subsequently treated with anti-H1 drugs for at least 2 months. In the case of failure or relapse after suspension of therapy, the patients were analyzed for immunological (antithyroid antibodies, antinuclear antibody, rheumatoid factor, complement levels, ENA, a-nDNA) and serological parameters (HbsAg, HbsAb, HCVAb, anti-*Helicobacter pylori* antibody) and radiological exams (chest and oral X-ray, abdominal ultrasonography) were performed. If these tests were negative, the patients were analyzed by ASST and treated with immunomodulants (steroids, cyclosporin). No patient had clinical symptoms of vasculitis. All the subjects provided written informed consent before admission to the study. Patients had not undergone antihistamine therapy for at least 5 days prior to the study.

Autologous Serum Skin Test

Serum was obtained from venous blood samples clotted at room temperature for 30 min in silicone-coated vacutainer tubes (Becton-Dickinson, Marlan, France), followed by centrifugation at 550 g for 10 min, and cell-free supernatant was collected. For skin testing, serum samples were used immediately after preparation. CIU patients were injected intradermally on the volar surface of the forearm with 50 μl of serum. Skin reactivity was evaluated in all individuals by a standard histamine prick test (histamine hydrochloride 1%, Bayer, Milan, Italy). A saline solution was utilized as negative control. All the injection sites were scored 30 min after inoculation. Reactions showing a mean wheal-flare diameter of less than 0.5 cm were considered negative [19].

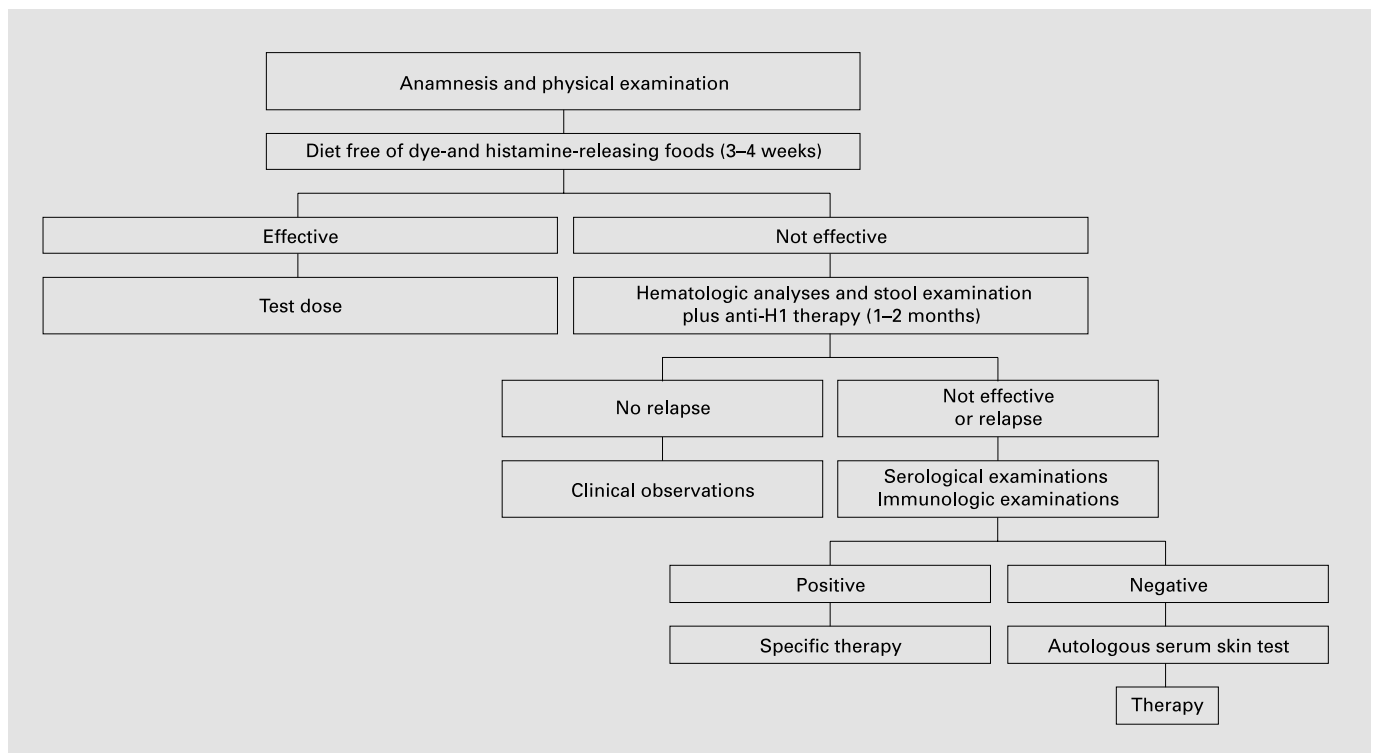


Fig. 1. Diagnostic flowchart.

Blood Sample Collection

Whole blood was collected by venipuncture in EDTA-containing vacutainer tubes (Becton-Dickinson). Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation on lymphocyte separation medium (Organon Teknika Corp., Durham, N.C., USA) and washed twice in PBS. The number of viable lymphocytes was determined by trypan blue exclusion and a hemocytometer.

In vitro Cytokine and Chemokine Production

For cytokine production, PBMCs were suspended at $3 \times 10^6/\text{ml}$ in RPMI-1640 and were either unstimulated or stimulated with phytohemagglutinin (Sigma, St. Louis, Mo., USA) ($12.5 \mu\text{g}/\text{ml}$) or lipopolysaccharide (Sigma) ($1 \mu\text{g}/\text{ml}$) at 37°C in a moist, 7% CO_2 atmosphere. Supernatants were harvested after 48 h. Production of IL-2, IFN- γ , IL-4, IL-10 and TNF- α by PBMCs was evaluated with commercially available ELISA kits (Endogen, Woburn, Mass., USA). All test kits were used following the procedures suggested by the manufacturer.

The production of beta chemokines (RANTES, MIP-1 α) was analyzed by stimulating 3×10^5 PBMCs with phytohemagglutinin in the presence of 20 IU/ml IL-2 for 48 h. The concentration of RANTES and MIP-1 α in the supernatants was determined by ELISA assay (R&D, Minneapolis, Minn., USA).

FACS Analyses

FACS analyses were performed using a Coulter counter (Coulter Electronics Inc., Miami Lakes, Fla., USA). Lymphocyte and monocyte subsets were evaluated by flow cytometric analysis using 50 μl of

EDTA-peripheral blood incubated for 30 min at 4°C with fluorochrome-labeled monoclonal antibodies. After incubation, erythrocyte lysis was performed using the immunoprep EPICS kit (Coulter Electronics) and Q prep Work station (Coulter Electronics).

Monoclonal Antibodies

The following monoclonal antibodies were used: anti-CD4-FITC (IgG2a), anti-CD8-TC (IgG2a), anti-CD-25-PE (IgG1), anti-DRII-PE (IgG2b), anti-CD44-PE (IgG1), anti-CD11a-PE (IgG1), anti-CD62L-PE (IgG1) and their isotype controls M α IgG1-PE and mIgG2a-TC (Caltag Laboratories Inc., Burlingame, Calif., USA).

Immunophenotypic Analyses

Lymphocytes and monocytes were analyzed using an EPICS XL flow cytometer (Coulter Electronics) equipped with a single 15-mW argon ion laser operating at 488 nm interfaced with a 486 DX2 IBM computer (IBM, Oxford, UK). Multiparametric data were acquired for 5,000 events and analyzed using Coulter System II software. Orange-red fluorescence from PE was collected through a 575-nm long-pass filter and TC (FL4) was measured through a 675-nm optical filter. Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL2 and FL3. Samples were first run using a single fluorochrome-stained preparation for color compensation. The lymphocytes were selectively analyzed using forward and side scatter properties. The absolute number of labeled cells was estimated by multiplying the absolute number of cells per microliter by the percentage of labeled cells.

Fig. 2. Mitogen-stimulated production of cytokines by PBMCs of 19 CIU patients and 15 HCs. Mean values, standard deviations and statistically significant differences are shown.

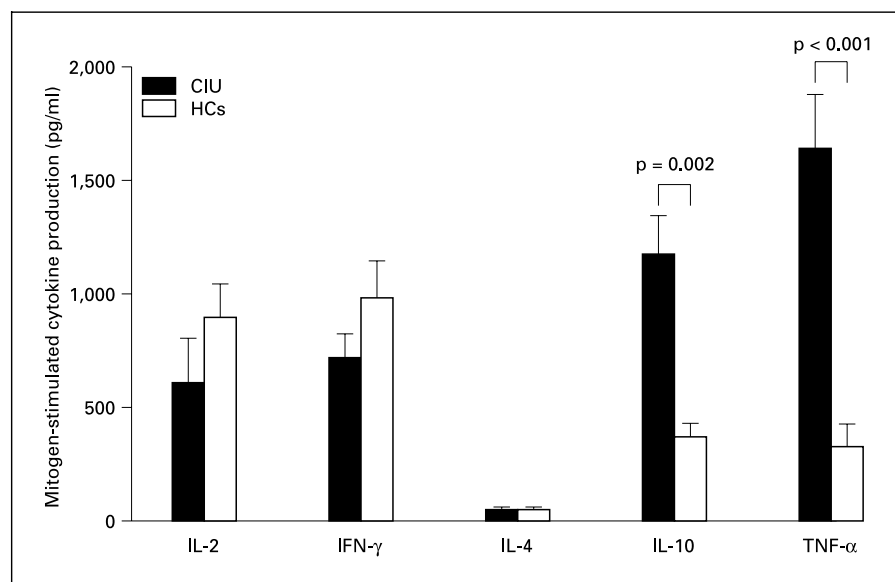


Table 2. Demographic characterization of CIU patients with a positive (p+ CIU) or negative (p- CIU) response to the ASST

Patient group	n	Sex (M/F)	Age, years
p+ CIU	9/19	3/6	40.8 (23–61)
p- CIU	10/19	2/8	37.6 (26–52)

Age is shown as the mean, with the range in parentheses.

Statistical Analyses

Statistical analysis was based on a nonparametric Kruskal-Wallis test, p values were two sided.

Results

Autologous Serum Skin Test

ASST was performed in all CIU patients. A positive response (p+ CIU) was detected in 9/19 subjects (47%), whereas the test was negative (p- CIU) in 10/19 individuals (53%). No clinical or epidemiological differences were observed when p+ and p- CIU patients were compared (table 2).

Mitogen-Stimulated Cytokine Production

PBMCs from CIU patients and HCs were stimulated with mitogen to evaluate IL-2, IFN-γ, IL-4, IL-10 and TNF-α production. CIU patients were characterized by a

lower production of IL-2 and IFN-γ compared to HCs, even if the differences did not reach statistical significance. IL-4 levels were similar in both groups. The production of IL-10 and TNF-α was significantly augmented in CIU patients compared to HCs (IL-10 p = 0.002; TNF-α p < 0.0001) (fig. 2).

When patients were divided based on the presence (p+) or absence (p-) of a positive response to the ASST, TNF-α production was significantly increased in both groups of CIU patients compared to HCs (p+ vs. HCs p = 0.003; p- vs. HCs p < 0.0001) and in p- compared to p+ individuals (p = 0.04). IL-10 production was more elevated in both groups of CIU patients compared to controls (p+ vs. HCs p = 0.009; p- vs. HCs p = 0.03). Finally, the IFN-γ concentration was reduced in both groups of CIU patients compared to HCs, but was significantly lower only in p+ CIU individuals (p+ vs. HCs p = 0.04 (fig. 3).

In vitro Chemokine Production

RANTES and MIP-1α production was evaluated upon stimulation of PBMCs from CIU patients and HCs with mitogens. The results showed that the production of these two chemokines is augmented in CIU patients compared to HCs (RANTES p = 0.04; MIP-1α p = 0.003) (fig. 4a). RANTES values were elevated in both p+ and p- CIU patients compared to controls, with the highest concentrations being detected in p- CIU individuals (p- vs. p+ CIU patients p = 0.06; p- vs. HCs p = 0.05). MIP-1α levels were significantly augmented as well in both groups of CIU patients compared to HCs. Once again, the highest

Fig. 3. Mitogen-stimulated production of cytokines by PBMCs of CIU patients divided in to two groups based on the presence (n = 9) (p+ CIU) or the absence (n = 10) (p- CIU) of a response to the ASST. Values obtained in 15 HCs are also presented. Mean values, standard deviations and statistically significant differences are shown.

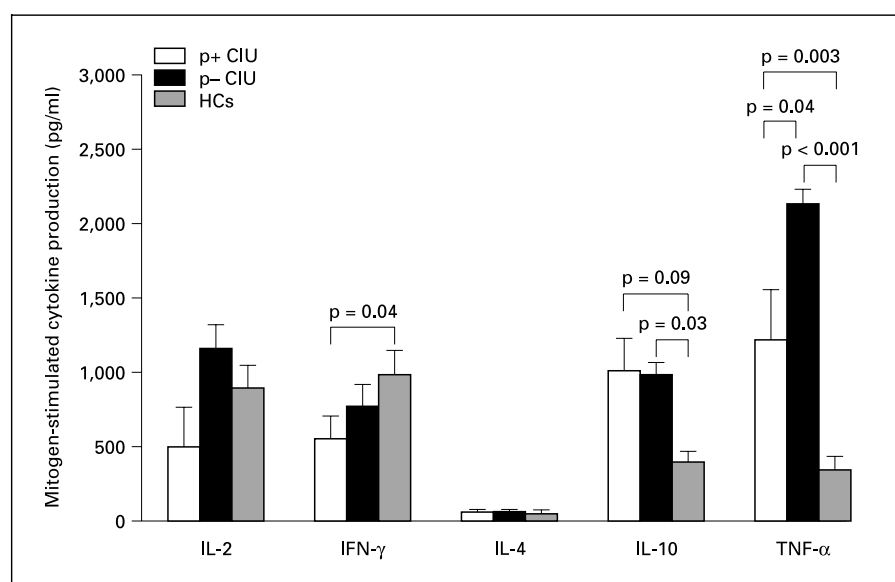
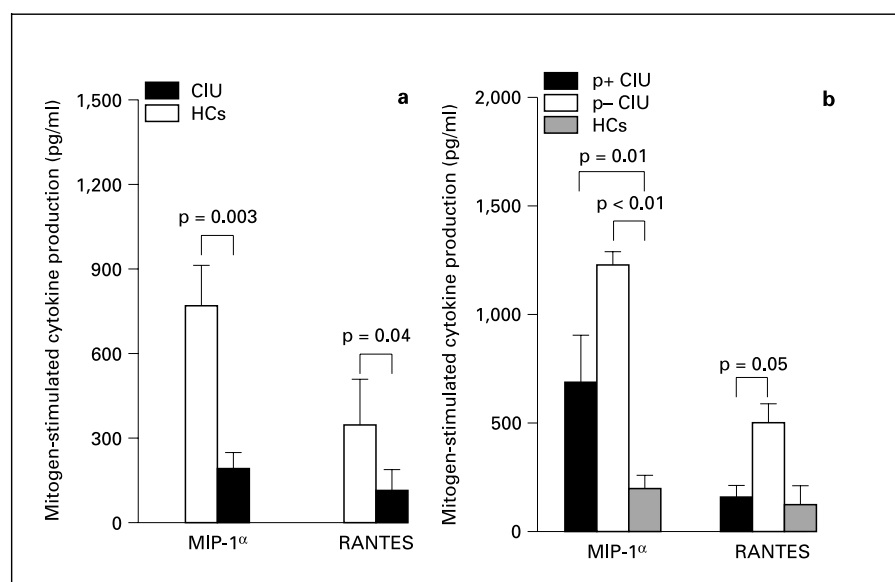


Fig. 4. a Mitogen-stimulated production of MIP-1α and RANTES by PBMCs of 19 CIU patients and 15 HCs. b Mitogen-stimulated production of MIP-1α and RANTES by PBMCs of CIU patients divided in to two groups based on the presence (n = 9) (p+ CIU) or the absence (n = 10) (p- CIU) of a response to the ASST. Values obtained in 15 HCs are also presented. Mean values, standard deviations and statistically significant differences are shown in both a and b.



production of MIP-1α was observed in p- CIU individuals (p+ vs. HCs p = 0.01; p- vs. HCs p < 0.0001) (fig. 4b).

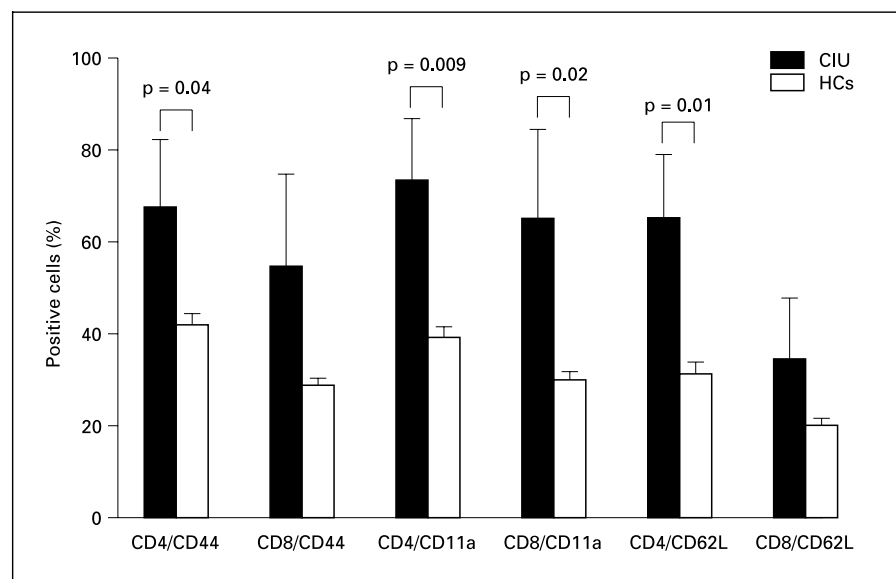
Immunophenotypic Analyses

The expression of all the adhesion molecules investigated (CD44+, Cd11a+, CD62L) was significantly higher in CIU patients compared to controls. In particular, the expression of CD11a – the ligand for ICAM-1 – was augmented in both CD4+ and CD8+ T lymphocytes of CIU individuals (CD4+/CD11a+ p = 0.009; CD8+/CD11a+ p = 0.02). The expression of CD62L, the L-selectin involved in leukocyte rolling, and of CD44, the ligand for

hyaluronic acid involved in cellular extravasation, was also increased in both CD4+ and CD8+ T lymphocytes from CIU patients. These differences reached statistical significance in the CD4+ T cell subpopulation (CD4+/CD62L+ p = 0.01; CD4+/CD44+ p = 0.04) (fig. 5).

No significant differences were observed when the expression of CD25 and DRII was analyzed in CIU patients and HCs (data not shown). Finally, the expression of all the molecules investigated was not significantly different when p+ and p- CIU patients were compared.

Fig. 5. Expression of adhesion molecules on CD4+ and CD8+ T cells of 19 CIU patients and 15 HCs. Mean values, standard deviations and statistically significant differences are shown.



Discussion

We evaluated a panel of immunologic parameters in the attempt to shed some light on the pathogenesis of CIU. The analysis of cytokine production showed that increased concentrations of TNF- α and IL-10 are present when CIU patients are compared to controls, suggesting that an inflammatory process is present in these patients. This suggestion is further reinforced by the observation that the expression of adhesion molecules is also augmented on lymphocytes of CIU patients. Interestingly, IL-4 production was comparable in patients and controls, suggesting that IgE-dependent mechanisms might not be primarily involved in the etiopathogenesis of CIU. The results of our analyses also showed that TNF- α and chemokine production is maximally augmented in CIU individuals with a negative result on the ASST. Thus, an important inflammatory process seems to characterize this subset of CIU individuals; inflammation may be less evident in those patients in whom the result of the ASST is positive.

The finding that TNF- α and chemokine production is maximally augmented in CIU individuals with a negative result on the ASST is puzzling, because ASST-negative patients often have a more severe clinical history, which is confirmed by the result of biooptical examination. This discrepancy could be explained if inflammation in CIU patients was supported by soluble factors that are different from the ones we have examined. Alternatively, it is possible to hypothesize that the serum of ASST-positive

patients contains one or more factors that inhibit PBMCs. The concentration of IL-10 is indeed slightly augmented in ASST p+ patients; we discovered the presence of high concentrations of soluble TNF receptors in ASST p+ individuals without noticing differences between ASST p+ and p- individuals. Additionally, preliminary results showed that antigen-stimulated proliferation and cytokine production of PBMCs from healthy controls is not impaired by the presence of serum of ASST p+ patients. Further experiments will be needed to clarify this issue.

Chronic urticaria is suggested by some authors to be an immediate hypersensitivity phenomenon appearing a few minutes after exposure to the antigen, but the presence of a delayed inflammatory phase is nevertheless observed in this pathology. Soluble factors must therefore be supposed to play a major role in the delayed inflammatory response observed in chronic urticaria [20, 21]. Cytokines have been suggested to be pivotal players in the pathogenesis of this delayed response [22], and amongst these cytokines, TNF- α might play a key role. TNF- α was previously shown to be augmented in serum and bioptic specimens of CIU patients [17, 18]; our data provide evidence that the production of this cytokine is also up-regulated when PBMCs of CIU individuals are analyzed. Because TNF- α stimulates cell-mediated immune responses and is a chemotactic agent for several cell types via the modulation of the expression of adhesion molecules on endothelial cells, leukocytes and mast cells [23], we analyzed the expression of a panel of these molecules in CIU patients. Results show that the expression of CD11a, CD62L and CD44 on

the surface of immune cells of CIU patients is indeed augmented.

ICAM-1 binds $\alpha 2$ -integrins (CD11a) on the surface of circulating immune cells [24] and favors the activation, endothelial adhesion and migration of these cells into inflammatory sites [25, 26]. L-selectin (CD62L) on lymphocytes is fundamental in allowing cell adhesion to the endothelium, cell extravasation and leukocyte activation [27]. This last function seems to be regulated mainly by CD44, which binds hyaluronic acid on endothelium and is involved in the accumulation of primed lymphocytes at inflammatory sites [28]. The increased production of TNF- α as well as the augmented expression of CD62L, CD11a and CD44 observed on lymphocytes of CIU patients thus support an inflammatory pathogenesis of CIU and suggest a possible role for CD4+ T lymphocytes in inducing and/or sustaining the chronic inflammatory process. These data also strengthen previous results obtained in histologic studies [29–32].

Analysis of chemokine production showed that the generation of these factors is also augmented in CIU patients compared to HCs. Chemokines are suggested to have a dual effect on eosinophils. Thus, chemokines have chemotactic properties and provoke histamine release from these cells independently of other factors. Hence, soluble factors – including chemokines – produced during an inflammatory response may sustain and induce mast cell degranulation and the release of histamine even in the absence of cellular recruitment [33]. In this inflammatory scenario, the increase in IL-10 production seen in CIU individuals is not easily understandable, as IL-10 is a known anti-inflammatory cytokine [34]. Other chronic

inflammatory diseases, including rheumatoid arthritis (RA), are characterized by augmented levels of both TNF- α and IL-10. TNF- α is known to be involved in the immunopathogenesis of RA, whereas IL-10 is suggested to be produced in the attempt to hamper the inflammatory milieu. The levels of IL-10 observed in vivo in RA patients are considered to be insufficient to counteract the inflammatory effects of TNF- α ; nevertheless, treatment of patients with exogenous IL-10 results in a decrease in TNF- α production and control over the inflammatory condition [35]. In contrast to the scenario observed in RA, the increased production of IL-10 seen in CIU patients is, at least in p+ CIU individuals, associated with lower TNF- α levels. Thus, increased levels of IL-10 might have a biological role in CIU patients.

In conclusion, our data support an inflammatory etio-pathogenesis of chronic urticaria and show that distinct immune profiles can be recognized within CIU patients. Thus, different patterns of response to the ASST are associated with different degrees of alterations of the biological scenario. These results could at least partially explain the heterogeneous response to therapy observed in CIU patients [33–40] and could be of help to the clinician facing the issue of selecting the adequate therapeutic protocol for chronic urticaria patients.

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