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Total and Biologically Active Serum-Soluble CD154 in Patients with Chronic Idiopathic Urticaria

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ABSTRACT

The pathogenesis of chronic idiopathic urticaria (CIU) is not understood completely; however, autoimmunity has been implicated. Because membrane and soluble forms of CD154 have been reported to be increased, in several autoimmune diseases, we have quantified the soluble CD154 (sCD154) molecule by a sandwich enzyme-linked immunosorbent assay in serum samples of 32 patients with CIU (aged 32 ± 12 years) and compared them with 32 age- and sex-matched nonallergic controls. A marked increase was observed in patients with CIU as compared with controls (4.8 ± 2.6 ng/mL versus. 2.9 ± 0.9 ng/mL; $p < 0.0005$). No significant differences were found between groups of patients with positive or negative autologous serum skin test. A biological assay to determine sCD154 showed that patients with positive autologous serum skin test have the highest levels (4.9 ± 1.2 ng/mL) of biologically active sCD154 as compared with their negative counterparts (2.2 ± 1.3 ng/mL; $p < .001$) and controls (0.6 ± 0.3 ng/mL; $p < 0.001$). Active sCD154 can be derived from mast cell activation or other leukocytes. It is concluded that active sCD154 may be involved in the immune activation observed in patients with CIU. (Allergy and Asthma Proc 25:1–00, 2004)

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Chronic idiopathic urticaria (CIU) is a common form of chronic urticaria, which is characterized by the occurrence of wheals for >6 weeks with no apparent cause.[1] It has been shown that in CIU, mast cells are activated as part of an inflammatory process. Several studies[2,3] have reported the presence of autoantibodies against the Fcε receptor I α-chain (FcεαRI), which are capable of activating basophils and mast cells at least in one subgroup of patients. Moreover, immunoglobulin G (IgG) anti-IgE-activating mast cell also has been reported in patients with chronic urticaria.[4] Even though other serum-associated factors like complement have been involved in mast cell degranulation,[3,5] autoimmunity is still questionable in patients with CIU.

Several hypotheses have been proposed to explain the genesis of autoimmune disorders. Moreover, hyperactive nonapoptotic memory cells against specific or nonspecific autoantigens have been a common characteristic of autoimmune disorders.[6] Certain *cell-to-cell* interactions play important roles in autoimmunity. Among them, the CD40-CD154 interaction have been involved in different key events in innate and acquired immune response.[7,8] In most autoimmune disorders, CD154 is overexpressed in CD4 memory T lymphocytes[7,8] and biologically active soluble CD154 (sCD154) also has been encountered and has been implicated with disease activity of systemic lupus erythematosus (SLE) and rheumatoid arthritis.[9,10] Overexpression of CD154 in the murine epidermis results in skin inflammation and systemic autoimmunity.[11] CD154 blockage with specific antibodies has been shown to be an experimental successful treatment in several diseases, from atherosclerosis to SLE.[12,13]

Toubi *et al.*[14] have shown a significantly higher expression of CD154 in activated T lymphocytes (PMA plus calcium ionomycin) of patients with chronic urticaria as compared with patients with atopic dermatitis or controls. This increased expression parallels an increased amount of bcl-2 in B and T cells of the same patients. Noteworthy, Confino-Cohen *et al.*[15] have shown an aberrant regulation of p21Ras in peripheral blood mononuclear cells in patients with urticaria probably through CD40 activation, as shown by Gulbins *et al.*[16] Because of the importance of the interaction CD40-CD154 in autoimmune diseases, along with the preliminary results of this interaction in CIU, the aim of the study was to detect soluble total and biologically active CD154 (sCD154) levels in CIU and its possible relevance in patients with positive or negative autologous serum skin tests (ASTs).

MATERIALS AND METHODS

Study Subjects

Blood samples from 32 patients (84% were women with a mean age of 32 ± 12 years), from the Allergic Diseases Clinic of the Institute of Immunology, and 32 controls (81% were women aged 31 ± 11 years) were obtained after each patient's written consent and the approval of the Institute Ethical Committee were obtained. The controls did not suffer from any chronic, viral, parasitic, allergic, or genetic diseases. A clinical evaluation and routine laboratory tests, hematology, IgE, prick test (household, plants, animals, food, and molds), and stool analyses were used to rule out allergic or parasitic diseases. Patients with autoimmune diseases, diabetes, or other chronic, viral, or systemic diseases, >60 years old or <16 years old, were excluded. Patients were treated mainly with cetirizine and eventually with loratadine. None of the patients were under steroid therapy (oral or topical). Patients did not receive any antihistamines for 120 hours and no medication was given, even mild anti-inflammatory drugs, at least 72 hours before the blood sample was taken. Even though the half-life of cetirizine is longer than other antihistamine drugs, 85% of the oral dose is eliminated after 48 hours.[17]

Patients were classified according to clinical (duration of wheals and time of urticaria), physical, and paraclinical parameters of urticaria (serum IgE levels and eosinophil count per cubic millimeter). Serum IgE levels were performed by a commercial enzyme-linked immunosorbent assay (ELISA; Binding Site, UK). The severity of urticaria was assessed using the scale employed by Claveau *et al.*[18] The AST was performed as described by Grattan *et al.*[19] and Gruber,[20] with minor modifications. Briefly, 0.05 mL of sterile autologous serum was injected intradermally. The wheal size was determined at 30 minutes. Positiveness was recorded when the wheal diameter was higher than 2 mm from the control (0.05 mL of saline solution). Histamine (1 mg/mL) as the prick test served as a positive control.

TABLE I

Effect of Biologically Active sCD154 in B-Cell Proliferation and Murine Macrophage Nitrite Production

CD154 (ng)	B-Cell Proliferation (cpm)	Nitrite Production (μmole/L)
0	246 ± 148	2.8 ± 1.1
0.1	1505 ± 252	6.9 ± 0.9
1	6260 ± 463	9.7 ± 2.2
10	18007 ± 2594	12.5 ± 2.1

Purified sCD154 was used to stimulate human B lymphocytes in parallel with the murine macrophage cell line as described in the Materials and Methods section. The results of five different assays are represented. A Pearson correlation ($R^2 = 0.59$; $p < 0.01$) was observed. It was concluded that both tests could be used for detecting biologically active sCD154.

ELISA Assay

Serum CD154 levels were assessed using a commercial sandwich ELISA assay (Chemicon International) according to the manufacturer's instructions. Two ELISA plates were run in parallel and the variation coefficient was <5%. It should be mentioned that this ELISA assay was unable to distinguish between biologically active and nonactive forms of CD154.

Biologically Active CD154

Biologically active sCD154 was assessed by a novel assay inducing nitrite production in a murine macrophage cell line RAW 264.7 (American Tissue Culture Collection, Manassas, VA) in similar fashion to that described by Imaizumi *et al.*[21] Briefly, active sCD154 was purified according to Vakkalanka *et al.*,[9] tested with human B cells according to Mazzei *et al.*,[22] and used to stimulate the murine macrophage cell line for 18 hours at 37°C. Nitrite production was considered easier and less time-consuming than B cell proliferation assays (3 days) and B cell differentiation assays (7 days). The correlation between B cell proliferation assays and nitrite production was $R^2 = 0.59$ ($n = 5$; $p < 0.01$; Table I).

Statistical analysis was performed using the Pearson coefficient, unpaired Student's *t*-test, and nonparametric analysis of variance assay. Significance was recorded when $p < 0.05$.

RESULTS

The characteristics of CIU patients were as follows: a long time period with urticaria (24 ± 42 months), duration of wheals after AST was 3.8 ± 3.8 hours, angioedema was observed in 22 (72%) patients, and dermographism was reported in 4 (13%) patients. Positive AST was observed in 18 (56%) patients, who also were positive for

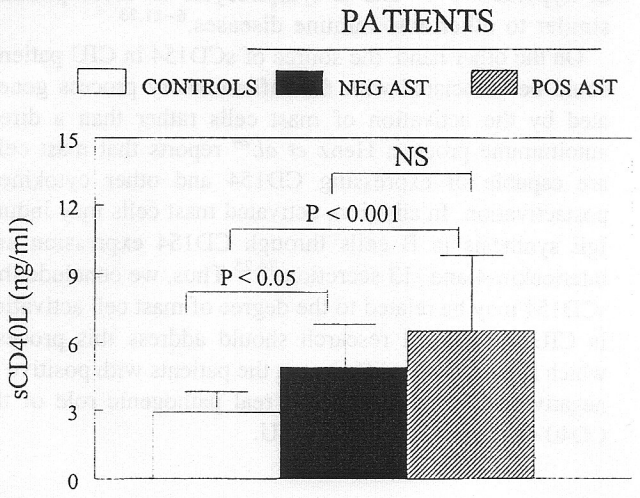

Figure 1. Serum sCD154 levels in controls and patients with negative or positive AST tests. The values obtained by the commercial ELISA assay are represented depending on the positiveness to the test. Significant differences, as compared with controls, are observed in the negative AST group ($p < 0.05$) and in the positive AST test ($p < 0.001$). No difference was observed between positive and negative AST groups.

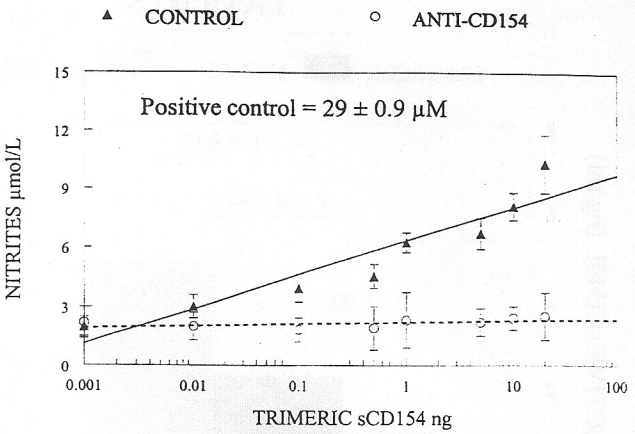

Figure 2. Production of nitrite by the murine macrophage cell line RAW 264.7. The figure represents the specific (subtracting background) effect of trimeric sCD154 on nitrite production in the absence (control, dark triangle) or treated with anti-CD154 (anti-CD154, circle) as described in the Materials and Methods section. Significant differences are recorded ($n = 40$; $p < 0.01$) for values above 1 ng of trimeric sCD154 as compared with anti-CD154. The baseline recorded with anti-CD154 was considered baseline value. The value of positive (1 µg of anti-CD40 plus 10 IU IFN-γ/mL) control is represented.

angioedema (90%). Familial history of autoimmune disorder was similar in both groups (patients, 16%; and controls, 13%).

The levels of IgE were higher than normal (355 ± 75 IU/mL) in 10 patients (31%). The levels of IgE did not have any relationship with positiveness to AST. In addition, the eosinophil count (per cubic millimeter) was not significantly higher than controls (146 ± 132 versus 140 ± 72).

A significant increase in total sCD154 levels was detected in CIU patients as compared with controls (4.8 ± 2.6 versus 2.9 ± 0.9; $p < 0.0005$). Furthermore, patients with positive AST had the highest values of sCD154 as compared with controls ($p < 0.001$; Fig. 1). However, no significant difference ($p = 0.3$) was observed between negative or positive AST groups.

To Ascertain the Amount of Biologically Active CD154, a Novel Biological Assay Was Developed. The production of nitrite by murine macrophages on active sCD154 stimulation was compared with the values obtained by the positive control of 1 µg of anti-CD40 (Serotec Corp., Oxford, UK) plus 10 IU/mL of murine recombinant interferon (rIFN) γ (Peprotech Corp., London, UK) as described by Imaizumi *et al.*[23] Specific sCD154 effect on nitrite production was assessed by precipitating sCD154 with 1 µg of anti-CD154 (~~tartrate-resistant acid phosphatase~~ 1; Beckman-Coulter Corp., FL) plus antimurine IgG-sepharose before the assay; alternatively, 1 µg of CD40-~~γ~~Ig fusion protein (Ancell Corp., Bayport, MN) was used for the same purpose. The results of the CD40 were similar to those of the antibody and were not included in Fig. 2 ($R = 0.9$; $n = 25$; $p < 0.01$). A net-specific (nitrites obtained with CD154 stimulation—background or anti-CD154 treated) significant increase ($n = 40$; $p < 0.01$ for values above 1 ng) in nitrite production was observed on sCD154 stimulation in cell culture (Fig. 2), which was markedly lower than the positive control (29 ± 4.3 µmoles/L; n = 30; $p < 0.0001$; 1 µg of anti-CD40 plus 10 IU/mL murine rIFN-γ). Despite this fact, biologically active sCD154 could be detected as low as 0.1 ng/mL (Fig. 2).

To assess active sCD154 in serum samples, sera were diluted fivefold with RMPI-1640, mixed with 1 ng of purified active sCD154, and added to 96-well plates that contained 1×10^5 macrophages and then incubated for 18 hours at 37°C. The specificity of sCD154 was assessed precipitating with anti-CD154 before the assay, as described previously, and the amount of nitrite detected in the absence of sCD154 was considered the basal value (Fig. 2).

The differences in the amount of biologically active sCD154 are depicted in Fig. 3. Patients with positive AST had the highest levels of biologically active sCD154 as compared with the negative counterparts ($p < 0.001$) and controls ($p < 0.0001$). A significant difference ($p < 0.01$) was observed between patients with positive AST compared with negative AST. There were no correlations between any other parameters measured and the total or biologically active sCD154.

DISCUSSION

Several authors have suggested that CIU should be classified as an autoimmune disorder because it exhibits many autoimmune characteristics.[2,3] In CIU, mast cell ac-

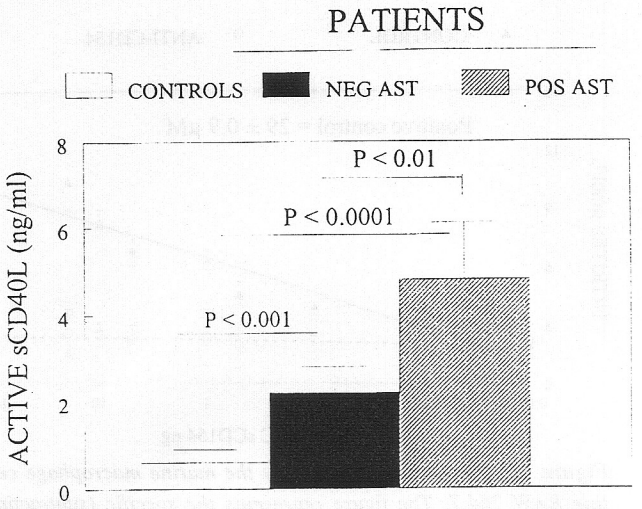

Figure 3. Serum biologically active sCD154 levels in controls and patients with positive or negative AST tests. The specific values obtained using the biological assay, as described in the Materials and Methods section, are represented depending on the positiveness to the test. Significant differences, as compared with controls, are observed in the negative AST group ($p < 0.001$) and in the positive AST test ($p < 0.0001$). The difference among patient groups was $p < 0.01$.

tivation has been associated with autoantibodies against FcεRI or IgE as well as other inflammatory mediators.[2,3] Efforts have been made to ascertain the possibility that leukocytes may be involved in the phenomenon and several studies have reported differences in activation pathways and antigen expression in these cells that support the aforementioned hypothesis.[2–5]

Toubi *et al.*[14] reported an increase expression of CD154 in activated T lymphocytes from patients with chronic urticaria in parallel with an increased proliferative response and bcl-2 expression in urticaria patients. However, no additional effort was made to measure sCD154 in these patients. In this report, high values of sCD154 were observed in patients with CIU as compared with controls, similar to what was reported in several autoimmune diseases.[7–11,23] Nonetheless, few reports have dealt with biologically active sCD154. Vakkalanka *et al.*[9] have shown that in SLE patients, there is an increased amount of circulating biologically active sCD154 as determined by CD95 induction in B-cell lines and a specific ELISA assay. Using a simple assay, we have been able to observe the effects of biologically active sCD154, which was crucial to differentiate between patients with positive or negative AST, which up to date has not been possible using other paraclinical and clinical parameters. Even though this sole criteria describes an increased nonspecific inflammatory response, with no clear definition of sCD154's origin, one might speculate, in concordance with other reports,[14–16] that this soluble molecule may be responsible for the presence of hyperactive T- and B-lymphocytes in these patients similar to other autoimmune diseases.[6–11,23]

On the other hand, the source of sCD154 in CIU patients could be associated with the inflammatory process generated by the activation of mast cells rather than a direct autoimmune process. Henz *et al.*[24] reports that mast cells are capable of expressing CD154 and other cytokines' postactivation. In addition, activated mast cells may induce IgE synthesis in B cells through CD154 expression and interleukin-4 and -13 secretion.[24,25] Thus, we conclude that sCD154 may be related to the degree of mast cell activation in CIU. Additional research should address this process, which is completely different in the patients with positive or negative AST, and define the real pathogenic role of the CD40-CD154 interaction in CIU.

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