

Immunophenotype characteristics of peripheral blood mononuclear leukocytes of Chronic Idiopathic Urticaria patients.

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Key words: Urticaria, cell immunophenotype, memory cells.

Abstract. The pathogenesis of chronic idiopathic urticaria (CIU) is not completely understood although autoimmunity has been proposed. The aim of the study was to assess the expression of different leukocyte antigens, by flow cytometry, assaying total blood of 29 patients with CIU and of 20 sex and age matched controls. Moreover, we assessed soluble CD154 a marker of immune cell activation, predominantly memory T cells. When patients were divided depending on their response to the autologous serum skin test (ASST), three different groups were encountered: group 1 (n = 11): with negative ASST -, group 2 (n = 11): positive ASST (ASST +) with normal lymphocyte counts and group 3 (n = 7): ASST + with low lymphocyte counts (< 1500 cells/mm³). A significant increase in CD19⁺ percentage and not in the absolute count (P < 0.05) was observed in group 1 as compared to controls and to the other groups. In contrast, CD30⁺, CD45RO⁺ and CD4⁺/CD45RO⁺ percentages and biologically active soluble CD154 levels were significantly higher (P < 0.05) in group 3 as compared to group 1 or to controls. In ASST positive groups, CD45RO⁺ and CD4⁺/CD45RO⁺ positiveness correlates with wheal diameter. In conclusion, memory cells may play a role in these different types of patients and in understanding CIU pathogenesis.

Características inmunofenotípicas de leucocitos mononucleares de sangre periférica de pacientes con Urticaria Crónica Idiopática.

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Palabras clave: Urticaria, inmunofenotipaje, células de memoria.

Resumen. La patogénesis de la urticaria crónica idiopática (CIU) no se conoce completamente; sin embargo, la autoinmunidad juega un papel importante en un subgrupo de pacientes. El objetivo de este estudio fue la determinación de antígenos leucocitarios en sangre total, utilizando citometría de flujo, de 29 pacientes con CIU y 20 controles de similar edad y sexo. Adicionalmente, se determinó el CD154 soluble como marcador de activación de células inmunes, predominantemente linfocitos T de memoria. Se describieron 3 grupos de pacientes de acuerdo al resultado de la prueba de suero antólogo (ASST): grupo 1: negativo (n = 11), grupo 2: prueba positiva y contaje linfocitario normal (n = 11) y grupo 3 prueba positiva y contaje linfocitario bajo (< 1500 células/mm³) (n = 7). En el grupo 1, se observó un aumento significativo (P < 0,05) en el porcentaje de células CD19+ aunque no en su número absoluto cuando se comparó con los controles y los pacientes con ASST +. En contrapartida, el porcentaje de células positivas para CD30, CD45RO y CD4/CD45RO y los niveles de CD154 soluble biológicamente activo fueron significativamente mayores (P < 0,05) en el grupo 3, en comparación con los controles y el grupo 1. Además, en los grupos con ASST positiva, los porcentajes de células CD45RO+ y CD4/CD45RO+ se correlacionan con el tamaño del habón a la ASST. En conclusión, las células de memoria pudieran jugar un papel importante en la patogénesis de la CIU.

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INTRODUCTION

Chronic idiopathic urticaria (CIU) is characterized by the occurrence of wheals for more than 6 weeks without an apparent cause (1-2). It has been demonstrated that in CIU, mast cells are activated as part of an inflammatory phenomenon. Several studies (3-8) have reported the presence of autoantibodies against the chain of the Fc receptor I which are capable of activating basophils and mast cells at least in one subgroup of patients. Autoantibodies anti-Fc RI without histamine releasing activity, have been found in the serum of patients

with probably non-allergic asthma and some autoimmune diseases (3-8). Furthermore, IgG anti IgE has been reported in different diseases (CIU, Atopic Dermatitis, Asthma, Rheumatoid Arthritis, Lupus Erythematosus, Systemic Sclerosis) and eventually, in normal individuals (3-8), but only in CIU they are capable of activating basophil and mast cells. Even though other serum-associated factors like complement have been involved in mast cell degranulation (1, 3, 4, 8), autoimmunity is still under investigation in patients with CIU.

Several hypotheses have been proposed to explain the genesis of autoimmune disorder-

ders (9). Hyperactive non apoptotic memory cells against specific or non-specific auto antigens have been a common characteristic of autoimmune disorders (9). In CIU, anti thyroid antibodies as well as other autoimmune markers have been reported (3-4).

In CIU, only few reports have analyzed peripheral blood leukocytes due to the fact that most authors restrict their research to skin and to antibody responses to damaged tissue (1-8). Some reports have dealt with the activation of lymphocytes either by the expression of certain antigens in T lymphocytes, as CD154, along with an increased expression of bcl-2 in B cells (10) or by the evidence of an aberrant regulation of p21Ras in peripheral blood mononuclear cells in patients with chronic urticaria (11). These reports suggest an abnormal activation of lymphocytes; however, none of these studies have analyzed the presence of memory cells in patients with CIU and the relationship with positive or negative autologous serum skin test.

Previously, our group described an increased amount of total and biologically active soluble CD154 in the sera of CIU patients (12). This molecule, a member of the TNF family, is present mostly in CD3⁺/CD4⁺/CD45RO⁺ cells and activated mast cells and may be released as a soluble active or inactive form (13). In sera of patients with Lupus erithematosus, a model of autoimmune disease, this soluble active molecule has been shown to be increased as compared to controls (14).

The aim of the study was to characterize the subpopulation of mononuclear cells as well as to assess the expression of some markers of cell activation as CD25, CD30 and CD45RO (memory cells) and to compare lymphocyte populations between patients with positive or negative serum autologous test. Additionally, we assessed the levels of soluble active CD154 to ascer-

tain possible memory T cell activation in CIU patients.

PATIENTS AND METHODS

Blood samples from 29 patients (86% female, age 33 ± 12 years), from the outpatient clinic of the Institute of Immunology, Central University of Venezuela, and 20 controls (85% female, age 34 ± 10 years) were obtained after each patient's written consent and the approval of the Local Ethical Committee. The controls did not suffer from any chronic, viral, parasitic or genetic disease. Patients with autoimmune diseases, diabetes or another chronic, viral or systemic disease, older than 60 years or younger than 16 years were excluded. Patients did not receive any medication at least 72 h before the blood sample was taken and steroid treatment was suspended one month prior to the study.

Patients were classified according with clinical (duration of wheals, time of urticaria), and paraclinical parameters (serum IgE levels and eosinophil count per mm³). Serum IgE levels were determined using a commercial ELISA assay (Binding Site, UK). The severity of urticaria was assessed using the scale employed by Claveau *et al.* (15), which evaluated three parameters (score from 0 to 3): number of wheals, sleep interruption or alteration of daily activity and intensity of the pruritus. All patients were hepatitis B and C negative and HIV negative.

The autologous serum skin test (ASST) was performed as described by Grattan *et al.* (16) and Gruber *et al.* (17) with minor modifications reported by Sabroe *et al.* (18). Briefly, 0.05 mL of sterile autologous serum was injected intradermally (volar area of the forearm). The wheal size was determined at 30 min as previously suggested (18) because minimal differences were observed as compared with 60 min. Positive-

ness was recorded when the wheal diameter was greater than 2 mm from the control (0.05 mL saline solution). Histamine by prick (1 mg/mL) served as a positive control to determine the reactivity of patients who received antihistamine treatment three or more days prior to the ASST test.

Immunophenotype analysis was performed with a set of double labeled cytoStat® antibodies purchased from Beckman-Coulter (Hialeah, FL, USA). One hundred μL of whole blood were labeled at room temperature with 20 μL of one of the following set of antibodies: CD3-FITC/CD4-RD, CD3-FITC/CD8-RD, CD19-FITC/CD14-RD, CD45RO-RD/CD4-FITC, CD30-RD/CD3-FITC, CD3-FITC/CD19-RD, CD25-FITC, CD16-FITC/CD3-RD and CD45RO-RD. The samples were automatically processed in a Q-prep® workstation (Coulter Corporation, Hialeah, FL) and analyzed in an EPICS ELITE flow cytometry (Coulter Corporation, Hialeah, FL) previously calibrated with fluorescent beads and with the compensation control. Samples were analyzed using electronic maps in the lymphocyte population and 5000 cells were counted for each test. Another electronic map was performed to assess monocytes. The values were either expressed in percentage of positiveness or in absolute numbers (mm^3) in relationship to the hematological counts. Hematological analysis was performed in a MicroDif 18 hematological counter (Coulter Corporation, Hialeah, FL) formerly rectified with the controls purchased from the manufacturer.

Leukopenia was not observed in CIU patients; however, lymphopenia was observed in some patients and it was defined as absolute count of lymphocytes lower than 1500 cells/ mm^3 . Three group of patients were defined according to ASST results and lymphocyte counts: group 1 (n = 11): patients with negative ASST, group 2 (n = 11) patients with positive ASST and normal lymphocyte values and group 3 (n = 7) pa-

tients with positive ASST and lymphopenic values.

Total soluble CD154 was assessed by a commercial kit (Chemicon, UK) following manufacturers instructions. Soluble biologically active CD154 was assessed by stimulating nitrite production in a murine macrophage cell line *in vitro* as described previously (12).

Statistical analysis was performed using unpaired Student's t test, ordinary one way ANOVA and Fisher's exact test. Significance was recorded when $P < 0.05$.

RESULTS

The general characteristics of the patients were as follows: long time period with urticaria (24 ± 42 months), duration of wheals: 3.8 ± 3.8 hr; angioedema was observed in 22 (79%) of the patients and dermographism was reported in 9 (31%) of the patients. Positive ASST was observed in 18 (62%) of the patients, similar to other studies (18-19) performed and 90% of them were also positive for angioedema. Angioedema was observed in 6 (54.5%) of the patients with negative ASST. There was no significant difference in reported familiar history of autoimmune diseases between patients and controls (patients 14% and controls 10%).

IgE levels were higher than normal (> 180 IU/mL) in 10 patients (34%) and it did not have any relationship with positiveness to ASST but it was related with mild respiratory allergy (asthma or rhinitis). In addition, the eosinophil count (per mm^3) was not significantly higher than in controls (146 ± 132 vs 140 ± 72).

There were no major differences in absolute values of monocyte and lymphocytes subpopulations between patients and controls (Table I). However, whole lymphocyte CD45RO⁺ percent expression was higher in CIU patients as compared to controls

TABLE I
ABSOLUTE NUMBER (Cell/mm³) OF LEUKOCYTE POPULATIONS AND SUBPOPULATIONS

	Controls	CIU Patients	P value
Total Leukocytes	7338 ± 2027	7655 ± 3016	0.66
Monocytes CD14 ⁺	486 ± 262	468 ± 321	0.84
Total Lymphocytes	2045 ± 575	1855 ± 597	0.53
CD3 ⁺	1415 ± 403	1310 ± 422	0.36
CD4 ⁺ /CD3 ⁺	915 ± 285	839 ± 280	0.36
CD8 ⁺ /CD3 ⁺	454 ± 157	439 ± 154	0.74
CD16 ⁺ /CD3 ⁺	216 ± 133	170 ± 91	0.22
CD19 ⁺	265 ± 119	285 ± 162	0.65
CD25 ⁺	49 ± 46	49 ± 37	0.98
CD30 ⁺	17 ± 22	13 ± 22	0.55
CD45RO ⁺	774 ± 285	830 ± 296	0.50
CD4 ⁺ /CD45RO ⁺	598 ± 298	628 ± 234	0.86

The table illustrates the cell populations and subpopulations from controls and CIU patients using total blood. The values were calculated using the total lymphocyte population for the absolute numbers of T, B and NK cells and the total leukocyte population for the absolute number of monocytes. The values are expressed as mean ± standard deviation. The P values reflect the statistical analysis performed using unpaired Student's t test.

(46.4 ± 8.8 vs. 40 ± 8.3%, P = 0.01). When absolute values were calculated, the effect was no longer observed due to a decrease, although not significant, in total lymphocyte numbers.

When patients were divided according to the autologous skin serum test, the percentage of CD19⁺ cells was significantly higher (P < 0.05) in patients with negative ASST as compared to those with positive ASST and to controls. Moreover, total absolute number of lymphocyte were lower (2031 ± 425 vs 1633 ± 495 cells per mm³, P = 0.03) in the positive ASST group despite similar amount of leukocytes (7200 ± 2000 vs. 7350 ± 2200 cells/mm³). Thus, absolute numbers of lymphocytes could not be used to calculate the amount of positive cells to the different antigens analyzed.

When the group of ASST + patients was separated according to lymphocyte

counts (> 1500 lymphocytes/mm³, group 2, and < 1500 lymphocytes/mm³, group 3), certain specific differences were observed as compared to the negative ASST and control groups (Table II). Total leukocyte counts, although in the normal range, were significantly lower in group 3 as compared to group 2 (6220 ± 1200 vs. 7800 ± 1700 cells/mm³, P < 0.05). The percentage expression of CD30⁺, CD45RO⁺ and CD4⁺/CD45RO⁺ positiveness, were significantly higher (P < 0.05) in group 3 as compared with the other groups and controls. Nevertheless, CD3⁺, CD3⁺/CD4⁺, CD3⁺/CD8⁺, CD3⁺/CD16⁺ and CD25⁺ were not different between group 1, 2 and 3.

The highest levels of biologically active sCD154 were observed in group 3 (6.4 ± 1.9 ng/mL) as compared to group 2 (4.1 ± 1.2 ng/mL), group 1 (1.9 ± 1.3 ng/mL), and controls (0.4 ± 0.3 ng/mL P < 0.0001).

TABLE II
LYMPHOCYTE POPULATIONS IN CIU PATIENTS

	ASST Negative		ASST Positive	
	Group 1 (n = 11)	Group 2 (n = 11)	Group 3 (n = 7)	ANOVA P value
Lymphocytes (cell/mm ³)	2031 ± 425	1940 ± 390	1200 ± 220	< 0.0001
CD3 ⁺ (%)	69.3 ± 6.2	67.6 ± 6.9	71.4 ± 7.9	0.53
CD4 ⁺ /CD3 ⁺ (%)	43.9 ± 4.9	41.8 ± 6.5	44.9 ± 6.2	0.53
CD8 ⁺ /CD3 ⁺ (%)	24.2 ± 4.3	24.0 ± 2.4	25.9 ± 5.1	0.56
CD16 ⁺ /CD3 ⁺ (%)	9.4 ± 3.0	10.9 ± 5.3	9.4 ± 5.8	0.72
CD19 ⁺ (%)	17.4 ± 3.7*	12.8 ± 4.1	14.3 ± 5.3	0.04
CD25 ⁺ (%)	3.0 ± 1.6	2.6 ± 2.3	4.0 ± 3.0	0.42
CD30 ⁺ (%)	0.6 ± 0.8	0.3 ± 0.3	1.2 ± 0.8*	0.03
CD45RO ⁺ (%)	39.8 ± 6.8	39.3 ± 6.0	46.5 ± 5.6*	0.04
CD4 ⁺ /CD45RO ⁺ (%)	30.8 ± 10.5	24.6 ± 5.3	34.8 ± 6.5*	0.04

The table illustrates the percentage expression of the different antigens in CIU patients: group 1: negative ASST (with normal lymphocyte counts), group 2 patients with positive ASST with normal lymphocyte values and group 3 patients with positive ASST with low (< 1500 mm³) lymphocyte values. The values are expressed as mean ± standard deviation. The P values reflect the statistical analysis performed using an ordinary ANOVA analysis. * As compared to controls P < 0.05 (Student's t-test).

Positive correlations were observed when biologically active sCD154 values were compared to CD4/CD45RO⁺ cell absolute numbers ($R^2 = 0.7$, $P = 0.005$) and to wheal diameter ($R^2 = 0.6$, $P = 0.01$).

When percentages of positiveness recorded for CD45RO⁺ and CD4⁺/CD45RO⁺ antigens, were compared to wheal size induced by serum in both ASST + groups, a significant correlation for each antigen was observed as shown in Fig. 1 ($r = 0.50$, $P = 0.04$ and $r = 0.65$, $P = 0.006$, respectively).

The general characteristic urticaria of the three groups is illustrated in Table III. Significant differences were observed in the time course of urticaria, which was markedly lower in group 2 as compared to other groups. Wheal size was also significantly lower ($P < 0.05$) in group 2 as compared to group 3. Interestingly, group 2 had the lowest, but non significant, frequency of

dermographism. No differences in IgE levels were recorded among these three groups.

DISCUSSION

CIU is one of the mast cell activation diseases in which autoimmune characteristics have been associated in a subgroup of patients, lymphocytes, cytokines and adhesion molecules have been involved in the pathogenesis (1-8). Oehling *et al.* (20) reported no differences in lymphocyte subpopulations among patients with chronic urticaria and controls. However, two studies have reported differences in lymphocyte activation pathways and antigen expression in chronic urticaria patients (10-11).

We could not demonstrate any differences in absolute values of monocytes and lymphocyte subpopulations between patients and controls. Nevertheless, the percentage of memory cells was significantly

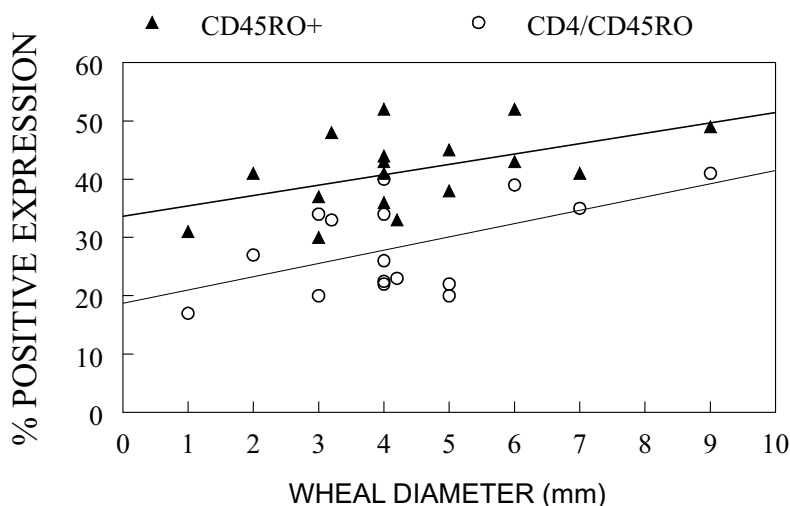


Fig. 1. The figure illustrates the correlation between the percentage of positiveness for CD45RO and CD4/CD45RO with wheal diameter observed in the group of patients with positive ASST (n = 18). A significant correlation was found with both markers (r = -0.50, P = 0.04 for CD45RO dark filled triangles and r = -0.65, P = 0.006 CD4/CD45RO, open circles).

TABLE III
GENERAL CHARACTERISTICS OF URTICARIA

	Group 1 (n = 11)	Group 2 (n = 11)	Group 3 (n = 7)	ANOVA P value
Evolution (months)	20.8 ± 41.5	5.3 ± 3.5	36.3 ± 52.7	0.04
Wheal Size ASST (mm)	-	3.6 ± 1.2	5.6 ± 2.0	0.02
Wheal duration (h)	2.9 ± 3.6	3.7 ± 2.5	4.5 ± 4.7	0.5
Urticaria severity score	3.5 ± 2.1	3.3 ± 1.6	4.0 ± 2.2	0.8
Dermographism (%)	5/11 (45.5%)	2/11 (18%)	2/7 (28.5%)	0.1
Angioedema (%)	6/11 (54.5%)	10/11 (91%)	6/7 (86%)	0.2

Table III depicts the general characteristics of CIU patients when they were divided by groups in accordance with results of ASST and absolute number of lymphocytes. Significant differences were observed in the time period of urticaria and wheal size (P < 0.05). No significant differences were observed in the other characteristics analyzed.

increased in CIU patients. When patients were divided by their response to ASST and lymphocyte counts, clear differences were observed between groups. Essentially, in the group in which the test was positive along with a decreased number of lymphocytes, there was an increase in the percentage of CD45RO+, CD3+/CD4+/CD45RO+ and CD30+ cells. One could propose that the low lymphocyte count, in group 3, suggests a redistribution of leukocytes to the

skin. The increased amount of memory cells may indicate an immune response to a recall, but yet unknown, antigen in these patients. In accordance to this hypothesis, previous studies refer CD3+/CD4+/HLADR+ cell infiltration in the lesions of patients with CIU (21-22). In addition, patients in group 3 had longer time with the disease and an increased wheal size in the ASST assay. These results suggest that despite the fact that patients of group 2 and 3

have positive ASST, they are two distinct groups with different clinical and laboratory characteristics which can not be attributed to atopy and neither to a possible direct pathogenic role of the anti-IgE or antiFc RI. Cell analysis in the skin may not record these differences.

No differences were observed with CD25 expression in contrast to CD30 in the three groups probably due to the fact that CD25 positiveness does not permit to distinguish activated cells from regulatory cells. In accordance with the proposed hypothesis, along with the increased expression of CD30, CD25 positiveness, in group 3, should represent activated memory cells.

Recently, we analyzed total soluble and biologically active serum CD154 (sCD154) levels in CIU patients (12). Since CD154 is expressed and released mostly in activated CD3+CD4+CD45RO+ cell population and activated mast cells (13) it was not surprising to find a be markedly increased amount of soluble total and biologically active CD154 in the sera of CIU patients as compared to controls (12). Thus, the marked difference in the levels of the biologically active molecule of group 3 as compared to groups 2, 1 and controls suggest a chronic memory T cell activation which correlates with ASST positiveness and wheal diameter induced by serum. Memory cell activation and redistribution may be increased in chronic ASST positive patients.

The results reported are in accordance to those of Toubi *et al.* (10) and Cofino-Cohen *et al.* (11) and support the proposed the hypothesis of patient heterogeneity and memory cell redistribution in these patients. However, future studies should ascertain the importance of memory cells in CIU along with autoantibodies against Fc receptor I and the possible effect of other overlaying conditions in the pathogenesis of CIU which may occlude the real characteristics of this clinical entity.

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