

Immune Aberrations in B and T Lymphocytes Derived from Chronic Urticaria Patients

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To investigate the pathophysiology of chronic urticaria (CU) in light of the abundant evidences that it is an autoimmune disease and to define some cellular markers in B/T lymphocytes that could be of pathogenic significance, we investigated 14 patients suffering from CU, compared to 7 contact dermatitis patients and 10 normal control individuals. We tested the expression of CD5, B7.1 (CD80), CD23, and CD25 on B cells and of CD40L and CD25 on T cells from all studied individuals. We also tested B cell proliferation upon their activation followed by dexamethazone induced inhibition of proliferation. The expression of bcl-2 protein in activated lymphocytes from normal individuals was compared to that of contact dermatitis and CU patients. CD40L expression was found significantly higher on *in vitro* activated CD3 [with phorbol myristate acetate (PMA) and ionomycine Ca²⁺ at 12 hr of culture] from CU patients compared to that of contact dermatitis and normal individuals. Whereas the proliferation of activated B cells from CU patients was higher, dexamethazone-induced inhibition of B cell proliferation was found statistically similar in both groups yet lower in B cells from most severe CU patients. We demonstrate a higher bcl-2 expression in activated B and T lymphocytes of severe CU patients compared to that of moderate CU and both contact dermatitis and normal individuals. The increased expression of CD40L on activated T cells might play a role in the polyclonal increased B cell proliferation of CU patients. This increased proliferation accompanies the finding that activated B and T lymphocytes from these patients demonstrate increased bcl-2 expression. The resistance of some B cells to dexamethazone-induced inhibition of proliferation encourages us to investigate the possibility that B cells from some CU patients might develop rescue mechanisms from activated cell death. These findings provide further evidence that CU is indeed an autoimmune disease, although its precise nature has yet to be fully elucidated.

KEY WORDS: Chronic urticaria; contact dermatitis; autoimmunity; bcl-2; proliferation.

INTRODUCTION

During the last decade our understanding of chronic urticaria (CU) has changed dramatically. More than half of CU cases previously regarded idiopathic subsequently have been identified as being autoimmune in origin. This finding is strongly supported by autoimmune thyroiditis, systemic lupus erythematosus (SLE), celiac disease, and others (1–3). Of most importance is the existence of autoantibodies (auto-Abs) against both IgE and FcεRIα on cutaneous mast cells. These are of particular interest since they have been shown to be capable of directly causing mast cell degranulation (4). The role of anti-IgE auto-Abs in the pathogenesis of CU is not clear. It is found in only 10% of all CU patients and also can be found in sera of patients suffering from atopic dermatitis, bronchial asthma, and even in normal individuals (5).

However anti-FcεRIα auto-Abs occur in a much higher significant percentage of CU patients, and this finding is probably more relevant to the pathogenesis of this entity. The incidence of autoimmunity to the IgE receptor in CU patients was studied in detail by Kaplans (6) group. Using Western immunoblotting (IB) technique, they documented functional anti-FcεRIα auto-Abs (capable of activating basophils or mast cells) in 60% of CU studied patient sera (6). In a later study they isolated skin mast cells and tested a large number of patient sera for their ability to activate these cells and isolated basophils (6–8). In addition they compared the results with those obtained by IB, using cloned alpha subunit of the IgE receptor. Sera from patients with chronic urticaria released significant amount of histamine (>15% of basal) on incubation with basophils (48%) and mast cells (46%). IB revealed positive results in 64% of tested individuals (7). Autologous serum test (AST), positive in 30–40% of CU patients, was shown

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to be attributed to the presence of mainly anti-Fc ϵ RI α auto-Abs but also IgG anti-IgE and other unidentified serum factors (4, 8). It appears likely therefore that many cases of CU ultimately will be considered an autoimmune disease rather than an allergic disease, and therefore should be called autoimmune chronic urticaria (ACU).

However, ACU differs from other known autoimmune diseases. It is a transient disorder (even when long lasting), and thus is intriguing from both a clinical and scientific prospective. Indeed, ACU has prompted many investigators to study and examine this still poorly understood phenomenon.

Polyclonal B-cell hyperreactivity and/or dysregulation of T-cell functions, extra- or intracellular signal(s) transduction defects could all play an important role in triggering and maintaining this autoimmune entity. This report is the first study that shows that B and T lymphocytes derived from active CU patients demonstrate an increased expression of various active/proliferation markers, and therefore might be prone to autoimmune responses. Our results further support the widespread believe that most cases of CU patients should be regarded as suffering from an autoimmune disorder and treated as such.

METHODS

Our study included 14 CU patients (12 women and 2 men), age range 28 to 52 years; 7 contact dermatitis patients (5 women and 2 men, age range 24 to 34) served as disease control group and 10 age- and sex-matched healthy individuals (served as the control group). All patients suffered from chronic urticaria with a disease duration lasting from 1 to 6 years. They were treated with antihistamines almost continuously and repetitive courses of prednisone (20–30 mg/day each 6–8 weeks). In both CU and contact dermatitis patients, steroids were stopped 4–6 weeks prior to analysis. The urticaria was considered to be of idiopathic character following a detailed investigation, without any positive results. This investigation included skin tests to food allergens, controlled elimination diet, a workup for auto-Abs against the thyroid and other autoantigens, and different infectious diseases. At this stage all individuals underwent AST and were studied for B- and T-lymphocyte activation state, their sensitivity to dexamethasone-induced inhibition of proliferation, and bcl-2 expression.

Table I. Patient's and Investigator's Rating Scale of Symptom Severity

Daily events	Points
No. of lesions	
0	0
10	1
11–20	2
>20	3
No. of separate episodes	
0	0
1	1
2 or 3	2
>3	3
Average size of lesion (inches)	
0	0
<1/2	1
1/2–1	2
>1	3
Average duration of lesions (hr)	
None	0
Up to 4	1
>4–12	2
>12	3
Pruritus	
None	0
Mild	1
Moderate	2
Severe	3

Urticaria Score

Patients recorded their urticaria symptoms daily, rated on a 4-point scale, as detailed previously by Breneman *et al.* (9): number of lesions, number of separate episodes, average size of lesions, average duration of lesions, and pruritus. Table I details the scoring system: 0 = no symptoms; 1 = mild urticaria (1–4 points); 2 = moderate urticaria (5–9 points); 3 = severe urticaria (≥ 10 points). On weekly follow-up visits, physicians used the same rating scale.

Autologus Serum Test (AST)

AST was performed in all studied individuals (in patients after antihistamine washout) as previously described (10), with minor modifications. Briefly, 0.1 cc of sterile autologus serum was injected intradermally. The wheal and flare size was determined at 30 min and followed for a total of 60 min. Saline was used as a negative control and histamine (1 mg/ml) served as a positive control. The flare/wheal diameter was measured and scored from 0 to +4 as follows: 0 = negative control; +1 = flare ≤ 15 mm, wheal = negative control; +2 = flare > 15 mm, wheal = 3–5 mm; +3 = wheal 6–10 mm; +4 = wheal = 10 mm with the presence of many pseudopods.

B- or T-Cell Separation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient separation. B lymphocytes were positively selected with magnetic microbeads conjugated to antihuman CD₂₂ (MAC's System, Melteni Biotech) according to the manufacturers instructions. Following isolation, CD₁₉-positive cells comprised 95% of the resulting population. The non-B population consisted of 80% CD₃-positive cells (enriched T cells), which were used for T-cell activation and marker studies.

B-Cell Activation

Purified B cells were cultured in RPMI-1640 supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin in 96- or 24-well plates (Corning Glass Works, Corning, NY). Cells were incubated in final concentration of 5×10^4 cells/well (96-well plates) or 1×10^6 cells/well (24-well plates) and activated during 3 days with CD_{40L} 1 μ g/ml, plus anti-IgM F(ab)₂ 1 μ g/ml (Dako, Glostrup-Denmark, Pharmaceutical Inc., OH) and anti-IL-4 10 U/ml (Genzyme Corporation, Cambridge, MA). Resting and activated B cells were assayed for the following: (a) extracellular markers: CD₅, CD₂₃, CD₂₅, CD₃₈, and CD₈₀; (b) intracellular markers: the expression of bcl-2 molecules was detected by immunoblotting; and (c) the proliferation of B cells and dexamethazone-induced inhibition of proliferation was analyzed by [³H]thymidine uptake.

T-Cell Activation

Enriched T cells were activated using two protocols: (a) in 24-well plates in final concentration of 1×10^6 cells/well and activated for 3 days with ConA 80 μ l/well; and (b) in 24-well plates in final concentration of 1×10^6 cells/well and activated with phorbol 12-myristate 13-acetate (PMA) 10 ng/ml and Ca ionomycin 500 ng/ml for 16 hr (Sigma, St. Louis, Missouri). Resting and activated T cells were assayed for the following: (a) CD_{40L} expression (PMA and Ca ionomycin-activated T cells), detected by fluorescence-activated cell sorter (FACS) scan; and (b) the expression of bcl-2 molecules in ConA-activated T cells by immunoblotting.

Flow Cytometry

Purified B cells and enriched T cells were immunostained directly (one step) with PE or/and FITC-conjugated monoclonal antibody (mAb) (Ancell, Bay-

portum MN). Flow cytometry was carried out with a FACS operating with Cellquest software (FACScan-Beckton Dickinson, Mountain View, CA). The total population of viable cells was gated according to their typical forward and right-angle light scatter. The fluorescence of cells treated with fluorescent isotype controls was evaluated in each experiment to determine the level of background fluorescence of negative cells. The percentage of stained cells was determined for each antibody taking into account only the positive cells.

Proliferation Assay

Purified B cells $5-10^4$ cells/well were incubated with 0.1 ml of culture medium in 96-well plated in triplicate and activated (CD_{40L}; anti-IgM F(ab)₂, anti-IL-4) during 72 hr with or without dexamethazone 10 ng/ml. The cells were labeled with 1 μ Ci of [³H]thymidine (Amersham, Buckinghamshire, UK) for the last 16 hr of the culture. Cultured cells were harvested and the radioactivity incorporated into DNA was determined using ready filter with Xtalscuit (Beckman Instruments, Palo Alto, CA).

Dexametasone Assay

Activated B cells were incubated from day 0 with dexamethasone 10 ng/ml for 72 hr [as described in Andreau *et al.* (11)] and then assayed for proliferation. The inhibition of proliferation (cell death) was assayed by [³H]thymidine incorporation assay.

Western Immunoblotting

Following B- and/or T-cell separation and incubation with or without activation, cells were washed with PBS and lysed in SDS-lysis buffer as described in Towbin *et al.* (12). Equal amounts of proteins (quantitated following cell lysis using a paper-dye staining method) were separated on 10% SDS-PAGE, electroblotted to nitrocellulose membrane, which thereafter was blocked with 3% skim milk and incubated with anti-bcl-2 (Ancell, Bayport, MN) overnight at room temperature. Visualization of bound proteins was performed following incubation with a second human anti-IgG-conjugated-alkaline phosphatase and a reaction stain (according to the manufacture recommendation). In order to determine the molecular length of the bound proteins, known molecular weight proteins (purchased from Sigma, St. Louis, Missouri) were cotreated and corun in two lanes in each PAGE.

Western Blot Analysis

The immunoblot bands were scanned using a Mustek 1200 ED scanner device (Hamburg-Germany) at an optical resolution of 2832×1864 pixels. The obtained image thereafter was analyzed by a software (Wscanaray-3 Galai Corporation, Migdal Ha'emek, Israel) for image processing. Briefly, the bands were outlined and an appropriate threshold was established. The average optical density of each band pixels was automatically measured. Density values range from 0 to 255 (the value of 0 represents the lightest pixel while the value of 255 represents the most hyperchromatic one).

Statistical Analysis

Western Blot Band Analysis. The comparison of continuous variables between urticaria, dermatitis, and control group was performed using the one-way analyses of variants followed by the Bonferroni post hoc test. Two-tailed *P* values of 0.05 or less were considered to be statistically significant.

FACSscan Analysis. The comparison between urticaria, dermatitis, and control group was performed using the students *t*-test.

Proliferation Assay. The comparison between urticaria and control group was performed using the students *t*-test.

RESULTS

Patients Characteristics

Whereas all normal and contact dermatitis individuals were AST negative, 5/14 (36%) CU patients were AST positive. All CU patients scored between moderate to severe urticaria, namely, six scored moderate and the remaining eight suffered from severe urticaria.

Extracellular Markers

B Cells. The expression of B-cell markers was assessed on isolated B lymphocytes derived from eight normal individuals in comparison to five contact dermatitis and eight CU patients (four moderately and four severely active). Using double staining we evaluated the expression of CD₅, CD₂₃, CD₂₅, and CD₈₀ on positive CD₁₉ lymphocytes and found them similarly expressed on resting and activated B lymphocytes from both normal and CU patients (data not shown). Since positive isolation of cells with microbeads is known to cause mild activation of cells, we compared B-lymphocytes activa-

tion pre- and postmicrobeads isolation. The obtained data did not show an elevation in any of the above studied markers. Thus, positive selection with microbeads does not contribute significantly to cell activation.

T Cells. Baseline expression of CD_{40L} was similar on CD₃ cells derived from either CU or contact dermatitis patients or normal individuals (10–15% of cells, data not shown). However, when T cells were activated *in vitro* (PMA 10 ng/ml + ionomycin Ca²⁺ 500 ng/ml during 12 hr), CD_{40L} expression increased significantly on CD₃ cells derived from CU patients in comparison to those derived from contact dermatitis or normal individuals (see Fig. 1). These findings were observed on activated T cells in six out of ten studied CU patients (one moderate, AST-negative CU and five with severe CU, two of whom were AST positive). The other four CU patients (all moderate AST negative) demonstrated similar CD_{40L} expression, in comparison to normals (see Fig. 2; *P* < 0.02). CD₂₅ was similarly expressed on resting CD₃ cells from eight CU and normal individuals. Following T-cell activation, the increased CD₂₅ expression was statistically similar in both groups. Yet T cells from three CU patients (severely active, one of whom AST positive) expressed it much higher than that from normal individuals.

Cell Proliferation. Cell proliferation of activated B cells was studied using [³H]thymidine incorporation into DNA (see Fig. 3). It was evident that DNA synthesis is elevated in B cells from nine CU patients in comparison to that from nine normal individuals (660 ± 324 ; 1698 ± 810 Count Per Minute (CPM) in B cells derived from healthy individuals or from CU patients, respectively, *P* = 0.004). Furthermore, the highest proliferation was detected in B cells derived from the most severe CU patients. The accumulative data, following incubation of cells with dexamethasone (a known inducer of B-cell apoptosis), show that the effect is statistically similar in both groups but less profound (rescue from apoptosis?) in B cells derived from three severe CU patients (two of whom were AST positive).

Bcl-2 Expression

The expression of bcl-2 was found similar in resting B and T cells of both contact dermatitis, CU, or normal individuals (data not shown). Using Western immunoblotting, we demonstrate that following B-cell activation (see Methods section) bcl-2 protein expression is statistically significantly increased in B lymphocytes of CU patients compared to bcl-2 levels in B cells derived from contact dermatitis and normal individuals. In order to assure that these results are quantitatively true, similar

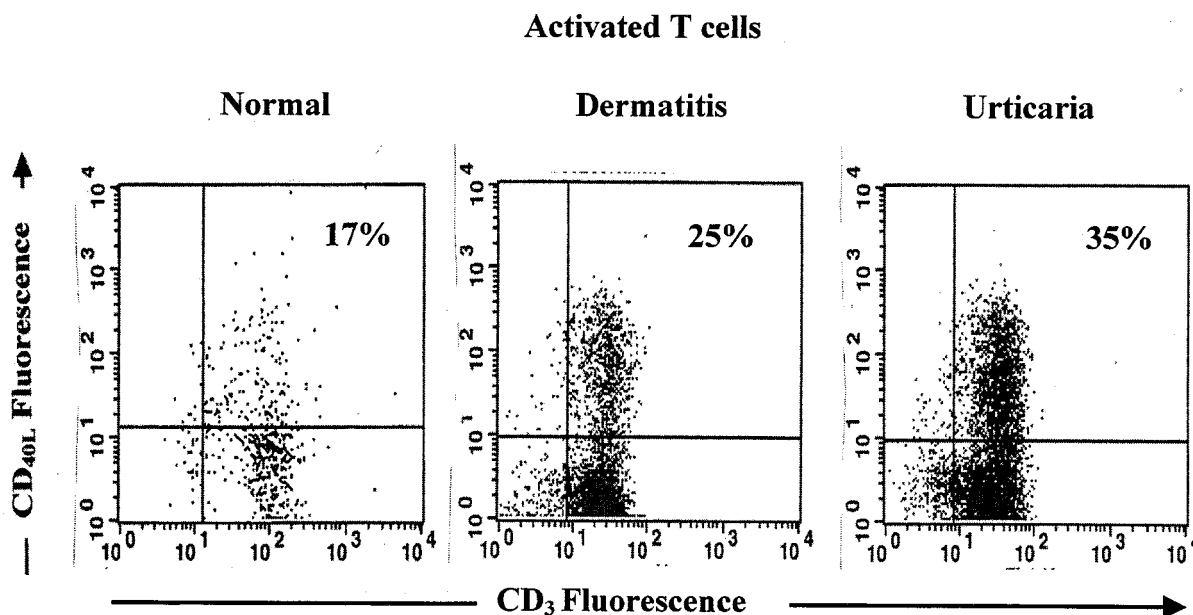


Fig. 1. A representative FACS analysis for CD_{40L}/CD₃ in activated T cells derived from normal individuals, contact dermatitis patients, and CU patients.

protein amount of lysed B cells (quantitated following cell lysis as described under Materials and Methods) were loaded in each lane. A representative Western blot is shown in Fig. 4a and the summary of all studied individuals in Fig. 4b. It is worth noticing that bcl-2 expression also was similarly higher in ConA-activated T cells of CU patients (mainly among the disease-active patients). A representative Western blot is shown in Fig. 5a and the summary of all studied individuals in Fig. 5b.

DISCUSSION

It is a decade since Greaves established the definition of autoimmune chronic urticaria (ACU), which was later

intensively evaluated by others (4, 13, 14). Since then, AST was demonstrated in many series to be positive in 30–40% of CU patients (15, 16). In attempt to identify the wheal-producing factor in the serum of patients with ACU, it was shown that in half such cases, there is evidence of functional auto-Abs against the high-affinity IgE receptor, IgE itself, or both. These auto-Abs were demonstrated to be capable of releasing histamine from basophils and mast cells. In the remaining patients whose serum causes wheals, auto-Abs or other histamine-releasing factors are yet to be unidentified (17, 18). Following this understanding of autoimmune origin of

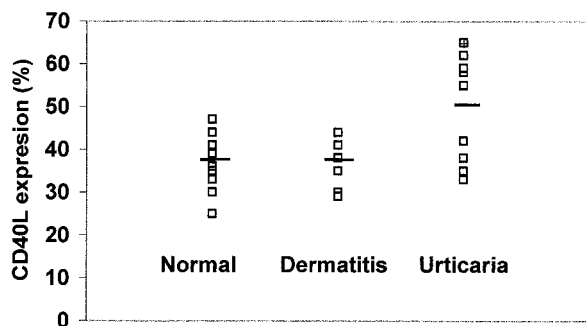


Fig. 2. The distribution of the cells expressing CD_{40L} among the three studied groups: normal ($n = 9$; mean $\pm$ SD = 36.7 ± 6.9), contact dermatitis ($n = 6$, mean $\pm$ SD = 36.2 ± 6), and urticaria ($n = 10$; mean $\pm$ SD = 51.2 ± 12.1).

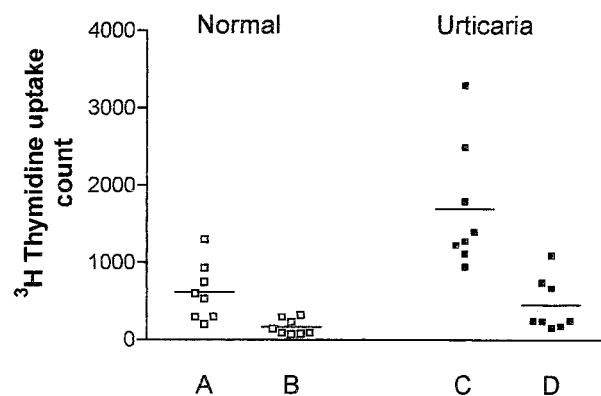


Fig. 3. Radioactive thymidine uptake into DNA in B cells derived from normal individuals or from CU patients: A, C, activated cell; B, D, resting cells, a (active normal) vs. c (active urticaria) $P = 0.004$.

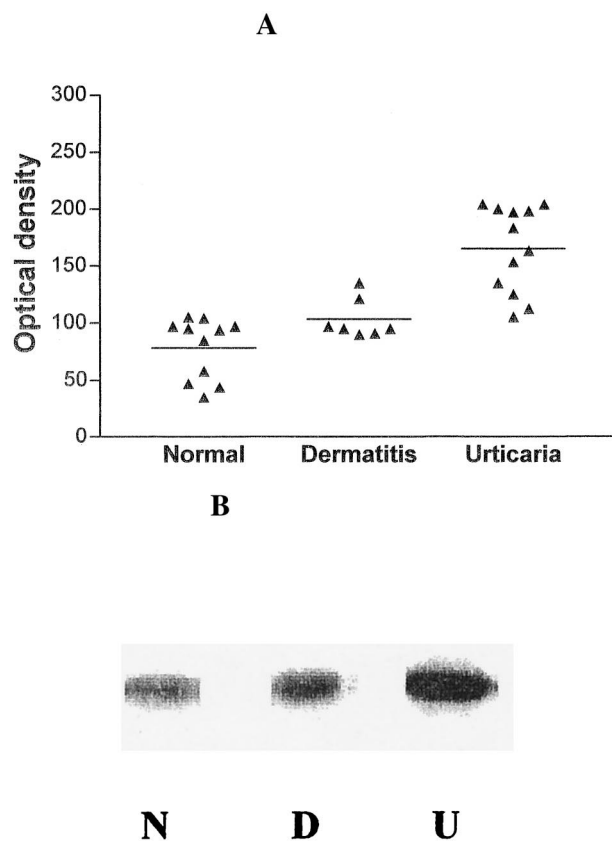


Fig. 4. (A) Bcl 2 expression in B cells. The Western blot analysis performed for all the studied individuals. Band bcl-2 optical density analysis: normal (mean 78 ± 26); dermatitis (mean 103 ± 17); urticaria (mean 165 ± 37). Statistical analysis: Normal vs. dermatitis, $P > 0.05$ (not significant); normal vs. urticaria, $P < 0.001$ (significant); D vs. U, $P < 0.001$ (significant). (B) Bcl 2 expression in T cells. Western blot analysis revealed increased expression of bcl-2 in B cells derived from U (urticaria), D (contact dermatitis), and N (normal). Note: Equal amounts of proteins were loaded in each lane.

this entity, we and others demonstrated the beneficial results of low-dose cyclosporin-A in the treatment of severe CU patients. This beneficial treatment occurred regardless of AST positivity, and due to its rapid onset cure effect was speculated to be the result of its immunomodulating cellular activity (19, 20).

The production of pathogenic IgG autoantibodies against the IgE FcR on mast cells seems to be the cause of mast cell degranulation and the long-standing CU in many cases. Such a pathogenic production might be driven by a polyclonal T-helper-cell activity, rather than a distinct monoclonal autoreactive T cell.

CD_{40L} usually is detected on peripheral blood T cells following their activation with PMA and ionomycin. It was shown that T cells from active SLE express CD_{40L} higher than that on inactive SLE or normal individuals,

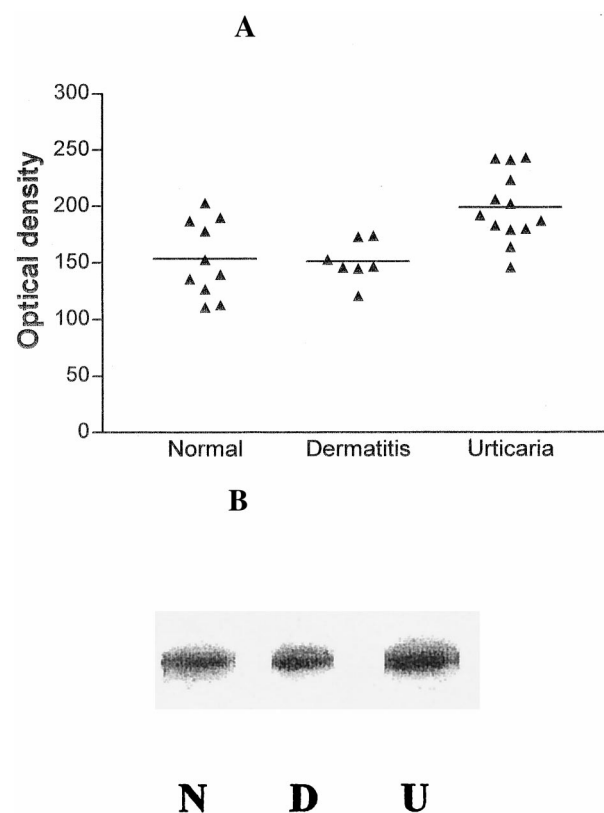


Fig. 5. (A) The Western blot analysis performed for all the studied individuals. Band bcl-2 optical density analysis: normal (mean 153 ± 33); dermatitis - (mean 151 ± 18); urticaria (mean 199 ± 30). Statistical analysis: normal vs. dermatitis, $P > 0.05$ (not significant); normal vs. urticaria, $P < 0.01$ (significant); dermatitis vs. urticaria, $P < 0.01$ (significant). (B) Western blot analysis: increased expression of bcl-2 in T cells derived from U (urticaria), D (contact dermatitis), N (normal). Note: Equal amounts of proteins were loaded in each lane.

suggesting that this plays a role in B-cell polyclonal hyperreactivity in this disease (21, 22). In our study CD_{40L} was found significantly increased on active T cells from CU patients, probably exerting its effect by up-regulating levels of the proto-oncogene bcl-2. Although CD_{40L} protein levels were slightly elevated in T cells derived from contact dermatitis patients (in comparison to normal individuals), they were much higher in T cells derived from CU patients. This hypothesis is favored by our finding, where elevated expression of bcl-2 was observed widely in activated B and T cells derived from CU patients, especially among those diagnosed as severe CU.

These findings are in contrast to the notion that the autoimmune reaction is of a monoclonal origin type, since one would not expect to find such a wide overexpression of bcl-2 on both active B and T lymphocytes. This may suggest that a nonspecific stimulus induces

higher levels of exaggeration, signal transduction, and proliferation in B and T lymphocytes derived from CU patients than in health (normal) individuals.

Although the exact mechanism of induction and clinically or physiological importance of bcl-2 overexpression in B and T lymphocytes in severe CU patients is still not clear, we herein suggest that such a reaction might turn those cells to be overprotected from apoptosis, a process that could contribute to the development of autoimmunity. Unlike patients with other autoimmune disorders or murine models of SLE-like disease, in whom an increase of CD₅⁺ B cells is responsible in part for the production of pathogenic auto-Abs, B cells from ACU patients did not show any different expression of this molecule. Similar to SLE, activated T cells from ACU patients were overexpressing with CD_{40L} in comparison to normal individuals. Such overexpression could contribute to the polyclonal hyperreactivity of B cells and might be involved in the rescue mechanisms from apoptosis, as hypothesized above. In this regard, we believe that many other cellular autocrine and paracrine factors are yet to be discovered.

Although the B7 antigens are playing an important role in T- and B-cell interactions, they are present mainly on antigen-presenting cells and serve as the ligand for CD28/CTLA4 expressed on T cells. The interaction between these two molecules allows T cells to respond to an activated B cell via induction and production of cytokines. Both B7.1 and B7.2 bind to CD₂₈ and to CTLA4; however, they use different binding determinants and have different binding kinetics.

Work done in mice suggests that B7.1 and B7.2 influence the type of T-helper-cell activation differently. Since we did not demonstrate any significant difference in the expression of B7.1 (CD₈₀) on activated B cells from both ACU and healthy (normal) individuals, it might be of importance to study the expression of B7.2 on these cells.

In our study, the effect of dexamethasone (a known inhibitor of cell proliferation) on proliferation was found statistically similar in both normal and CU B lymphocytes, yet it is worth mentioning that in 3 out of 11 patients studied (most severe CU), dexamethasone-induced inhibition was less than that demonstrated in normal B lymphocytes. It is our intention to investigate (using different assays) the sensitivity of B lymphocytes from ACU patients to the induction of apoptosis.

Environmental factors are important modulators to autoimmune responses; among these are infectious agents, which often precede or exacerbate these disorders. Although the exact mechanisms by which infectious agents trigger such responses are incompletely

understood, increasing evidence suggest that peptidoglycan and superantigens may induce the stimulation of B or T lymphocytes (or both). In ACU one could speculate that some of these superantigens may indirectly activate autoreactive B cells, in a T-cell-dependent way, to produce antibodies to FcεRIα on mast cells, which induces their degranulation. Experiment to verify or exclude such a possibility are now in progress.

Our data, although preliminary, further extend our understanding and add strength to the notion that CU is indeed an autoimmune disorder.

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