

Ph.D. THESES

**CLINICAL AND IMMUNOLOGY EXAMINATIONS IN
PATIENTS WITH CHRONIC URTICARIA**

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**UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
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1. INTRODUCTION

1.1 Definition and classification of urticaria

Urticaria is characterized by different sized, usually hyperaemic, itching or burning wheals. In case of chronic urticaria symptoms are present for six weeks daily or almost daily. Chronic urticaria is a frequent dermatologic disease and approximately 0,1-3% of the population are affected. Wheals developing in shubs, causing severe itching and the subsequent sleeping disorders impair quality of life. In almost half of the cases angioedema is also present and the skin symptoms last for several years. Due to the difficult treatment of this disease it's still a great challenge for the physicians.

The etiology of chronic urticaria is heterogenous. It can be caused by different immunological and non-immunological factors, but in many cases the etiology remains unexplained. Due to a classification created for clinical use in 2002 in the background of chronic urticaria pseudoallergic reaction (4%), local infection (4%) or urticaria vasculitis (5%) can also be found. In more than one third of CU cases physical urticaria (PU) was diagnosed (35%), while autoimmun urticaria (AIU) was present in 24%. If the cause of the disease remains unexplained despite extensive workup the diagnosis is chronic idiopathic urticaria (CIU) (28%).

Physical urticaria

According to the literature PU is constituted more than one third of CU. Wheals appear just after physical stimuli and lasting for 1-2 hours. Most frequent forms of FU are cholinerg urticaria and urticaria factitia which fulfills mostly the diagnostic criteria of CU, because symptoms occur daily causing severe complaints. Cholinerg urticaria is responsible for 30% of PU cases. It's precipitating factor is the increase of body temperature by 1C, which can be caused by physical exercise, mental stress, or due to a hot shower. Symptoms are caused by release of acetylcholin due to increase in body temperature resulting in degranulation of mast cells

Introduction

and subsequent histamin release.

The prevalence of urticaria factitia is quite similar, which is also mentioned as symptomatic dermographism in the literature. Shortly after the stimulus at the site of pressure or scratching urticaria appear belted by an erythemic zone accompanied by severe itching.

Rare forms are the cold-, warm-, solar- and delayed pressure urticarias.

Autoimmun urticaria

Several studies investigated autoimmun urticaria, a special form of CU in the last decade, which constitutes almost one third of all CU cases. According to the results of these investigations in 58% of CIU patients wheals develop at the site of injection of autolog serum in the active phase of the disease, while in the symptom-free periods this can not be observed. This finding served as a prove for the usefulness of a currently used diagnostic method, the autolog serum skin test (ASST). Somewhat later it was also reported that the development of urticarias in AIU is due to IgG type anti-IgE autoantibodies and have pathological roles of 10% of patients. Several studies proved that anti-IgE antibody can not be alone responsible for all the histamin release in the CU patients' serum. While others reported that in some cases in the sera of CU patients' high affinity IgG type antibodies against the Fcepsilon-alfa (FcεR α) chain can also be found, which on the basophils of healthy individuals in vitro show histamin release. Female dominance, more severe symptoms presenting mostly at night, coincidence with other autoimmun diseases and effective response to immunosuppressive therapy are all characteristics of autoimmun urticaria.

1.2. Roles of citokines in CIU

In CIU patients' histological samples the number of eosinophil, neutrophil, basophil cells, macrophags are significantly higher, while the number of triptase positive mast cells is significantly lower than in healty individuals'. The number of CD3+, CD4+, CD8+ and CD25+ cells are markedly increased in CIU than in healty

Introduction

controls and a similar cellular infiltratum was observed in atopic patients following allergen exposition still after 24 hours. Immunofluorescence double-staining verified that most of the cells are CD25+ and CD4+ T cells. Autoantibody positivity or negativity based on basophil histamine release did not show difference in any type of cells. According to the literature in CIU patients' histological samples besides IL4, IL5 the IFN-gamma expression is also increased compared to healthy individuals applying in situ hybridisation methods. Based on these results phenotype Th0 was found to be common in the skin, but cytokines released from a mixture of Th1 and Th2 cells is also possible. There were not publications regarding cytokine pattern of peripheral T cells in CIU at the starting of our investigation.

1.3 Diagnosis of AIU

It is impossible to distinguish between CIU and AIU based on symptoms. Although the symptoms of AIU are more severe, eosinophil degranulation is increased and in the peripheral blood the number of basophils is decreased, based on these observations the exact diagnosis is not possible. Diagnosing AIU ASST is a common method, used also for screening. The test's sensitivity is 70% and specificity is 80%. ASST positivity can be found in 50-60% of CIU patients. To reduce the proportion of false positive results some authors suggest the use of 1:10 diluted serum for ASST.

For the certain diagnosis of AIU usually further examinations, other functional or binding tests are necessary. One of the most commonly used tests is the histamine release test (HR), nowadays it is the most accepted method in diagnosing autoantibodies in AIU. It is regarded as a gold standard, although time consuming and difficult to standardise. In different studies CIU sera were positive in 27-52% using HR tests, depending on the characteristics of patients and the donor cells.

1.4 Therapy of chronic urticaria

Management of chronic urticaria is also a challenge for the physicians. If the precipitating factor is identified, its elimination is the most important. In general

Introduction

avoiding the provoking drugs, diet, elimination of local infections and in cases of PU the elimination of provoking factors are suggested. In the pharmacological therapy the first step is to relieve symptoms due to released histamin. H1 receptor antagonists covering the histamin receptors inhibit histamin effects and first line of therapy in this disease. In case of inefficiency two H1 receptor antagonists or one H1 and one H2 receptor antagonists can be combined.

In case of inadequate response for antihistamin therapy several study suggested low or medium doses of systemic corticosteroid as second-line of therapy. Corticosteroids influences mainly the inflammation during the late phase reaction. Corticosteriods side-effects are well-known so their use is indicated only for short term.

If these therapies did not result in improvement some authors reported the favourable effect of plasmapheresis, cyclosporin and high dose immunoglobulin therapy.

2. OBJECTIVES

We compared two functional tests in the diagnostics of AIU: the gold standard HR test and the basophil CD63 expression method.

2. Then we examined the clinical relevance of the modified basophil CD63 expression test. We compared the three most common urticaria forms (PU, CIU, AIU) in our investigation, in which AIU was diagnosed by CD63 assay. PU patients were diagnosed with physical tests and first in the literature were compared with CIU and AIU patients.

2/a. We compared the demographic data of patients with chronic urticaria and the severity of urticaria applying urticaria score.

2/b. We investigated the prevalence of atopic, allergic and autoimmun diseases in the chronic urtiacaria patients' own and family history, moreover the presence of certain autoantibodies.

2/c. We evaluated the efficiency of the therapy in all subgroups of chronic urticaria.

3. Immunological examinations were carried out at CIU patients: we determined the lymphocyte subpopulations, the level of intracitoplasmatic cytokins and examined the soluble cytokins also.

3. MATERIALS AND METHODS

3.1. Patients

1. 72 CIU patients (16 males, 56 females; mean age 40.4 years, range 13-74 years) with almost daily attacks of urticaria and with mean continuous disease duration of 8.4 months (range 3-32 months) were investigated. Sera of patients with dermatomyositis (DM, n=11) and systemic lupus erythematosus (SLE, n=15) were also tested in the HR and CD63 surface expression assays.

2. 22 CIU patients (4 men, 18 women, mean age: 40 years, range 11-76) with almost daily attacks urticaria and a mean continuous disease duration of 16.3 months (range: 3-48) were investigated.

3. We examined 109 patients with chronic urticaria. (82 females, 27 males, age 39.8 years, range 13-74 years). From the history of the 109 patients the mean duration of skin symptoms was 24.7 ± 36.4 months (2-231 months).

After informed consent had been obtained, the patients were subjected to a diagnostic protocol, which included detailed history taking, physical examination, routine laboratory tests and provocation tests for physical urticaria. Urticaria patients with focal infection, drug or food allergy as aetiological background were excluded. Biopsy was performed if the possibility of urticaria vasculitis existed. ASST and CD63 assay were performed on every patient. The 109 patients suffering from PU, CIU and AIU were then asked to fill in an internationally accepted questionnaire. Patients suffering from more than one form of chronic urticaria were excluded.

Symptomatic dermatographism (D) was diagnosed in 48.1% (13/27) of PU patients, with 33.3% (9/27) of the PU patients having cholinergic urticaria (C), 11.1% (3/27) delayed pressure urticaria (DPU) and 7.4% (2/27) cold urticaria. DPU patients with associated AIU were excluded from the study. The skin symptoms of patients

suffering from D and C appeared several times a week.

Antihistamines were withdrawn 72 hours before provocation tests, while corticosteroid and other immunosuppressant drugs were withdrawn at least 4 weeks prior to provocation tests, ASST and laboratory examinations. After the completion of the aforementioned investigations a treatment protocol was started on all patients.

3.2. Methods

3.2.1. Stimulation of donor basophils with different agents

Cells from one highly sensitized atopic donor (D_A) and one nonatopic, healthy person (D_{NA}) were used. Peripheral blood leukocytes from two donors were separated on 6% Macrodex (Baxter, München, Germany) as described earlier¹⁵ and stimulated with the sera of patients. Fifty microliters of cells were incubated with 50 μ L serum diluted 1:1, or buffer, for 40 minutes at 37 °C. The reaction was terminated by cooling the tubes on ice and addition of 900 μ L ice-cold Hepes buffer without Ca, Mg and bovine serum albumin (BSA). Cells were sedimented by centrifugation at 500 g for 5 min at 4 °C and used for basophil surface CD63 expression assay. The supernatants were collected, stored at -70 °C and analysed for their histamine content. For determining total histamine, three replicates of aliquot of cells were lysed by adding 950 μ L distilled water, with repeated freezing and thawing 2 times.

3.2.2. Histamine release from basophils

The histamine content of the duplicate samples was determined by ELISA method (Immunotech, Marseille, France). After subtraction of spontaneous release, the results were expressed as the net percentage of total histamine content. The spontaneous histamine release was less than 5% of the total histamine content. The cut-off values for different donors were determined as 95 percentiles of histamine release induced by sera of 20 healthy controls (11.6 % for D_A and 7.3 % for D_{NA}).

3.2.3. CD63 surface expression assay

The CD63 expression on the surface of donor basophils was determined as described earlier. Briefly, after incubation for 40 minutes with CIU sera the cells

were labelled with goat anti-human IgE-FITC and R-phycoerythrin conjugated anti-human CD63 monoclonal antibody (CD63-PE) (both from Caltag Laboratories, Burlingame, CA) for 60 min at 4 °C. The remaining erythrocytes were lysed, washed and the percentage of IgE and CD63 double positive basophils measured on Becton Dickinson FacsCalibur flow cytometer. Setting an FL1 threshold, cells presenting brightly fluorescent anti-IgE FITC were gated and CD63 positive cells were determined on FL2. At least 500-1000 basophils were counted.

Values above the 95 percentiles of CD63 positive cells induced by 20 negative healthy control sera were considered as positive (cut-off values for different donors were the following: D_A: 5.1%; D_{NA}: 2.2%).

3.2.4. Detection of autoantibodies

Levels of autoantibodies against thyroglobulin (a-TG), thyroid peroxidase (a-TPO) and extractable nuclear antigen (a-ENA) were measured by a commercial enzyme-linked immunosorbent assay (ELISA; Hycor Biomedical GmbH, Kassel, Germany) according to the instructions of the manufacturer. The serum concentrations of antiendothelial cell antibody (AECA) was analysed by ELISA (Cogent Diagnostic, Edinburgh, UK) according to the manufacturer's instructions.

3.2.5. 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). Erythrocytes were lysed and stained cells were fixed by the Coulter QPrep protocol. The fluorescence analysis was performed on a Coulter EPICS XL-4 flow cytometer (Coulter Hialeah, FL, USA) with an 488 nm argon ion laser. Data of 5000 cells were acquired and analyzed by the System II. software. Gates were set on lymphocytes according to forward and side scatter properties. Results were expressed as the percentage of surface CD markers in the lymphocyte gate.

3.2.6. Measurement of intracytoplasmic cytokines

Heparinized whole blood was diluted in 1 to 2 volume rate in RPMI (Sigma). T cells were stimulated with PMA (Sigma, 25 ng/ml) and ionomycin (Sigma, 1 µg/ml) for 4 hours at 37°C in humidified CO₂ incubator. Blockage of cytokine secretion and intracellular accumulation is achieved by treatment 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. Two ml of diluted FACS lysing solution was added to each tube for 10 minutes in order to lyse the erythrocytes and to fix the plasma membrane of leukocytes. Samples were centrifuged at 500g for 5 min. and supernatants were removed. Diluted FACS permeabilizing solution was added to each sample and were incubated for 10 minutes. After a washing step the permeabilized cells were stained with FITC-labelled anti-human IFN-γ and 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 minutes at room temperature in dark. The samples were washed again and were 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 according to 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.

3.2.9. Autologous serum skin test (ASST)

The ASST was carried out according to the literature. Positive reaction was defined when the serum-induced wheal was red and had a diameter ≥ 1.5 mm than the saline-induced response at 30 minutes.

3.2.10. Questionnaire

All patients filled in a questionnaire, which was kindly provided by M. Kozel (Department of Dermatology of the Academic Medical Center, University of Amsterdam), and which was supplemented with a few questions of ours. From this form, more accurate information was gained about the sexual distribution, the severity of symptoms and the disease course. Personal and family history was

thoroughly investigated regarding the occurrence of other types of urticaria, allergic and autoimmune diseases.

3.2.11. Measurement of urticaria score parameters

The urticaria score introduced by Breneman et al. with slight modification in order to be suitable for scoring PUs was used for assessing the severity of the disease. A four-grade scale was used for describing the localization, the size of lesions, the duration of lesions, the frequency of episodes and the intensity of pruritus. Localization of wheals was classified; 0: no wheals, 1: wheals were localized to one part of the body, 2: two-three parts of the body were involved, 3: generalized wheals. Average size of lesions; 0: no wheals, 1: 1-2 cm; 2: 3-4 cm; 3: >4 cm. Average duration of lesions; 0: no symptoms; 1: <60 minutes; 2: 1-4 hours; 3: >4 hours. The frequency of lesions was scored as follows; 0: no wheals, 1: lesions appearing 2 times a week, 2: 3 times a week, 3: >4 times a week. Intensity of pruritus; 0: none; 1: mild; 2: moderate; 3: severe. Severity of pruritus was measured using a visual, analog ten-point scale, and the results were converted into the above mentioned four-point scale. By summing up the points we got the total severity score of a patient.

3.2.12. Therapeutical protocol

All patients got antihistamines as a first line of therapy. Patients received low sedation, second generation H1 receptor antagonist antihistamine (cetirizine 10 mg/day) monotherapy first. In case of inadequate response cetirizine was given in higher dose (20 mg/day), than sedative antihistamine (hydroxyzine 25 mg twice a day) and H2 receptor antagonist (cimetidine 400 mg twice a day) were combined. We regarded the therapy ineffective if the patient's severe complaints persisted or the urticaria score index did not show an at least 50 % reduction. In CIU and AIU patients a short course of oral corticosteroid treatment (methylprednisolone 32 mg/day, reduced by 4 mg every 5 days to zero) was used as a second line of therapy. If this therapy resulted only in temporary improvement, as a third line of therapy, cyclosporine (3 mg/kg/day for three months) was added for AIU patients.

3.2.13. Statistical analysis

For characterization of donor cells and group data that had normal distribution, the results were expressed the Student's t-test was used for comparisons between two groups. Nonparametric testing for non-normally distributed data was performed by Mann-Whitney rank sum test between two groups and by Kruskal-Wallis one way ANOVA on ranks among several (more than two) groups, as transformation failed to generate normally distributed data.

Fisher's exact test was used to analyze the relationship between categorical variables.

A P value of < 0.05 was regarded as significant.

4. RESULTS

4.1. Meaurement of HR and CD 63 assay

In the ASST positive patient group 85% of their sera induced HR from cells from D_A while 55% induced HR from D_{NA} . Sera of CIU patients with positive ASST mediated significantly higher net HR compared with sera of CU patients with negative ASST results following the use of basophils from both donors. Sera of patients with DM and SLE did not produce HR from basophils from either D_A or from D_{NA} .

When the sera were divided into two groups according to their reactivity in the HR assay, 97% of histamine release positive sera resulted in CD63 induction on the surface of basophils from D_A and 83% resulted in CD63 induction if basophils from D_{NA} were used

On the surface of basophils from both donors stimulation with ASST positive CIU sera resulted in significantly higher induction of basophil activation marker CD63 expression compared with stimulation with ASST negative CIU sera. Sera of patients with DM and SLE did not induce CD63 expression on donor cells.

4.2. Correlation of histamine release assay, CD63 expression assay and ASST

The results of the HR assay and the CD63 expression assay showed significant correlation (Spearman Rank correlation, D_A : $r=0.91$, D_{NA} : $r=0.70$, $P<0.001$ for both) when individual patients were assessed. When the results of the HR assay and the ASST were compared, correlation was found only when basophils from D_A were used (D_A : positive HR: 51.4%, positive ASST: 55%, double positive: 92%, double negative: 83%; D_{NA} : positive HR: 32%, positive ASST: 55%, double positive: 96% double negative: 63%).

4.3. Clinical tests

Among the 109 patients 53.2% had a positive ASST and 43% had a positive CD63 assay. Positivity of the CD63 assay was needed for a diagnosis of AIU.

4.4. Demographical data

Among the 109 patients with chronic urticaria 47 patients (43%) had AIU, 35 (32%) had CIU and 27 (25%) had PU. The mean age of patients with chronic urticaria was 39.8 ± 15.8 (13-74) years. The mean age in the AIU subgroup was 43.1 ± 15.8 years, in the CIU subgroup 38 ± 13.7 years and in the PU subgroup 32.7 ± 17.8 years. The female/male ratio in the whole group was 3.0:1, which meant a statistically significant female preponderance. Female/male ratio in the AIU group was 6.9:1, in the CIU group 2.5:1, and in the PU group 1.5:1. We found that the female preponderance was significantly different only between the AIU and PU groups.

4.5. Evaluation of urticaria score parameters

4.5.1. The localization score of AIU patients' was 2.6, for CIU 2.0, 1.23 for D patients and 1.55 for C patients. The differences were significant between AIU and CIU patients and between AIU and D patients and between AIU and C patients. We also found significant differences between CIU and C patients and also between CIU and D patients.

4.5.2. We investigated the duration of wheals (from appearance to vanishing). The mean duration was 9 hours in the AIU subgroup, with a mean score of 2.74. Among CIU patients the mean duration of wheals was 4.45 hours with a mean score of 2.14, while for D patients the mean duration and score was 0.81 hours, and 1.0 respectively, and in C patients 0.79 hours, that indicated 1.0 score. The results show significant differences between the AIU and CIU patients, between AIU and D, and between AIU and C patients. Also differences were significant between CIU and D and between CIU and C patients.

4.5.3. Mean size of lesions was 2.36 ± 1.54 cm for all patients enrolled. The mean size was 3.1 ± 1.8 cm for patients with AIU, which meant a score of 1.91, 1.9 ± 1.1 cm for patients with CIU, meaning a score of 1.3, and 2.4 ± 1.5 cm for patients with D, with a score 1.76, while in C patients the average size was 1.1 ± 0.7 cm, that is 1.1 score. The differences were significant between AIU and CIU and also between AIU and C patients. Comparing CIU to D patients the size of wheals was significantly higher in D patients. The differences were significant between D and C patients.

4.5.4. The frequency of urticaria episodes showed significant differences between the AIU and CIU patients on one hand and between AIU and D and C patients on the other. Accordingly, the frequency of urticaria episodes score for AIU patients was 2.85, while this was 2.17 for CIU and 1.7 for D and 1.7 for C patients

4.5.5. According to the pruritus scale the AIU patients' mean score was 2.80, while for CIU, D and C patients it was 2.37, 2.0 and 2.1 respectively. The differences were significant between AIU and CIU patients, and between AIU and D patients and also between AIU and C patients. The pruritus score was significantly different between CIU and D patients.

4.5.6. Total scores counted from the parameters mentioned above were summed up to give the individual score index of each patient (the severity of disease). These mean score index was 12.89 for AIU patients, 9.98 for CIU patients, and 7.69 for D and 7.45 for C patients. The total urticaria score was significantly different between the AIU and D patients and between AIU and C patients and

between AIU and CIU patients.

4.6. Accompanying diseases in chronic urticaria

Twenty three percent of chronic urticaria patients mentioned the occurrence of some type of urticaria in their family history. 48.9% of AIU, 5.7% of CIU patients and 3.7% of PU patients had a positive family history of urticaria. This meant a significant difference between AIU and CIU groups, and between AIU and PU groups.

Twenty-two percent of patients reported autoimmune diseases in their history. Autoimmune disease was present in the history of 51% of AIU patients' but none in the history of CIU and PU patients. This meant a significant difference between these groups. Most often, autoimmune thyroiditis (44.6%), rheumatoid arthritis (4.2%) and vitiligo (17%) were associated with AIU.

4.7. Detection of autoantibodies

Patients with AIU had a significantly higher occurrence of autoantibodies against the thyroid gland, than patients with chronic urticaria of other origin. We found anti-thyroid autoantibodies (a-TG, a-TPO) in 11% of chronic urticaria patients, and in 23% of AIU patients. Among CIU patients we detected a-TPO positivity in only one case (2.8%), but no anti-thyroid antibodies were found in the sera of PU patients. This meant a significantly higher occurrence of anti-thyroid antibodies in AIU than in the other subgroups. We did not observe any differences between the subgroups while investigating other autoantibodies (a-ENA and AECA).

4.8. Response to the therapeutical protocol

Antihistamine therapy was effective in 45% of chronic urticaria patients. To the first line of therapy 70.3% of PU patients and 68.6% of CIU patients responded, in contrast with 12.8% response rate of AIU patients. In the PU group antihistamine therapy was equally effective in D (77%) and C (77.7%) patients, but DPU (33%) and cold urticaria (50%) patients responded poorly. Oral corticosteroids were used in 31.4% of CIU patients, and in 87.2% of AIU patients, and were found to be effective in 63.6% of CIU patients and in 53.6% of AIU patients. Nineteen

AIU patients were ultimately given oral cyclosporine therapy, which relieved the symptoms in 15 of 19 patients (78.9%). Corticosteroid and cyclosporine therapy was not introduced in the PU patient group.

4.9. 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 certain lymphocyte subpopulations were separated on the basis of the cell surface markers (CD molecules). In the case of CIU patients the proportion of the CD3+, CD4+, CD8+ and CD19+ T and B lymphocytes did not show any significant deviation as compared with the control group. We also examined the percentage of the activated T cells within the T lymphocyte population. While examining T lymphocytes producing surface HLA-DR molecule, a significant increase was observed as compared with the control subjects. In the case of the other activation marker (CD3+/CD69+) we could not observe any deviation as compared with the control group.

4.10. 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. In contrast, the frequency of IFN- γ producing helper T cells from patients with CIU was significantly lower than that of control subjects. The proportion of IL-4 producing CD4+ T cells from patients with CIU was also significantly lower than that of healthy control groups. The ratio of IL-13 producing CD4+ lymphocytes was higher in patients than in the control group, but the difference was not.

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 subject. The ratio of IFN- γ producing CD8+ T cells in patients with CIU was significantly lower than in healthy control subjects. The frequency of IL-4 producing CD8+ T cells was higher in

patients compared with the control group, and the ratio of IL-13 producing CD8+ T cells from patients with CIU was also higher than that of healthy controls, but neither difference was statistically significant.

5. DISCUSSION

In the last decade several observations and experimental data proved that a new chronic urticaria subgroup, the AIU can be separated from CIU. Laboratorial diagnosis of this disease is difficult, mostly because of the imperfection of the laboratorial methods. Currently the basophil HR is regarded as gold standard in identifying patients with functional autoantibodies, but this test is difficult to be carried out and in some laboratories the conditions are not appropriate for the routine use of this method.

In 2004 Gyimesi et al. applied first in the literature a modified method based on CD63 identification for the diagnosis of autoimmune urticaria. Applying this functional test they demonstrated that without adding IL-3 the strongly sensitised D_A basophil cells can be successfully used in the diagnosis of autoimmune urticaria. Positive correlation was found between the basophil CD63 expression test and the ASST.

Among our aims was to investigate the correlation between the CD63 expression test and the gold standard HR. According to our results comparing the tests we found positive correlation using either D_A or D_{NA} basophil cells. Most recent studies also found positive correlation between ASST and CD63 expression assay in chronic urticaria patients. It was demonstrated that IgG fraction of sera is responsible for activation observed in CD63 expression test and was suggested the simultaneous use of both tests, which finding was supported by our work. CD63 expression test is favourably accepted in the diagnosis of AIU and recent studies also widely use it.

Our further aim was to investigate the clinical relevance of CD63 test. We examined a great population of AIU patients diagnosed by the CD63 assay. According to the literature differentiating AIU from CU patients applying HR test

AIU was found to be significantly more severe than CIU. On the other hand applying ASST there was no significant difference comparing these CU subgroups, therefore ASST alone is not convenient in diagnosing AIU. We compared the PU subgroup with CIU and AIU first in the literature.

In our study among CU patients 43% had AIU, 32% had CIU and 25% had PU. We found a 75% female preponderance (3.0:1.0) in the total chronic urticaria patient group and an even more significant female preponderance was detected in the AIU group. Several other studies have confirmed our results. Assessing the severity of disease we applied the summarized urticaria score and we found the AIU more severe than CIU and PU. In conformity with the literature the serum autoantibody positive patients had significantly higher urticaria score. Although the total urticaria score was higher in the CIU subgroup, but we did not find significant difference between CIU, PU and CU patients' total urticaria score. Several studies have investigated the presence of autoantibodies in the sera of AIU patients. Originally in 1983 an increased incidence of anti-thyroid antibodies (a-TG, a-TPO). was observed among CU patients and since then several groups detected this type of autoantibodies. In conformity with these findings we also detected a significantly higher occurrence of anti-thyroid autoantibodies in AIU than in the other two subgroups. Our results showed no significantly increased prevalence of a-ENA or AECA in either subgroup of chronic urticaria patients.

In our present study the occurrence of urticaria in the family history of AIU patients was significantly more frequent, compared to CIU or PU subgroups. No prior studies examined this question. According to the literature regarding other atopic and allergic diseases we did not find significant difference between the subgroups.

We used a three-line therapy protocol in the therapy of CU. All chronic urticaria patients were treated first with antihistamin and altogether 45% responded well. In our study this first-line therapy was effective in 68,6% of CIU, 70,3% of PU and in 12,8% of AIU patients, which suggests that AIU is not just the most severe form of CU but responds less to conventional antihistamin therapy. This therapy

resistance can be the major cause of the higher proportion of AIU patients in the regional centers. For patients in whom the first-line treatment was ineffective, oral corticosteroid therapy was started. 63,6% of CIU and 53,6% of AIU patients responded well to this second-line therapy. For AIU patients not responding to corticosteroids cyclosporin therapy was introduced, which proved to be effective at 78,9%.

First in the literature we also addressed the question if T1 or T2 dominance can be observed in the peripheral blood of CU patients. In our study the distribution of the lymphocyte subpopulation did not show significant difference comparing to healthy controls. Among the CD3+ T lymphocyte, we examined the proportion of activated T cells and the proportion of HLA-DR expressing T cells was significantly higher comparing to healthy individuals, while regarding the early activation markers there were no difference.

Several studies have investigated the intracellular cytokine production of T cells in the skin samples of CIU patients', but observed neither Th1 nor Th2 dominance, because the expression of IL-4, IL-5 and Gamma-INF mRNA were also elevated comparing to healthy controls. The cytokine production of mononuclear cells in the peripheral blood was not investigated earlier. Among the peripheral blood T cells - we reported first – neither T1, nor T2 dominated cytokine production was detected, albeit significantly decreased IFN-gamma in case of CD4+ and CD8+ T cells and significantly decreased IL-4 expression in case of CD4+ T cells was found in the CIU patients. Moreover the absolute number of CD4+ and CD8+ T cells expressing IL-13 and also the number of CD8+ cells producing IL-4 did not show significant increase comparing to healthy controls. On the other hand the IL-10 production of both CD4+ Th and CD8+ Tc cells significantly increased in CIU patients.

IL-10 is an immunoregulatory cytokine produced by Tr1 cells and has an inhibitory effect for the proliferation and cytokine production of both Th1 and Th2 cells. Based on our results the inhibition of IFN-gamma production of Th and Tc cells can be due to the increased IL-10 production. Due to the inhibitory effect of IL-

10 the IL-4 expression of Th2 cells is also decreased.

6. New observations

1. According to our examinations we described first that the basophil CD63 assay shows significant correlation with the basophil HR test on atopic and non-atopic donor cells. Our results proved the usefulness and reliability of a method based on measuring the CD63 expression in the diagnosis of autoimmune chronic urticaria.
2. Among 109 chronic urticaria patients we separated AIU patients using CD63 assay. First in the literature we compared the heterogeneous PU group to AIU and CIU groups. According to our results we found significant female dominance in the AIU group. Based on clinical parameters AIU proved to be the most severe form of CU. At AIU patients we found significantly higher prevalence of other autoimmune diseases and in their sera autoimmune autoantibodies were detected significantly more frequently. AIU patients responded less to antihistamin therapy, which also shows that it is a more severe disease. First in the literature we also detected the accompanying of different urticaria types in the family history of AIU patients. CIU patients had more severe symptoms comparing to PU patients, but the difference is not significant.
3. We reported first that in the sera of CIU patients neither Th1, nor Th2 dominance is present, but the proportion of IL-10 producing regulatory T cells is higher. Investigating the CIU patients' lymphocyte subpopulations the proportion of HLA-DR expressing T lymphocytes was significantly higher comparing to healthy individuals.

7. PUBLICATIONS:

Publications on the subject of the thesis:

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Total IF: 20,3

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