

Brief Definitive Report

Different serum cytokine levels in chronic vs. acute *Anisakis simplex* sensitization-associated urticariaA. DASCHNER,¹ M. RODERO,² C. DE FRUTOS,¹ A. VALLS,¹ F. VEGA,¹ C. BLANCO¹ & C. CUÉLLAR²¹Servicio de Alergia, Instituto de Investigación Sanitaria – Hospital Universitario de la Princesa, Diego de León, Madrid, Spain,²Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, Madrid, Spain

SUMMARY

The knowledge on immune mechanisms of chronic urticaria (CU) at the cytokine level is widely scarce. We compared pro- and anti-inflammatory as well as Th1- and Th2-associated serum cytokine levels in two phenotypes of CU: associated with (CU+) and without (CU-) sensitization against *Anisakis simplex*, a ubiquitous fish parasite, that has been associated with acute urticaria in gastro-allergic anisakiasis (GAA) and with CU+. Thirteen CU+ and 19 CU- patients were compared with 13 GAA patients and 15 control subjects for cytokines, measured by cytometric bead array. Urticaria activity score was positively correlated with IL-6 in CU-. Serum levels of IL-10 were lower in CU+ and CU- with respect to the control group. Median IFN- γ was lower in all urticaria groups. Patients with previous parasitism by *A. simplex* displayed higher TGF- β levels than subjects without previous parasitism. The main finding was lower levels of IL-17 in CU+ with respect to GAA or controls, with a further tendency to even lower levels in CU-. Different urticaria phenotypes are associated with distinct serum cytokine levels.

Keywords allergy, *Anisakis simplex*, cytokine, flow cytometry, human

Abbreviations: CU, chronic urticaria; CU+, *Anisakis simplex* sensitization-associated chronic urticaria; CU-, chronic urticaria without sensitization against *Anisakis simplex*; GAA, gastro-allergic anisakiasis

INTRODUCTION

Chronic urticaria (CU) is a frequent and disabling illness occurring worldwide in 0.1% of the population and has repeatedly been shown to affect quality of life (1). While CU induced by infectious agents has been proposed over the last 30 years, publications describing these associations are anecdotic (2).

Whereas CU is not a typical feature of allergic disease, it is frequently assessed in a clinical allergological setting. Acute urticaria of the immediate-type reaction is frequently IgE-mediated, but CU is only rarely associated with an IgE-mediated immunologic feature. Otherwise, one frequent phenotype of CU in our area (Central Spain) is its association with sensitization against the ubiquitous fish parasite *Anisakis simplex* (3). In our area, this phenotype of patients with CU constitutes up to 50% of patients attended for allergological evaluation, and in a relevant subgroup of these patients, sensitization against *A. simplex* was explained by a previous parasitism episode by *A. simplex* (4). Gastro-allergic anisakiasis (GAA) is a well-established clinical entity, where acute short-lived, IgE-mediated urticaria, angio-oedema or anaphylaxis accompanies acute *A. simplex* infection (5,6). It has been shown that the immunologic response accompanying this short-lived parasitism is accompanied by an important polyclonal stimulation of different isotypes of immunoglobulins comprising a mixed Th1- and Th2-mediated reaction (7). Otherwise, the possible immunologic mechanisms in *A. simplex* sensitization-associated CU (from now on CU+) are not clearly established.

Previous studies did not establish CU to be associated with a polarized Th1 or Th2 milieu but demonstrated a mainly Th0 or mixed Th1/Th2 profile (8). Our hypothesis was that CU could be associated with the Th17 or regulatory, rather than the Th1/Th2, axis. Further, in the model of *A. simplex* sensitization-associated urticaria, the phenotype of urticaria (acute vs. chronic) could be attributed to

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different expression of regulatory cytokines. We thus assessed serum pro- and anti-inflammatory, as well as Th1-, Th2- and Th17-associated, cytokines for its possible implication in CU with a special emphasis on possible differences in CU+ vs. CU-.

MATERIAL AND METHODS

Study population

Patients were recruited prospectively if they matched the selection criteria and gave written consent to participate. The study protocol was approved by the University Hospital La Princesa Ethical Committee.

Gastro-allergic anisakiasis was suspected clinically by acute urticaria/angio-oedema or anaphylaxis appearing within 24 h after eating raw or undercooked fish meal. Patients were finally diagnosed as GAA if they displayed positive serum-specific IgE against *A. simplex* and a positive skin prick test against *A. simplex*, and other possible causes were excluded (9).

Patients with CU were included with at least twice a week spontaneously occurring wheals for at least 6 weeks, independent of other conditions known to be associated with CU. Only patients with suspected food-induced urticaria and urticaria elicited exclusively by physical stimuli were excluded from recruitment.

Within the group of CU, two phenotypes were differentiated: Criteria to include patients in the CU+ group were a positive skin prick tests (SPT) against *A. simplex* and detectable specific IgE against *A. simplex* (>0.7 kU/L) by CAP-System (PHADIA, Uppsala, Sweden).

Patients with CU did not have any previous fish-induced allergic and/or abdominal symptoms and did not show any detectable specific IgE by means of SPT nor by serum-specific IgE against *A. simplex*.

We further assessed a control group of voluntary subjects, who did not have any history of urticaria and were not sensitized against *A. simplex*. As patients with CU can have concomitant respiratory allergy (10), and the prevalence of atopy is high in our area, contrary to other studies in the field, we chose to select these control subjects only on the basis of a missing urticaria history, independently of their possible atopy status or allergic respiratory symptoms.

Urticaria activity score

Urticaria activity score (UAS) was assessed as previously described. Shortly, severity of urticaria in patients with CU was clinically assessed after withdrawing antihistamines for 5 days. The mean score of the last 4 days was calculated as sum of the wheal number score (between 0

and 3: 0; 0–9; 10–50; >50) and the itch severity score (between 0 and 3: no; mild; moderate; severe).

Assessment of respiratory allergy

Patients were interviewed about the presence of seasonal or persistent rhinoconjunctivitis or bronchial asthma in the last two years and were labelled allergic respiratory allergy if they displayed at least one positive SPT against one of the tested aeroallergens.

Skin prick tests

Skin prick tests were performed on the patient's arm: SPT with *A. simplex* and against the most frequent aeroallergens in our area: animal dander (cat, dog), house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*), pollen of *Cupressus arizonica*, *Olea europea*, *Lolium perenne*, weed mix and mould *Alternaria alternata* (Lab. ALK-Abelló, Madrid, Spain). Further, all patients were assessed for other IgE-mediated food allergies by SPT test against a set of food agents, including egg, milk, fish and vegetables.

Skin prick tests was performed by standard technique and was considered positive with a mean wheal diameter of 3 mm or more. Histamine at 1% concentration and saline solution 0.9% (NaCl) were positive and negative controls, respectively. Wheal diameter was measured 15 min after treatment.

Cytokines measurement

Serum levels of IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ and IL-17A were quantified using a multiplex assay in accordance with the instructions of the manufacturer [BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine kit; BD Biosciences, San Jose, CA, USA]. TGF- β levels in serum were assessed by a single plex assay [BD™ Cytometric Bead Array (CBA) Human TGF- β 1 Single Plex Flex Set; BD Biosciences] as indicated by the manufacturer. All samples were analysed with BD FACSCalibur Flow cytometer™ (BD Biosciences), and the results were expressed in pg/mL using the FCAP Array™ software (BD Biosciences). Detection limits for the measured cytokines were as follows (pg/mL): IL-2: 0.52; IL-4: 5.2; IL-6: 0.58; IL-10: 0.58; TNF- α : 0.96; IFN- γ : 0.5; IL-17: 1.1; TGF- β : 2356.

Statistics

Statistical analysis was performed using SPSS ver. 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Prevalences were

calculated for sex, respiratory allergy and atopy status in all studied groups and compared by chi-square test. Mean ages, UAS and serum TGF- β were calculated in all studied groups and compared by ANOVA (normal distribution of data). Other cytokine levels as well as specific IgE displayed no normal distribution. Here, median and interquartile ranges (IQR) were calculated and compared by Mann–Whitney test. Spearman correlation coefficient was used for correlation studies.

RESULTS

We studied a total of 60 subjects. The general data (number of patients in each group, mean age, female proportion and concomitant respiratory allergy) are listed in Table 1. Only mean age was found statistically different between CU+ and CU–.

The number of samples with detectable serum cytokine levels was too low for IL-2 and IL-4; thus, no comparative analysis could be performed with them.

Mean UAS was 3.2 in CU+ and 4.4 in CU– ($P < 0.05$). UAS showed a positive correlation with pro-inflammatory IL-6, but only in the CU– group ($r = 0.48$; $P = 0.04$). No other cytokines were correlated with UAS.

Serum IL-10 levels were low in all studied groups but significantly higher in subjects without urticaria (3.2; IQR 2.8–4.1 pg/mL) compared with both CU groups [CU+ (0.7; IQR 0–3.2 pg/mL) and CU– (0; IQR 0–2.1 pg/mL) $P < 0.03$; Figure 1a]. On the other side, patients without history of urticaria displayed higher serum IFN- γ than all groups of urticaria ($P < 0.001$; Figure 1b). Serum IL-17 was similar in patients with a history of GAA (49; IQR 8.2–192 pg/mL) and subjects without urticaria history (40.6; IQR 8.3–61.2 pg/mL) and significantly higher than in patients with CU ($P < 0.05$). Interestingly, there was a tendency to even lower and mainly undetectable serum IL-17 in CU– (only three samples positive) compared with CU+ (Median 1.1; IQR 0–26.9 pg/mL; $P = 0.99$; Figure 1c).

The distribution of circulating TGF- β displayed more homogeneity than the other studied cytokines (Figure 1d).

Mean levels showed a descending gradient from GAA over CU+, CU– and controls. Higher values were detected, when all subjects with sensitization against *A. simplex* were compared with those without sensitization (6584 ± 2434 pg/mL vs. 5112 ± 1626 pg/mL; $P = 0.008$).

DISCUSSION

Anisakis simplex is a ubiquitous fish parasite, which is not adapted to humans. Whereas it is not able to survive in the host more than a few hours or days, the allergenicity of its excretory/secretory proteins gives rise to different phenotypes of urticaria in its acute and chronic forms (3). A previous study showed that *A. simplex* is also able to induce a prolonged acute urticarial reaction (11). Whereas CU is labelled idiopathic or autoimmune in most cases, at least a proportion of CU+ has been claimed to be associated with a previous contact with the live larva of *A. simplex* and is thus related to fish-eating habits. This could be the explanation for our different epidemiologic data on age and sex distribution. Previous studies on cytokine profiles in CU demonstrated a mainly Th0 or mixed Th1/Th2 profile (8). The justification for assessing cytokines in serum samples is the possibility that their finding in the circulation could be a feature of an amplified inflammation in chronic inflammatory processes as in CU (12–15). On the other side, low circulating levels of cytokines could depend on their rapid consumption.

IL-4 has previously been shown to be higher in patients with CU than in healthy donors. In the study by Ferrer *et al.* (8), the median levels of 1 pg/mL in CU were higher than those in healthy donors, where median values were 0.2 pg/mL. These levels were overall very low and lower sensitivity of our own method did not give us the opportunity to corroborate these findings. On the other side, even if IFN- γ levels were overall low, they were higher in our control group than in all urticaria groups. This feature is of special interest when taking into account the fact that our control group, subjects without sensitization against *A. simplex* and without any history of urticaria, displayed a high percentage of atopy as measured by SPT, similar to

Table 1 General data of the studied groups

	GAA	CU+	CU–	CONTROL	P
n	13	13	19	15	
Mean age (years)	48.8	53.2	38.7	40.1	0.01
Female proportion (%)	46.2	76.9	47.4	66.7	n.s
Rhinoconjunctivitis or bronchial asthma (%)	7.7	46.2	26.3	40	n.s
Positive SPT against aeroallergens (%)	38.5	76.9	68.4	61.7	n.s

GAA, Gastro-allergic anisakiasis (acute urticaria), CU+, *Anisakis simplex* sensitization-associated chronic urticaria, CU–, chronic urticaria without sensitization against *A. simplex*, SPT, Skin prick tests.

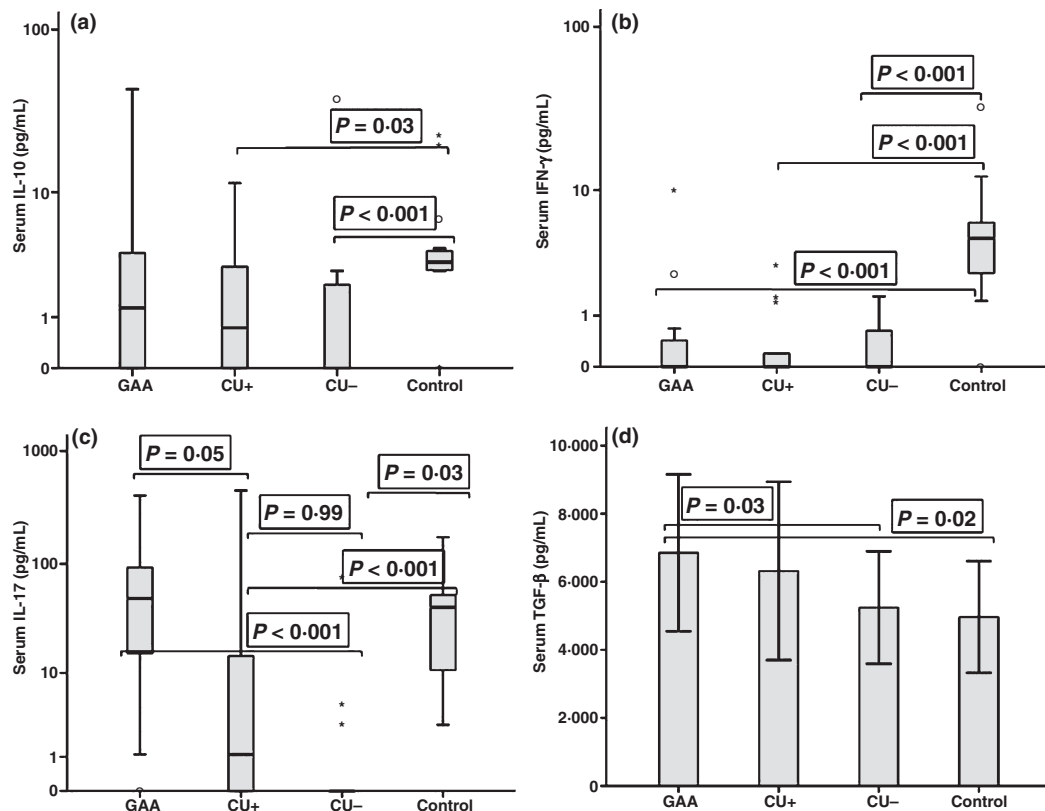


Figure 1 Serum levels of significant cytokines. Due to distribution patterns, IL-10, IFN- γ and IL-17 are shown as boxplots and TGF- β as mean bars $\pm$ SD. GAA: Gastro-allergic Anisakiasis (acute urticaria), CU+: *Anisakis simplex* (*A. simplex*) sensitization associated chronic urticaria, CU-: chronic urticaria without sensitization against *A. simplex*.

both CU groups. Nevertheless, the levels of IFN in all studied subjects did not show any association with respect to presence or absence of atopy.

Previous reports on circulating pro-inflammatory cytokines in CU have been highly variable. Whereas in one study, cytokines such as TNF- α , IL-1 β , IL12p70 and IL-6 have been found to be elevated in most patients with CU (12), no evidence of detectable TNF- α in serum could be stated in another study (14). Our own results were not consistent with these findings with respect to TNF- α and IL-6. The fact that IL-6 levels were associated with disease severity in CU- and the finding that CU- had overall a higher disease severity as assessed by UAS than CU+ suggest that the discordant finding with respect to the study by Dos Santos *et al.* (12) could be because of differences in the disease severity of the studied patients. The fact that TNF- α is able to induce mediator release from skin mast cells underpins the possibility of being cause and not effect in CU.

IL-6 can be produced by mast cells, and it has been speculated that an increased number of total mast cells, as

can be observed in the skin of patients with CU, might lead to the increase in proinflammatory cytokines, including IL-6 (16). As this cytokine is secreted by a wide range of inflammatory processes, including hypersensitivity reactions, we cannot rule out the possibility not to find an association owing to the selection criteria of our control group, where the inclusion of patients with atopy and/or respiratory allergy could drive higher IL-6, so that no differences were found.

Serum IL-10 levels have not previously been associated with CU, but two studies addressed the production of this cytokine by lymphocytes. In a study by Piconi *et al.* (17), IL-10 production after stimulation with phytohemagglutinin was elevated together with TNF- α , MIP-1 α and RANTES. In another study, CU patients with positive autologous skin test had also increased IL-10 production after pokeweed mitogen stimulation (12). In our study, circulating IL-10 levels were lower in both CU groups, especially in CU-, compared with our control population. Contrary to the previous reports arguing that a higher production of IL-10 could be a secondary response to the

inflammatory milieu, in our study, the lower circulating levels are consistent with a lack in anti-inflammatory cytokines being associated with CU. Our results have to be analysed in the context of our control population including patients with respiratory allergy, where altered IL-10 production as well as IL-10 serum levels could be biased.

TGF- β is a multifunctional polypeptide implicated in the regulation of a variety of cellular processes; it has been reported to inhibit Th1 and Th2 differentiation pathways (18,19). Whereas there were no significant differences between CU and control subjects, it was interesting to note that patients with previous parasitism by *A. simplex* displayed higher levels than subjects without previous parasitism. Chronic helminth infection has been associated with higher production of anti-inflammatory cytokines (20). *Anisakis simplex* parasitism in humans is only acute, as the third-stage larva is not able to complete its life cycle, but human contact with *A. simplex* has a special feature: whereas the acute allergic reaction is produced only by the live third-stage larva of *A. simplex*, patients are subsequently exposed to other antigens of *A. simplex* when continuing to eat frequently parasitized fish. It would therefore be interesting to explore further the possibility that there exists a continuing stimulus by some antigens or *A. simplex*-derived mediators, which could drive the upregulation of TGF- β (21).

Our most significant and interesting result was the lower serum IL-17 levels in patients with CU+ and CU-, compared with patients without history of urticaria and patients with past GAA. Levels of the latter group were in the same range as in the control group. Thus, it seems that it is not the fact of a previous parasitic episode as in GAA or CU+, which is associated with IL-17 levels, but the present activity of urticaria when the serum sample is taken. Interestingly, there is a tendency to even lower levels of IL-17 in CU- compared with CU+. One possibility

of this result is that IL-17 is associated with disease activity, as CU+ patients displayed a significantly lower disease activity than CU- patients.

Up to now, no study has reported diminished levels of this cytokine in CU. Interestingly, a possible regulatory role of this cytokine in CU could be underlined by the fact that it shows diminished levels.

We must be cautious when interpreting serum levels of these cytokines, as their levels do not necessarily reflect a diminished production. Rapid consumption of increased production could also be an explanation, as patients with CU+ show higher production of IL-17 than patients with GAA when stimulated with concanavalin A (data not shown). Further, a study by Piconi *et al.* (17) showed also simultaneous elevated production of IL-10 and TNF- α , and IL-10 production was interpreted to be secondary to the attempt to hamper the inflammatory milieu. Taken together, it seems that diminished IL-17 levels are because of consumption in the inflammatory process and possibly associated with disease activity.

If we therefore summarize our significant results, analysis of serum samples is not able to give sufficient information on a possible deviation of the Th1 or Th2 milieu but shows diminished serum IL-10 as well as IL-17 in CU, especially in CU- patients, both cytokines representing markers of a different immunoregulatory axis. Further, previous contact with *A. simplex* is associated with higher TGF- β serum levels.

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