

ORIGINAL ARTICLE

Serum total tryptase levels are increased in patients with active chronic urticaria

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Summary

Background We have demonstrated previously mast cell histamine release upon incubation with chronic urticaria (CU) sera, presumably by degranulation.**Objective** To explore total and mature tryptase in order to assess whether any increase in total tryptase levels is due in part to mast cell degranulation or to mast cell burden. We also wanted to explore differences between the autoimmune groups called idiopathic (serum unable to activate basophils), and to correlate total and mature tryptase levels with different urticaria features.**Methods** We measured total and mature tryptase serum levels in 81 CU patients, 16 atopic donors and 21 healthy control sera. We assessed autoimmunity by measuring the CD63 expression in normal basophil donors upon incubation with CU sera.**Results** We found significantly higher levels of total tryptase in the sera of CU patients ($6.6 \pm 4.1 \mu\text{g/L}$) than in sera from healthy non-atopic subjects ($4.4 \pm 2.8 \mu\text{g/L}$) and from atopic subjects ($4.5 \pm 1.7 \mu\text{g/L}$). Mature tryptase levels were undetectable ($< 1 \text{ ng/mL}$). Total tryptase levels in the autoimmune urticaria group were significantly higher ($9.8 \pm 5.4 \mu\text{g/L}$) than the idiopathic urticaria group ($4.4 \pm 2.2 \mu\text{g/L}$). A significant difference in total tryptase was found between symptomatic patients ($7.3 \pm 4.1 \mu\text{g/L}$) compared with asymptomatic ones ($5.7 \pm 4.1 \mu\text{g/L}$) at the time of venesection. No difference was found in mature tryptase levels either.**Conclusion** Total elevated tryptase levels are not accompanied by an elevated mature tryptase levels, as might be expected if the serum levels reflected mast cell degranulation.**Keywords** chronic urticaria, mast cells, tryptase

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Introduction

β -Tryptase is the major protein component of human mast cell secretory granules, accounting for as much as 20% of the total cell protein [1]. It is stored in secretory granules as a proteolytically active tetramer of 120 kDa that is bound to heparin or other proteoglycans through its cationic groove. α -Tryptase is the other form of soluble tryptase abundantly expressed by human mast cells. Both α - and β -protryptases are spontaneously released by resting cells, whereas primarily mature β -tryptase is stored in secretory granules and released by activated mast cells. Because α -protryptase does not undergo au-

toprocessing, it may remain as a zymogen. Also, experimental preparations of mature recombinant α -tryptase are essentially inactive as a protease; and little if any mature or pro forms of α -tryptase have been detected in mast cell secretory granules. Two genes exist in the normal haploid genome for these tryptases: TPSB2, which is monomorphic for β -tryptase, and TPSAB1, which is dimorphic, encoding an α -tryptase haplotype ~50% of the time and a β -tryptase haplotype in the remaining subjects. Thus, about 25% of human subjects are completely deficient for the α -tryptase gene. Furthermore, portions of α - and β -protryptases are spontaneously released by resting mast cells. In contrast, mature β -tryptase is released when mast

cells are activated to degranulate. When released in tissues by degranulation, the large mature tryptase : proteoglycan complex limits diffusion of tryptase through tissue into the circulation, in contrast to histamine, which rapidly appears in the circulation after its release. During anaphylaxis, plasma histamine levels peak about 5 min after symptom onset and then decline with a half-life of a few minutes, whereas mature tryptase levels peak in serum or plasma 30–60 min after symptom onset and decline with a half-life of 2–3 h. Basophils are the only other cell type that normally expresses α -/ β -tryptases, but do so in much lower amounts than mast cells [2]. Baseline serum levels of total tryptase (a mixture of pro and mature forms of α -/ β -tryptases) largely reflect the body burden of mast cells and are elevated in systemic mastocytosis and other disorders associated with either with hyperplasia or clonal expansion of mast cells, or with aberrant production by transformed cells, such as AML blasts [3]. Elevated baseline levels of serum tryptase are also associated with an increased risk of severe systemic anaphylaxis from insect stings [4–5].

Two additional genes are related to those that encode α -/ β -tryptases, and encode γ - and δ -tryptases. γ -Tryptase is a membrane-bound monomeric protease that is unlikely to cross-react with the antibodies against α -/ β -tryptases, which do not label the surface of cells expressing γ -tryptase. δ -Tryptase has a truncation mutation that eliminates 40 amino acids from the C-terminus; has little if any proteolytic activity; and appears to be expressed at much lower levels than α -/ β -tryptases [6].

Chronic spontaneous urticaria (CU) can be defined as the occurrence of widespread daily or almost daily weals for at least 6 weeks [7]. We calculated 0.6% (95% CI: 0.4–0.8) prevalence in a population study [8]. Currently, the presence and possible role of IgG autoantibodies against the epitopes expressed on the α -chain of the IgE receptor [9] and to a lesser extent against IgE in a subset of patients is generally acknowledged [10–12]. Autoimmunity was first demonstrated *in vitro* by the ability of CU sera to induce histamine release in basophils and human skin mast cells [9]. This ability of CU to sera to show functional activity has also been demonstrated by CD63 [13–15] and CD203c [16] basophil expression upon incubation of atopic and non-atopic basophils with CU sera. This expression showed a good correlation with the basophil histamine release assay [14].

The aim of the present study is to further explore serum tryptase levels in CU using two well-characterized assays for α -/ β -tryptases. One measures total tryptase (α - and β -protryptases and α - and β -mature tryptases); the other measures mature tryptase (α - and β -tryptases). Thus, throughout the present study, mature tryptase will always comprise both α - and β -tryptase, even though it is likely that most if not all of the mature form is β -tryptase. The purpose of measuring mature tryptase apart from total

tryptase is to assess whether any increase in total tryptase levels is due in part to mast cell degranulation or to mast cell burden. We wanted also to explore the differences, if any, between the autoimmune group of CU patients and the group called idiopathic (serum unable to activate basophils) of mast cell burden.

Material and methods

We studied sera from 81 patients with CU (52 females, mean age 44.8 years), 16 atopic donors (six females, mean age 29.4 years) and 21 healthy control sera (20 females, mean age 31 years).

We defined CU patients as having recurrent spontaneous hives for more than 6 weeks' duration, occurring at least four times a week, with individual lesions lasting < 24 h. Patients with clinical evidence of urticarial vasculitis or physically induced urticaria (e.g. cold urticaria, cholinergic urticaria, dermatographism) were excluded. No medication was withdrawn during the study.

We defined patients as being symptomatic if hives, with or without angioedema, were present at the time of the venepuncture.

We considered atopic controls as those patients with rhinitis and/or asthma, or eczema, and a positive skin test to at least one allergen, and specific IgE (CAP-FEIA, Phadia, Uppsala, Sweden) against that allergen. We included as healthy donors those with negative skin prick tests to a standard pool of common allergens such as common inhalants (house dust mites, pollens, animal dander, and moulds), food panel allergens as fish, shell fish, nuts, fruits, legumes, wheat, egg and milk; latex and anisakis, no history of mastocytosis, hives, rhinitis, asthma, or eczema, and normal IgE levels.

All sera were separated by centrifugation at 215 g for 10 min and stored at -80°C .

Flow cytometry

We incubated all 30 sera with basophils from three different non-atopic healthy donors, as described previously [17]. After cell separation, we incubated 50 μL of cell suspension with 50 μL of CU sera at 37°C for 30 min. We used sera from 30 patients suffering from CU. As controls, we used 10 sera, five sera from non-atopic donors and five sera from atopic donors.

In order to assess the role of complement, we decompartmented all sera by heating at 56°C for 30 min before incubation with the three basophil preparations.

Briefly, blood was collected in 6 mL ACD tubes (Vacutainer, Becton Dickinson, Meylan Cedex, France). The tubes were centrifuged for 10 min at 4°C , 53 g. Cells from the layer above the red blood cells were collected and centrifuged again for 10 min at 4°C , 53 g. The supernatant was decanted and 500 μL of HEPES calcium buffer, denominated

stimulation buffer (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, CaCl₂ 7 mM, MgCl₂ 3.5 mM, HSA 1 mg/mL, pH 7.4), containing IL-3 (2 ng/mL) and 10 µL of heparin (5000 UI/mL (ROVI, Madrid, Spain) were added to each tube, mixed and gently shaken. The basophils from the pellet were double labelled by adding 20 µL of anti-CD63 PE-labelled antibody (Caltag Laboratories, Burlingame, CA, USA), diluted 1 : 80 in tubes (Falcon) and anti-IgE FITC-labelled antibody (Caltag Laboratories) diluted 1 : 60 in washing buffer. After incubation for 30 min at 4 °C (protected from light exposure), we added 4 mL of erythrolytic reagent (Ortho-Mune lytic reagent, Ortho Diagnostic System Inc., San Fernando de Henares, Madrid, Spain) in each tube and left them at room temperature (18–20 °C) for 5 min. Cell lysis was stopped with 1 mL of washing buffer. After centrifugation for another 5 min at 215 g, the supernatant was decanted and 500 µL of washing buffer was added to each tube, which was then gently shaken before flow cytometric analysis.

Flow cytometric analysis of surface markers was performed at 488 nm using a FACScan flow cytometer equipped with a 15 nW argon ion laser (Becton Dickinson, BD Biosciences, San Jose, CA, USA) and analysed by CellQuest software.

On the histogram defined by the forward scatter and side scatter, the initial cell gate was defined by a bit map around lymphocytes. The second gate was defined around cells showing high-density anti-IgE-label, identifying them as basophils. In each assay, we assessed at least 500 basophils. The other parameter analysed on the identified basophils was CD63. In 10 cases, all samples were run in duplicate, in order to assess the reproducibility of the technique.

Tryptase measurement

We measured total tryptase serum levels with UniCAP from Phadia following the manufacturer's instructions. The Phadia total tryptase assay measures mature and pro forms of α - and β -tryptases with the B12 capture and β -galactosidase-G4 detector monoclonal antibodies (mAbs), and uses mature β -tryptase purified from lung to generate the standard curve.

A sandwich ELISA described previously was used [18] to determine the concentration of mature tryptase (both α and β) in serum, which was modified to include a 1% (V:V) heterophilic antibody blocking reagent (HBR1, Scantibodies Laboratory Inc., Santee, CA, USA). The mature tryptase assay measures mature α - and β -tryptases with the B12 capture and biotin-G5 detector mAbs and has a lower limit of sensitivity with serum of 1 ng/mL of mature tryptase. A negative control with a biotinylated isotype-matched murine mAb was included. Tryptase purified from lung (mature β -tryptase) was diluted in HEPES buffer (0.01 M HEPES, pH 7.4, containing 0.5 M NaCl, 25 mM EDTA, 0.1% BSA and 0.05% sodium azide) and

added to tryptase-depleted serum to generate the standard curve.

Statistical method

To assess significant differences between mean serum tryptase levels among CU, atopic and healthy controls, we used a non-parametric test for independent samples, the Kruskal–Wallis test followed by the Mann–Whitney test with Bonferroni's correction. To compare mean serum tryptase levels between each group, we utilized a paired *t*-test with the limit for normal range levels set at 11.5 µg/L.

The Spearman's rank correlation test was used to examine both the relationship between serum total tryptase levels and both CU duration (months) and age.

We used the Mann–Whitney test to compare serum total tryptase levels between those CU patients whose urticaria was associated with angioedema and those with no angioedema accompanying the urticaria as well as to study differences between the group whose urticaria was exacerbated by non-steroidal anti-inflammatory drugs (NSAIDs) and those patients whose urticaria was not exacerbated when taking NSAIDs.

All data were analysed using SPSS v15 software.

Results

Total and mature tryptase levels

Total tryptase. We found significantly ($P = 0.015$, Kruskal–Wallis test) higher levels of total tryptase (mean $\pm$ SD) in the sera of CU patients (6.6 ± 4.1 µg/L) than in sera from healthy non-atopic subjects (4.4 ± 2.8 µg/L) and from atopic subjects (4.5 ± 1.7 µg/L) (Table 1).

Mature tryptase. We then measured mature tryptase levels (α and β) in 52 sera and found in each case (except two) that the level was undetectable (< 1 ng/mL). In two

Table 1. Comparison between serum tryptase levels of CU patients, atopic controls and healthy controls

	Healthy controls	Atopics	CU patients	<i>P</i> -value [†]
<i>N</i>	21	16	81	
Age (years)*	31.2 (12.4)	29.4 (18.4)	44.8 (12.6)	
Sex (% women)	95.2	37.5	64.2	
Tryptase levels (µg/L)*	4.4 (2.8)	4.5 (1.7)	6.6 (4.1)	0.015

*Mean (SD).

[†]Kruskal–Wallis Test. Total tryptase levels: Atopic vs. healthy subjects ($P = 0.64$) and CU vs. healthy subjects ($P = 0.006$) using Mann–Whitney *U*-tests (Bonferroni correction).

CU, chronic urticaria.

subjects, a value could not be determined because the negative control test with their sera gave a false-positive value, suggesting heterophilic antibody activity.

In two subjects, insufficient sera were available for measuring mature tryptase.

Tryptase levels in chronic urticaria subsets

Autoimmune urticaria. We examined a subset of sera from 27 CU patients for their ability to stimulate basophils from healthy non-atopic donors. Five of the 27 CU sera were able to induce the CD63 expression upon incubation with normal basophils (Fig. 1). Decomplementing sera neither increased nor decreased this effect.

Basophils from each of the three donors responded similarly with respect to the CD63 expression to CU sera (see data included in Fig. 1a).

Total and mature tryptase levels in CU subsets (Table 2)

Total tryptase levels in the autoimmune urticaria group were significantly higher ($9.8 \pm 5.4 \mu\text{g/L}$, $n = 5$) than the idiopathic urticaria group ($4.4 \pm 2.2 \mu\text{g/L}$, $n = 22$) ($P = 0.039$). Owing to sera availability, we only measured mature tryptase in six sera of this group, one autoimmune and five idiopathic, also finding undetectable levels. A significant difference in total tryptase was found between patients who were symptomatic ($7.3 \pm 4.1 \mu\text{g/L}$) compared with those who were asymptomatic ($5.7 \pm 4.1 \mu\text{g/L}$) at the time of venesection ($P = 0.02$, Mann–Whitney test). No difference was found as well in mature tryptase levels.

We found no significant difference between total tryptase levels in the group of CU patients, whose urticaria was accompanied by angioedema and those whose urticaria was not associated with angioedema.

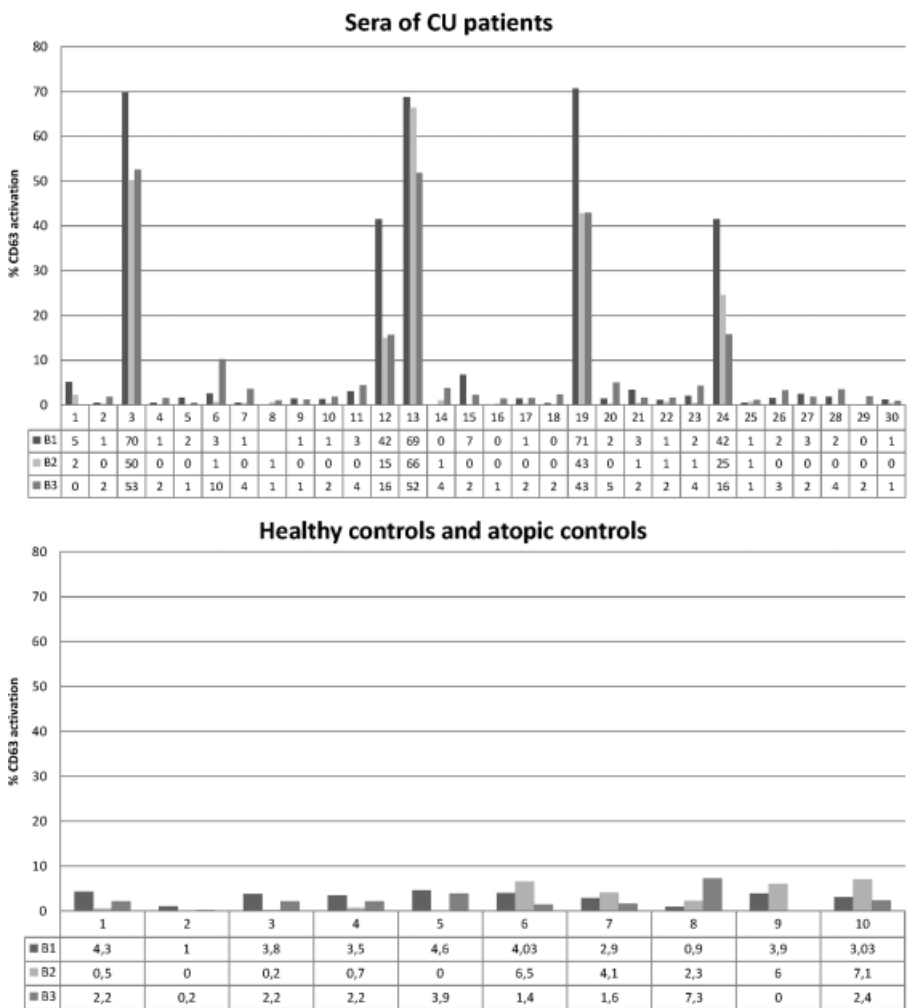


Fig. 1. Percentage of basophils from healthy donors expressing CD63 upon incubation with sera from chronic urticaria (CU) and control subjects. Beneath the x-axis is shown the percentage of basophils from each donor (B1, B2, B3) expressing elevated CD63 levels for each serum. (a) Sera from 27 CU patients. (b) Sera from five healthy (1–5) and five atopic (6–10) subjects.

Table 2. Comparison between serum total tryptase levels according to age, gender and the presence or absence of clinical conditions in CU patients

	N	Tryptase levels (µg/L)*	P-value†
Age (years)			
<Median‡	42	6.4 (4.8)	0.2
>Median	39	6.7 (3.3)	
Sex			
Men	29	6.9 (4.6)	0.7
Women	52	6.4 (3.9)	
Symptomatic			
Yes	41	7.3 (4.1)	0.02
No	39	5.7 (4.1)	
Angioedema			
Yes	51	6.7 (4.5)	0.9
No	29	6.2 (3.4)	
NSAID intolerance			
Yes	50	6.7 (3.9)	0.4
No	28	6.3 (4.6)	

*Mean (SD).

†Mann-Whitney *U*-test.

‡Median of age = 44 years.

CU, chronic urticaria; NSAID, non-steroidal anti-inflammatory drug.

Total tryptase levels did not correlate with gender or urticaria duration.

Finally, total tryptase levels in sera from urticaria patients, whose urticaria was exacerbated by NSAIDs and those who tolerate NSAIDs, revealed no significant difference.

Discussion

The current study finds that patients suffering from CU have significantly higher total serum tryptase levels than healthy and atopic controls, with no rise of mature β -tryptase levels. These differences in tryptase levels, although statistically significance, should be considered as a necessary first step only, and a proper interpretation should also take into account the clinical relevance. It has been demonstrated previously that mast cell histamine is released upon incubation with CU sera, presumably by degranulation [9]. Consequently, one might have expected that degranulation would account for the elevated total tryptase levels in serum obtained from CU patients. But the lack of a corresponding increase in mature tryptase levels suggests this is not the case unless there are other confounding factors such as slow diffusion into the blood and subsequent dilution after an acute release. Instead, the increase in total tryptase levels may reflect either an elevated mast cell burden or an elevated baseline production of tryptase per mast cell. Indeed, some studies have found elevated mast cell numbers in the skin of CU subjects [19, 20]. But other studies have not found mast cell numbers to be increased [21, 22]. It would be of

interest to determine whether cytokines and chemokines that attract mast cells, such as SCF, are elevated in the skin of CU patients. Also, whether an elevated baseline total tryptase level places such patients at risk for more severe anaphylaxis to allergens needs to be considered, because elevated baseline tryptase levels have been reported as a risk factor for severe anaphylaxis to insect venoms [4–5].

Another consideration is the involvement of basophils in CU pathophysiology. Basophils are the only other cell type that normally expresses tryptase, but contain approximately 1/500th the amount in mast cells [2]. Further, nearly all of the tryptase found in basophils is mature, and hence, it appears unlikely that the source of this total tryptase serum levels is basophils. However, basophils could have a role because a typical feature of the CU is basopenia [23] and this basopenia is probably due to chemotaxis of basophils from the circulation into the skin.

The elevated level of serum total tryptase in CU patients found in the current study is consistent with a study by Hidvegi et al. [24] who reported a mean level in 50 CU patients of 7.1 µg/L, very similar to the level of 6.6 ± 4.1 µg/L reported herein, and also significantly higher than that of the control group. By contrast, the observation that total tryptase levels are higher in symptomatic patients with active lesions than asymptomatic patients without weals supports the involvement of mast cells in the pathophysiology of CU. In a previous study [17], when cutaneous mast cells from healthy donors were incubated with sera, those sera from CU patients who were symptomatic at the time of sample collection induced significantly higher histamine secretion than those collected from asymptomatic CU patients, somewhat analogous to the current data for higher serum total tryptase levels in such subjects. It would be interesting in the future to measure tryptase over time comparing levels in individual patients during symptomatic and asymptomatic periods. However, this higher total serum tryptase level does not imply elevated mature tryptase levels. As stated previously, chemokines and cytokines could be responsible for attracting mast cells and causing symptoms, it would have also been interesting to explore whether higher tryptase levels were related to the atopic condition.

The observation that total tryptase levels were also slightly elevated in patients asymptomatic at the time of venesection agrees with studies that have demonstrated an increased histamine [25] in suction-blister fluid patients with urticaria and the up-regulation of cytokine expression [26] in the non-lesional skin of CU patients. Interestingly, the same results were confirmed by Deleuran et al. [27] who demonstrated higher tryptase levels in the suction-blister fluid patients with urticaria in both the lesional and non-lesional areas of skin. The Hermes study [26] performed immunohistochemical staining of several cytokines in tryptase-positive cells in different subtypes of urticaria and found higher TNF- α and IL-3 in all

urticaria lesions, with TNF- α also being up-regulated in non-lesional skin in certain types of urticaria. All these studies suggest an increase in mast cell number, or an increase in mast cell releasability. Interestingly enough, recently increased levels of metalloproteinase-9 have been reported in CU patients with a positive correlation with the urticaria severity score [28] as the authors suggest, we should further investigate the role of T lymphocytes (or other cells) as cutaneous mast cells activators.

Another goal of the current study was to explore the ability of CU sera to activate basophils. Certain sera were consistently able to activate basophils, as reflected by an increased surface CD63 expression with basophils from each of the three donors. We also checked serum from patients drawn at separate occasions and obtained reproducible data. In previous studies, other authors [13, 29] compared percentages of CD63 expression among different basophil donors or groups studied with no side by side comparison. Szegedi et al. [14] performed the same study utilizing one non-atopic and two atopic basophil donors, and showed data comparing only the two atopic donors. Wedi et al. [15] did not find correlation comparing results obtained from incubating CU sera with one atopic donor and a non-atopic donor. We think that the discrepancies might be due to use of atopic basophils donors that could render high baseline. We suggest using only non-atopic basophils donors in order to avoid unspecific activation. In our hands this consistency was striking and showed in previous studies analyzing histamine release, leukotrienes or IL-4 [17] Although none of the sera tested from 10 healthy and atopic controls activated basophils, only five of the 30 sera tested from CU patients do so. Interestingly enough, this basophil activating activity corresponded to higher serum total tryptase levels. This feature agrees with the fact that autoimmune urticaria is more severe than idiopathic urticaria [15] However, it should be noted that the limitation of the relatively small *n*-value for the autoimmune group.

In conclusion, our data show that patients suffering from active CU have significantly higher total tryptase levels than asymptomatic CU, atopics without CU and healthy subjects. Further, elevated total tryptase levels are not accompanied by elevated mature tryptase levels, as might be expected if it were due to mast cell degranulation. Our data suggest that the cause of the results presented herein is an increased body burden of mast cells. Our data confirm previous findings [19, 20] reporting increased mast cell numbers in CU skin biopsies. As it was suggested in a recent editorial [30], inflammation in CU is not limited to the consequences of mast cell degranulation. Our work supports a need to re-examine the nature of mast cell infiltration and activation within the skin and its relationship to the perivascular cellular infiltration observed.

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