

No evidence of increased serum substance P levels in chronic urticaria patients with and without demonstrable circulating vasoactive factors

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Summary

The neuropeptide substance P is a possible candidate as histamine-releasing factor in some patients with chronic ordinary urticaria (COU), particularly in those with evidence of a nonautoantibody circulating histamine-releasing factor. In this study, serum substance P levels were measured by enzyme immunoassay in 117 COU patients, 40 atopic subjects and 24 normal subjects. *In vivo* and *in vitro* assays for histamine-releasing factors, autologous serum skin test (ASST) and basophil histamine release (BHR) assay, respectively, were performed in all COU patients. Mean serum substance P concentration was not significantly different in COU patients and in normal subjects; however, significantly higher levels were detected in atopic subjects than in COU patients ($P < 0.003$). ASST and BHR assays allowed us to distinguish different subsets of COU patients. Mean serum substance P concentration did not vary significantly in the different subsets of patients. Nevertheless, interestingly three patients with positive ASST and negative BHR assay showed very high substance P levels. These results suggest that substance P does not play an important role as histamine-releasing factor in COU in general but only in occasional patients in whom it might act as a trigger of urticarial symptoms.

Introduction

The demonstration that the intradermal injection of autologous serum causes a wheal and flare reaction in 30–60% of chronic ordinary urticaria (COU) patients has led to important advances in the understanding of this disorder.^{1–3} The fact that a significant proportion of patients with COU have an autoimmune disorder, due to circulating autoantibodies against high affinity IgE receptors (FcεRI) or IgE, is now largely accepted.⁴ These autoantibodies can induce mast cell activation mimicking the effect of allergen stimulation in allergic patients. Up to 60% of COU patients have a positive autologous serum skin test (ASST), but circulating autoantibodies

against FcεRI or anti-IgE can be demonstrated only in about half of them.⁵ The mechanism involved in the remaining 50% of ASST-positive patients is still undefined. Furthermore, it has been demonstrated that sera from COU patients containing anti-FcεRI antibodies retain their *in vivo* ability to induce a wheal and flare reaction also after depletion of IgG.⁶ It is therefore conceivable that histamine-releasing factors other than anti-FcεRI and anti-IgE autoantibodies can account for the *in vivo* activity of sera from COU patients. A preliminary, partial characterization of a mast cell-specific histamine-releasing factor with a molecular weight less than 30 kDa was reported by Kermani *et al.*;⁷ the nature of the serum histamine-releasing factor, however, remains elusive in a consistent proportion of COU patients.

In the attempt to find the serological mediator of whealing in COU patients with negative *in vitro* functional assays for histamine-releasing autoantibodies, we

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decided to measure serum substance P in different groups of patients with COU. Substance P is a peptide with a molecular weight of 1.34 kDa, which is released from nerve endings and has the capacity to provoke histamine release from skin mast cells, but not from human basophils;^{8,9} therefore, it may be a mediator of histamine release in ASST-positive COU patients when release from basophils can not be demonstrated.

Subjects and methods

Subjects

One-hundred and seventeen adult patients diagnosed as having COU at the allergy centre of Clinica S. Carlo (Paderno Dugnano, Milano, Italy) during the last year were selected for the study. COU was diagnosed on the basis of the recurrence of hives with or without angio-oedema for more than 6 weeks. All patients had active urticaria at the time of the study. In all cases, known causes of chronic or recurrent urticaria were ruled out by appropriate investigations. Physical urticarias were excluded as well. Five days after antihistamine therapy (cetirizine, loratadine, or fexofenadine in all cases) was stopped, an intradermal test with 0.05 mL of fresh autologous serum was carried out. ASST was performed and read at 30 min following the method by Sabroe *et al.*¹⁰ Intradermal injection of saline solution (0.9% w/v NaCl) was performed as negative control and skin prick test with 1 mg/mL histamine as positive control. Patients showing a wheal with a diameter at least 1.5 mm greater than the control saline solution were considered positive. Eighty-seven patients were strongly positive on ASST and 30 were negative. Sera from 40 atopic subjects (20 with allergic rhinitis, 20 with allergic bronchial asthma) and 24 healthy subjects were used as control.

Basophil histamine release assay

Leucocyte suspensions from normal blood donors were prepared by dextran sedimentation of peripheral venous blood, anticoagulated with 0.01 M EDTA, and mixed with 6% dextran in saline solution (Plander 70, Fresenius Kabi Italia, Isola della Scala, Verona, Italy) and 30 mM dextrose (Sigma Chemical Co., St Louis, MO, USA). The cells were allowed to settle for 60–90 min at room temperature, according to Lichtenstein *et al.*¹¹ The leucocyte-rich plasma was aspirated and centrifuged at $300 \times g$ for 15 min at 4 °C, and the cell button was washed twice in Tyrode's buffer, pH 7.4, containing 140 mM NaCl, 5.5 mM dextrose, 2.7 mM KCl, 0.36 mM

NaH₂PO₄, and 12 mM NaHCO₃. Leucocytes (with $\approx 7 \times 10^4$ basophils) were then resuspended in 100 µL volume of Tyrode's buffer with 1.8 mM CaCl₂ and 0.5 mM MgCl₂, and incubated with 100 µL of the serum under examination, making a final volume of 200 µL. After incubation for 40 min at 37 °C, the reaction was stopped by addition of 800 µL of ice-cold buffer solution and centrifugation at $1000 \times g$ for 10 min at 4 °C. After centrifugation, the supernatants were aspirated, mixed with an equal volume of 6% HClO₄ and centrifuged at $1000 \times g$ for 10 min at 4 °C. Histamine concentration in the supernatants was measured by an automated fluorometric method, according to Ruff *et al.*¹² Spontaneous histamine release was evaluated by measuring histamine concentration in the supernatant of unstimulated cells, incubated for 40 min at 37 °C. Total histamine content was obtained by adding 100 µL of 6% HClO₄ to 100 µL of cell suspension. Net histamine release was calculated as percentage of total histamine content, after subtraction of spontaneous release. A 5% release cut-off value was used. Sera were tested with leucocyte suspensions from three normal donors whose basophils were previously shown to release 30% of total histamine content on challenge with an optimal dose of goat polyclonal antihuman IgE (Sigma Chemical Co.; 10 µg/mL). One assay per serum was performed to detect histamine-releasing activity. Histamine release assay was carried out using the same sera used for ASST.

Substance P determination

Serum substance P concentration was measured by a competitive enzyme immunoassay (R & D Systems, Minneapolis, MN, USA) with a sensitivity of less than 8 pg/mL. After drawing venous blood from the subjects under examination, serum was frozen at –80 °C until assayed for substance P concentration.

Statistical analysis

Results were expressed as mean $\pm$ SEM. Mann–Whitney *U*-test and Spearman rank correlation test were used for statistical analysis. *P*-values < 0.05 were considered significant.

Results

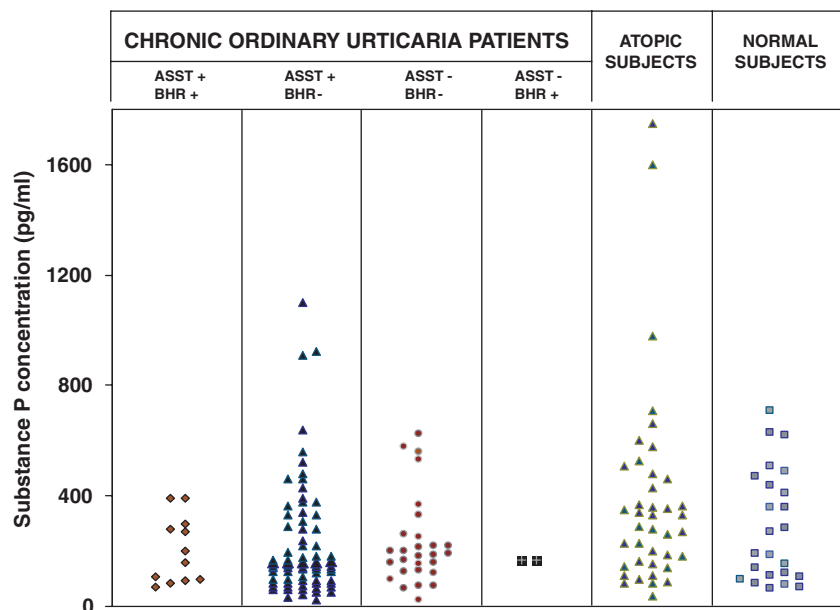
Mean substance P concentration was 221.94 ± 17.28 pg/mL in COU patients and 290.29 ± 41.36 pg/mL in normal subjects (*P* non-significant; Table 1). Basophil histamine release (BHR) assay was

Table 1 Serum substance P levels (pg/mL) in patients with chronic ordinary urticaria (COU), atopic subjects and normal subjects.

	Patient category	<i>n</i>	Mean $\pm$ SEM	Median (range)	<i>P</i>
1	COU patients	117	221.94 $\pm$ 17.28	160 (10–1100)	1 vs. 2: < 0.003 1 vs. 3: n.s.
2	Atopic subjects	40	386.25 $\pm$ 57.01	310 (36–1750)	2 vs. 3: n.s.
3	Normal subjects	24	290.29 $\pm$ 41.36	230 (65–620)	

Table 2 Serum substance P levels (pg/mL) in patients with COU divided into different subsets according to results of ASST and BHR assays.

	Patient subset	<i>n</i>	Mean $\pm$ SEM	Median (range)	<i>P</i>
1	ASST-positive	87	220.33 $\pm$ 21.12	156 (31–1100)	1 vs. 2: n.s.
2	ASST-negative	30	226.6 $\pm$ 28.7	182.5 (10–625)	
3	ASST-positive/BHR-positive	12	203.25 $\pm$ 34.42	180 (70–390)	3 vs. 4: n.s. 3 vs. 5: n.s. 3 vs. 6: n.s.
4	ASST-positive/BHR-negative	75	223.07 $\pm$ 23.92	155 (10–1100)	
5	ASST-negative/BHR-negative	28	230.64 $\pm$ 30.64	187.5 (10–1100)	5 vs. 6: n.s.
6	ASST-negative/BHR-positive	2	170	170	

Figure 1 Serum substance P levels in patients with chronic ordinary urticaria (*n* = 117), divided in subgroups according to results of ASST and BHR assays, in atopic (*n* = 40) and normal subjects (*n* = 24).

positive in 12 ASST-positive patients (13.7%) and in two ASST-negative patients (6.6%); mean percent release was $7.3 \pm 0.7\%$ Table 2. No significant correlation was found between percent histamine release and serum substance P concentration in ASST-positive patients with a positive BHR assay. If COU patients were grouped according to results of ASST and BHR assay, no difference in serum substance P concentration was observed between patients who tested positive or negative on ASST or on BHR assay. Nevertheless, interestingly 3/75 patients positive on ASST and

negative on BHR assay showed high levels of serum substance P (1100, 925 and 910 pg/mL, respectively) (Fig. 1).

Altogether mean serum substance P level in atopic subjects was 386.25 ± 57.01 pg/mL, significantly higher than in COU patients ($P < 0.003$). However, rhinitic subjects showed higher levels than COU patients (470.95 ± 98.37 pg/mL, $P < 0.0003$), whereas levels in asthmatic subjects were not significantly different from those in COU patients (301.55 ± 53.93 pg/mL; P non-significant).

Discussion

There are several data suggesting that the pathogenesis of COU may be heterogeneous.^{2,13} Some patients are negative on ASST, and only part of those positive on ASST have histamine-releasing autoantibodies. We have recently found that sera from nearly all ASST-positive patients with COU are able to induce histamine release *in vitro* either from basophils or from skin mast cells.¹⁴ However, the serum from each single patient seems to show its maximal activity on autologous mast cells, and functional *in vitro* tests seem less sensitive than ASST in the detection of patients with autoimmune COU and show much variability. Therefore, the pathogenetic mechanism of COU remains elusive in a substantial fraction of patients. In the present study, the proportion of ASST-positive patients showing significant histamine release on the BHR assay was lower than in other published series, probably because only one *in vitro* assay/serum was performed.^{3,13,15} This confirms that functional *in vitro* tests are less sensitive than ASST in the detection of serum histamine-releasing factors and give quite variable results in relation to variations in density of free IgE receptors, surface IgE and individual basophil releasability.

The COU patients with positive ASST and negative BHR assay were good candidates for having a circulating histamine-releasing factor other than anti-FcεRI or anti-IgE autoantibodies. In a previous work we excluded a role for stem cell factor, a growth factor able to induce histamine release from human skin mast cells, but not from human basophils.¹⁶ In the present study we looked for a possible role played by substance P, another substance able to induce histamine release from human skin mast cells but not from human basophils.^{8,9} Possible substance P involvement in COU was also suggested by the enhanced cutaneous response to its intradermal injection in COU patients.¹⁷

Substance P concentrations did not differ significantly in COU patients and in normal subjects, nor did they differ in COU patients according to ASST and BHR assay results. These results do not support an important role for substance P as histamine-releasing factor in COU patients, and are in line with the findings of Kermani *et al.*⁷ who showed that a substance P receptor antagonist did not inhibit *in vitro* histamine release induced by sera from COU patients.

However, high levels of circulating substance P were observed in three patients showing positive ASST and negative BHR assay. This suggests that, at least in occasional COU patients, substance P might contribute to the triggering of symptoms of urticaria. It is also

interesting that atopic subjects had higher serum substance P levels than COU patients, in agreement with previous observations showing increased levels in patients with allergic rhinitis, bronchial asthma and atopic dermatitis.^{18–20} Furthermore, the lack of raised serum levels of this neuropeptide in the majority of COU patients does not definitely exclude a pathogenetic role as a direct contact between nerve fibres and mast cells has been demonstrated,²¹ and substance P released from nerve fibres might cause skin mast cell degranulation in certain conditions. Substance P release in the skin microenvironment might be insufficient to cause its increase in blood concentration, and this might account for our results. Finally, the absence of raised serum levels might be also explained either by rapid binding of substance P to mast cell receptors or by its rapid inactivation.²²

A previous study on the effects of spantide, a competitive inhibitor of substance P, suggested the involvement of this neuropeptide in allergic contact urticaria and in the reaction to tuberculin.²³ Definite elucidation of the role of substance P in COU requires testing of specific antagonists on ASST and disease activity. These clinical assays might be worthwhile as clear evidence of histamine-releasing autoantibodies can be provided only in a minority of COU patients and histamine-releasing factors other than immunoglobulins might be involved in the disease.

References

- Grattan CE, Wallington TB, Warin RP *et al.* A serological mediator in chronic idiopathic urticaria: a clinical, immunological, and histological evaluation. *Br J Dermatol* 1986; **114**: 583–90.
- Greaves MW. Chronic urticaria. *N Engl J Med* 1995; **332**: 1767–72.
- Asero R, Tedeschi A, Lorini M *et al.* Chronic urticaria: novel clinical and serological aspects. *Clin Exp Allergy* 2001; **31**: 1105–10.
- Hide M, Francis DM, Grattan CE *et al.* Autoantibodies against the high affinity IgE receptor as a cause of histamine release in chronic urticaria. *N Engl J Med* 1993; **328**: 1599–604.
- Greaves MW, O'Donnell BF. Not all chronic urticaria is 'idiopathic'. *Exp Dermatol* 1998; **7**: 11–3.
- Fagiolo U, Kricek F, Ruf C *et al.* Effects of complement inactivation and IgG depletion on skin reactivity to autologous serum in chronic idiopathic urticaria. *J Allergy Clin Immunol* 2000; **106**: 567–72.
- Kermani F, Niimi N, Francis DM *et al.* Characterization of a novel mast-cell specific histamine releasing activity in chronic idiopathic urticaria (CIU) [Abstract]. *J Invest Dermatol* 1995; **105**: 452.

- 8 Miadonna A, Tedeschi A, Leggieri E *et al.* Activity of substance P on human skin and nasal airways. *Ann Allergy* 1988; **61**: 220–3.
- 9 Miadonna A, Leggieri E, Tedeschi A *et al.* Study of the effect of some neuropeptides and endogenous opioid peptides on in vitro histamine release from human lung mast cells and peripheral blood basophils. *Agents Actions* 1988; **25**: 11–6.
- 10 Sabroe RA, Grattan CE, Frances DM *et al.* The autologous serum skin test: a screening test for autoantibodies in chronic idiopathic urticaria. *Br J Dermatol* 1999; **140**: 446–52.
- 11 Lichtenstein LM, Osler AG. Study on the mechanism of hypersensitivity phenomena. IX. Histamine release from human leukocytes by ragweed pollen antigen. *J Exp Med* 1964; **120**: 507–30.
- 12 Ruff F, Saindelle A, Dutripon E, Parrot JL. Continuous automatic fluorometric evaluation of total blood histamine. *Nature* 1967; **214**: 279–81.
- 13 Sabroe RA, Fiebiger E, Francis DM *et al.* Classification of anti-Fc γ RI and anti-IgE autoantibodies in chronic idiopathic urticaria and correlation with disease severity. *J Allergy Clin Immunol* 2002; **110**: 492–9.
- 14 Asero R, Lorini M, Chong SU *et al.* Assessment of histamine-releasing activity of sera from patients with chronic urticaria showing positive autologous skin test on human basophils and mast cells. *Clin Exp Allergy* 2004; **34**: 1111–4.
- 15 Zweiman B, Valenzano M, Atkins PC *et al.* Characteristics of histamine-releasing activity in the sera of patients with chronic idiopathic urticaria. *J Allergy Clin Immunol* 1996; **98**: 89–98.
- 16 Asero R, Tedeschi A, Lorini M *et al.* Circulating stem cell factor in patients with chronic idiopathic urticaria. *Ann Allergy Asthma Immunol* 2003; **91**: 79–81.
- 17 Borici-Mazi R, Kouridakis S, Kontou-Fili K. Cutaneous responses to substance P and calcitonin gene-related peptide in chronic urticaria. the effect of cetirizine and dimethindene. *Allergy* 1999; **54**: 46–56.
- 18 Zhang RX. Substance P in plasma of patients with allergic rhinitis and its clinical significance. *Zhonghua Er Bi Yan Hou Ke Za Zhi* 1993; **28**: 72–3.
- 19 Cardell LO, Uddman R, Edvinsson L. Low plasma concentrations of VIP and elevated levels of other neuropeptides during exacerbations of asthma. *Eur Respir J* 1994; **7**: 2169–73.
- 20 Toyoda M, Nakamura M, Makin T *et al.* Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. *Br J Dermatol* 2002; **147**: 71–9.
- 21 Wiesner-Menzel L, Schulz B, Vakilzadel F, Czarnetzki NM. Electron microscopical evidence for a direct contact between nerve fibres and mast cells. *Acta Dermatovener* 1981; **61**: 465–9.
- 22 Schaffalitzky De Muckadell OB, Aggestrup S, Stentoft P. Flushing and plasma substance P concentration during infusion of synthetic substance P in normal man. *Scand J Gastroenterol* 1986; **21**: 498–502.
- 23 Wallengren J. Substance P antagonist inhibits immediate and delayed type cutaneous hypersensitivity reactions. *Br J Dermatol* 1991; **124**: 324–8.