

Increased Plasma Levels of Substance P in Vernal Keratoconjunctivitis

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Purpose. The increase of nerve growth factor (NGF) plasma levels in vernal keratoconjunctivitis (VKC) patients has been demonstrated previously. Results of numerous studies in vitro and in vivo have shown that NGF modulates the synthesis of substance P (SP), a neuropeptide involved in the pathogenesis of human allergic diseases. In this study the involvement of SP in this allergic conjunctivitis is investigated, along with its relationship with NGF and other systemic and local markers of VKC.

Methods. Competitive radioimmunoassays were used to detect the levels of SP in plasma, the levels of eosinophil cationic protein, and the total and specific immunoglobulin E in the serum of 11 patients with VKC and in 11 healthy matched controls. Plasma levels of nerve growth factor (NGF) were measured in all VKC patients and controls using an immunoenzymatic assay. Histologic evaluation was performed in tarsal and bulbar conjunctival specimens obtained in biopsies from 8 VKC patients and 4 control subjects.

Results. Patients with VKC show a significant increase of SP and NGF plasma levels ($P < 0.003$ and $P < 0.001$, respectively), and an increase of eosinophil cationic protein and immunoglobulin E levels in the serum ($P < 0.001$ and $P < 0.002$, respectively). Mast cells, eosinophils, and lymphocytes were also significantly increased in the conjunctiva of VKC patients. Interestingly enough, VKC patients with the highest NGF plasma levels also showed the highest SP levels.

Conclusions. The data show the involvement of SP in VKC and suggest that SP with NGF could modulate the allergic response in this disease, probably through an interaction with inflammatory cytokines. Invest Ophthalmol Vis Sci. 1997;38:2161–2164.

Vernal keratoconjunctivitis (VKC) is a chronic, bilateral, allergic inflammation of the conjunctiva

with seasonal recrudescence of symptoms, characterized by itching, hyperemia, chemosis, photophobia, sticky mucous discharge, and giant papillary hypertrophy of the upper tarsal conjunctiva. Although several aspects of this allergic inflammation have been elucidated, the complex interaction between cells and inflammatory mediators has not been clarified yet. In conjunctival tissue, eosinophils, mast cells, and lymphocytes are active participants in promoting and perpetuating the inflammation through the complex action of cell-derived products and cytokines. However, the cell-to-cell signal interaction does not seem to be limited to the eye, and recent evidence shows that the immune and nervous systems also seem to be involved in the modulation of the inflammatory process in VKC. Total and specific immunoglobulin E (IgE), as well as eosinophil cationic protein (ECP) and eosinophil derived neurotoxin are increased in the serum of patients with VKC, demonstrating the systemic involvement of the immune system.¹ Moreover, we reported increased plasma levels of nerve growth factor (NGF) in patients with VKC and a significant correlation between NGF levels and the number of mast cells infiltrating the conjunctiva.² Knowing that NGF affects immune cells, which play a key role in allergic inflammation, we hypothesized that this neurotrophin could be involved in the pathogenesis of VKC, acting together with inflammatory cytokines and neuropeptides. Results of several studies have shown that NGF regulates the synthesis and release of substance P (SP), one of the most studied neuropeptides.³ Substance P is an undecapeptide of the tachykinin family (peptides with the common C-terminal sequence *Phe-X-Gly-Leu Met-NH₂*); that is, it is involved in the ocular inflammatory events through its release in the anterior segment. Recently, the involvement of SP in the pathogenesis of the allergic reaction was also hypothesized. In vitro, SP induces histamine release by mast cell degranulation; enhances migration, mediator release, and cytotoxic activity of eosinophils; and stimulates T-cell proliferation.⁴ Moreover, it has been demonstrated that this neuropeptide is released during allergen challenge in patients with asthma and rhinitis and that SP treatment influences the allergic process in patients with rhinitis and atopic dermatitis, inducing a wheal-and-flare reaction and the recruitment of inflammatory cells.^{5,6} Taken together, these findings suggest the possible involvement of SP in the inflammatory process of VKC. To gain further insight

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into the possible involvement of SP in ocular allergic inflammation, in the current study, we analyzed the SP plasma levels and their relations to the other systemic factors (NGF, ECP, and total IgE levels) and to local markers of VKC.

MATERIALS AND METHODS. Subjects and Procedure. Eleven patients (6 men, 5 women; age range, 4 to 32 years), with acute symptoms of VKC, and 11 matched control subjects (6 men, 5 women; age range, 9 to 27 years) with no history of atopic disease were included in the study. The diagnosis of VKC was based on the presence in early spring of recurrent symptoms of itching, photophobia, and tearing, associated with a mild to very severe cobblestone-like appearance of the upper tarsal conjunctiva, mucous discharge, epithelial keratopathy, and the presence of at least two eosinophils per field (magnification, $\times 100$) in the conjunctival scraping. All patients remained without treatment for at least 1 week before the study was performed. The number of circulating peripheral eosinophils, the ECP serum levels, and the total (paper-radioimmunosorbent test) and specific (radioallergosorbent test) IgE in a panel of common allergens were measured in patients and in control subjects. Venous peripheral blood was collected in spring during the most symptomatic phase of the disease. After centrifugation, the plasma was stored at -70°C until assay for NGF and SP. Conjunctival biopsy was performed for histologic examination in 8 patients and 4 control subjects. The tenets of the Declaration of Helsinki were followed, informed consent was obtained, and institutional human experimentation committee approval was granted.

Determination of Substance P Levels. A highly specific radioimmunoassay (RIA) was used (detection limit $1.5\text{ fmol} = 2\text{ pg}$ per incubate; detectable concentration $15\text{ fmol/l} = 20\text{ pg/ml}$). Plasma samples were extracted twice with two volumes of acidic acetone (97% acetone and 3% hydrochloric acid), according to Chang and Leeman's modified procedure.⁷ After centrifugation, the supernatant was vacuum treated to remove the organic phase, and the lyophilization of the aqueous phase was carried out overnight. The lyophilized samples were taken up with $300\text{ }\mu\text{l}$ of assay buffer (0.1 M phosphate buffer, pH 7.4, containing 0.1% wt/vol bovine serum albumin, 0.01% sodium azide, 0.09% sodium chloride and 1 mM EDTA) and diluted as needed to perform the radioimmunoassay using ^{125}I -SP. A volume of standard SP or unknown samples was incubated with a volume of antiserum and a volume of tracer (5000 cpm) and the tubes incubated further for 20 hours at 4°C . The antigen-antibody complex was separated from free antigen by adding a peg/gar/nrs solution. After centrifugation, the supernatant was discarded, and the radioactivity

of the bound fraction was measured in a gamma counter for 60 seconds. Recovery of SP in this procedure was approximately 60%. Unknown values were calculated by comparing the mean counts per minute of duplicate samples with a calibration curve of standard samples ranging from 20 to 1200 pg/ml. ^{125}I -(p-HP)-Argyl-SP was used as a tracer, with a specific activity of 60 to 65 TBq/mol with 50% to 60% binding to the antibody in a dilution of 1:240,000. The antibody (M27 from guinea pig) was directed against the C-terminal and the N-terminal sequence of SP. The results were expressed as picograms per milliliter of SP, and all results were provided as mean values $\pm$ SEM. All assays were performed in triplicate.

Determination of Nerve Growth Factor Levels.

The NGF level was measured by a highly sensitive, two-site, immunoenzymatic assay that recognizes human and murine NGF and is capable of detecting levels as low as 5 pg/ml, as previously described.² Data were presented as picograms per milliliter, and all results were provided as mean values $\pm$ SEM. All assays were performed in triplicate.

Histologic Evaluation. Conjunctival biopsy specimens were obtained from eight patients (four men, four women; age range, 8 to 32 years) with VKC and from four control subjects (two men, two women; age range, 3 to 19 years). Tarsal and bulbar conjunctival specimens were taken from each patient after anesthesia with subconjunctival 2% mepivacaine hydrochloride. Specimens were fixed in 100 mM phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde. The specimens were stored in 20% sucrose, 0.1 M PBS (pH 7.4) and sectioned at $6\text{ }\mu\text{m}$ with a freezing microtome. The slides were then stained with hematoxylin-eosin (to identify lymphocytes and eosinophils) or toluidine blue acidic solution (to identify mast cells) and evaluated by light microscopy. Lymphocytes, eosinophils, and mast cells were counted in three fields (magnification, $\times 40$) for each of three randomly selected sections. Data are presented as mean values $\pm$ SEM.

Statistical Analysis. Nonparametric statistical analysis (Mann-Whitney test) was performed to compare in VKC patients and control subjects the levels of SP, NGF, ECP, and total IgE; the number of eosinophils in the circulating blood; and the number of mast cells, eosinophils, and lymphocytes infiltrating the conjunctiva. The Spearman rho test was performed to correlate SP plasma levels with NGF plasma levels, total IgE serum levels, and with the number of eosinophils, mast cells, and lymphocytes in the conjunctiva of VKC patients. A probability of 0.05 or less was considered statistically significant.

RESULTS. In analyzing the data collected in our study, we observed that SP plasma levels were higher in

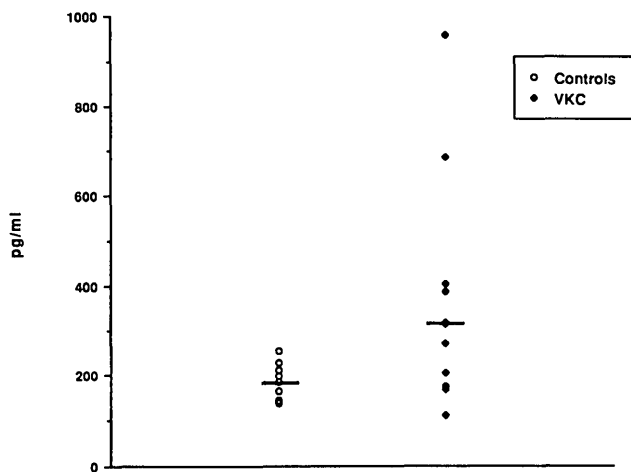


FIGURE 1. Substance P (SP) plasma concentration in vernal keratoconjunctivitis (VKC) patients and control subjects. The horizontal bars represent the medians of the values. Readings in the two groups differ significantly ($P < 0.001$).

patients affected by VKC (362.3 ± 75.9 pg/ml; median, 314 pg/ml) than those in healthy control subjects (184.4 ± 12.4 pg/ml; median, 188.5 pg/ml; $P < 0.003$; Fig. 1).

Compared with levels in healthy subjects, the VKC patients also exhibited an increased number of circulating eosinophils, (381.2 ± 84.6 versus $193.5 \pm 26.4 \times \mu\text{l}$; $P < 0.02$) and enhanced serum levels of total IgE (116.4 ± 36.8 KU/l versus 50.5 ± 10.7 KU/l, $P < 0.002$) and ECP (45.4 ± 11.3 $\mu\text{g/ml}$ versus 6.7 ± 1.95 $\mu\text{g/ml}$, $P < 0.001$; Table 1). Moreover, NGF levels were dramatically increased in the plasma of VKC patients ($11,037 \pm 10,641$ pg/ml; median, 130 pg/ml; $P < 0.001$) compared with levels in the plasma of control subjects (47.5 ± 8.5 pg/ml; median, 42.5 pg/ml).

Although there was no significant correlation between SP and NGF plasma levels ($r = 0.57$; $P = 0.07$), it is interesting to note that two VKC patients with the high-

est SP levels also showed the highest NGF levels (956 pg/ml of SP and 17,430 pg/ml of NGF; and 684 pg/ml of SP and 2,363 pg/ml of NGF, respectively; Table 1). There was no significant correlation between SP plasma levels and the other systemic markers, including total IgE, ECP serum levels, and circulating eosinophils.

Results of histologic analysis showed that patients with VKC displayed a significant increase in the number of lymphocytes (29.6 ± 4.2 per field versus 15 ± 2.1 per field; $P < 0.01$), eosinophils (7.1 ± 1.6 per field versus 0.2 ± 0.2 per field; $P < 0.001$), and mast cells (6.7 ± 1.5 per field versus 3.1 ± 1.1 per field; $P < 0.05$) in the bulbar conjunctiva when compared with controls. Similar significant differences were found in the number of lymphocytes (61.4 ± 12.7 per field versus 20 ± 3.2 per field), eosinophils (26.4 ± 11.1 per field versus 0.5 ± 0.5 per field), and mast cells (18.2 ± 2.2 per field versus 4 ± 0.7 per field) in the tarsal conjunctiva of VKC patients compared with those seen in control subjects ($P < 0.01$; $P < 0.001$; $P < 0.01$, respectively). Results of statistical analysis showed no significant correlation between the SP plasma levels and the inflammatory cell types infiltrating the conjunctiva.

DISCUSSION. Our findings demonstrate that SP plasma levels are increased in patients with VKC. The hypothesis of SP's involvement in the inflammatory reaction of VKC is strongly suggested by evidence of the biologic effects exerted by this neuropeptide in vitro and in vivo on the cells that play a major role in VKC. In vitro, SP induces degranulation of mast cells; enhances migration, mediator release, and cytotoxic activity of eosinophils; and stimulates T-cell proliferation and activation.⁴ Moreover, in vitro, SP induces fibroblasts, endothelial, and smooth muscle cell proliferation, suggesting that SP may be involved in fibrovascular proliferation (papillae), a clinical feature in

TABLE 1. Characteristics of Patients With Vernal Keratoconjunctivitis

Patient Number	Age (yr), Sex	Previous and Associated Atopic Diseases	Substance P (pg/ml)	NGF (pg/ml)	Total IgE (kU/l)	RAST Positive*	ECP ($\mu\text{g/l}$)	Circulating Eosinophils ($\times \mu\text{l}$)	Biopsy
1	15, M	—	956	117,430	142	—	23.5	220	No
2	23, F	Asthma, rhinitis, eczema	684	2363	133	Par. off., Dph.	21.8	200	Yes
3	16, M	Rhinitis	404	121	39	Gram., Par. off.	47.5	540	Yes
4	7, M	—	387	77	44	—	53.9	600	Yes
5	21, F	Rhinitis	318	130	37	—	8	100	Yes
6	4, F	—	314	114	15	—	ND	ND	No
7	8, M	—	270	701	13	Gram., Par. off.	32.4	290	Yes
8	8, M	Eczema	203	10	407	Gram., Par. off., Dph.	108.6	800	Yes
9	32, F	—	174	167	196	Dph.	ND	ND	Yes
10	10, F	—	165	274	30	Par. off., Dph.	ND	ND	Yes
11	7, M	Asthma	110	20	224	Gram., Par. off., Dph.	67.3	300	No

ECP = eosinophil cationic protein; NGF = nerve growth factor; ND = not done.

*Serum measurements of specific IgE include rye grass (Gram.), Dermatophagoides pteronyssinus (Dph.), Parietaria officinalis (Par. off.), Olea Europaea, milk, and egg proteins. We considered patients RAST positive who showed even a class 1 positivity for at least one of the allergens tested.

VKC that still has to be clarified. Substance P is released during allergen challenge in patients with asthma and rhinitis and induces a wheal-and-flare reaction and recruitment of inflammatory cells in patients with rhinitis and atopic dermatitis.^{5,6} Indeed, findings in studies in vitro and in vivo underline the biologic role of SP in the modulation of cytokine synthesis. In particular, in vitro, SP stimulates the release of interleukin (IL)-1-like activity from the murine macrophage cell line and induces human peripheral blood monocytes to release IL-1, IL-6, and tumor necrosis factor- α (TNF- α);⁸ in vivo, it provokes an enhanced expression of the messenger RNA for IL-1 β , IL-3, IL-5, and IL-6; TNF- α ; and interferon- γ in all mucosal samples of allergic subjects.⁹ These cytokines exert a well-known effect in allergic inflammation: In fact, IL-3 and IL-5 activate eosinophils and basophils, and TNF- α has a proinflammatory role in regulating eosinophil function. Interestingly enough, IL-1, IL-6, TNF- α , and interferon- γ are also potent inducers of NGF synthesis in peripheral tissues and in the central nervous system.¹⁰

Our data confirm those reported previously, showing the production of SP by several immune cells (eosinophils and mast cells), which are the principal effectors in the allergic inflammatory process.⁴

Moreover, results of several studies in vivo and in vitro have shown that NGF, a neurotrophin involved in the inflammatory process and in several human immune diseases, stimulates the synthesis and release of SP in the central and peripheral nervous systems. We have demonstrated previously that VKC patients have a notable increase of circulating NGF, which correlates significantly with the number of conjunctival mast cells and correlates inversely with ECP serum levels and the number of circulating eosinophils.² In this context, it is worth mentioning that two patients with the highest NGF plasma levels showed the highest SP levels.

However, in our current findings, there was no significant correlation between serum levels of NGF, ECP, and circulating eosinophils and SP serum levels. Two hypotheses may be advanced for the lack of correlation. First, it is possible that the increased SP levels are an epiphenomenon of the inflammatory reactions related to other physiological events not directly associated with the pathogenesis of VKC. Second, the increased SP levels may be modulated by a complex network of several neuroimmune factors involved in

the pathogenesis of VKC, including NGF and the different inflammatory cytokines.

In conclusion, our results showing that patients with VKC display high plasma levels of SP represent, to the best of our knowledge, a novel observation, suggesting that this neuropeptide plays a functional role in the pathogenesis of this ocular disease, probably acting in concert with cytokines and neurotrophins to modulate the allergic inflammation.

Key Words

allergic disease, nerve growth factor, substance P, vernal keratoconjunctivitis

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