Altered Expression of Neurotransmitter Receptors and Neuromediators in Vernal Keratoconjunctivitis
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Background: There is growing evidence that autonomic innervation is involved in the pathogenesis of mucous hypersecretion, goblet cell hyperplasia, and conjunctival hyperreactivity.

Objective: To determine the expression of neurotransmitters and neurotransmitter receptors in vernal keratoconjunctivitis (VKC) tissues to evaluate whether neurogenic inflammation plays a role in this ocular atopic-related disorder.

Methods: Biopsy specimens of upper tarsal conjunctiva from 8 VKC patients with active inflammation and from 4 healthy subjects were processed for immunohistochemistry using anti-M1, anti-M2, and anti-M3 muscarinic receptors; β1-adrenergic receptor; vasoactive intestinal peptide; nerve growth factor; and protein gene product 9.5, a marker of nerve fibers.

Results: In the conjunctival epithelium of VKC patients, M1 muscarinic receptor, nerve growth factor, and protein gene product 9.5 expression were decreased, whereas M2 and M3 muscarinic receptors and β1-adrenergic receptor were irregularly distributed, compared with control subjects. Neurotransmitter receptors and vasoactive intestinal peptide expression were increased in the substantia propria–localized infiltrate of VKC compared with healthy tissue. Nerve growth factor and protein gene product 9.5 staining was also enhanced in the conjunctival stroma of VKC vs healthy conjunctiva.

Conclusions: The inflamed conjunctiva of VKC patients demonstrated an obvious alteration in muscarinic and β1-adrenergic receptor, vasoactive intestinal peptide, protein gene product 9.5, and nerve growth factor expression. These results substantiate the involvement of an autonomic dysfunction in the pathogenesis of VKC.

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In recent years, the role of the nervous system in immunology has been under investigation, and new data have accumulated with regard to the neurogenic control of physiologic and pathological conditions, referring especially to allergic and autoimmune diseases.1-7 The endocrine system also participates in this exchange of information, with interaction and integration of these 3 systems ensuring a constant control of homeostasis. This neuroendocrine-immunological interaction occurs at the systemic level through activation of the hypothalamic-hypophysio-adrenal axis, at the regional level through interaction of nerve fibers with primary and secondary immune organs, and at the local level through release of neuromediators into the inflamed tissue.8

Several studies report interplay of neural and endocrine systems in the eye, particularly for control of tear fluid secretion by the lacrimal gland and of mucous secretion by conjunctival goblet cells9-11 and in the control of corneal epithelial proliferation in rats.12 Recently, the existence of a lacrimal functional unit, composed of the lacrimal gland, cornea, conjunctiva, and their related innervation, was proposed to play a major role in the pathogenesis of dry eye disease.13,14

Vernal keratoconjunctivitis (VKC) is the clinical manifestation of complex pathogenic mechanisms, some leading to systemic neuroendocrine changes.15 Patients are usually boys or young adults whose clinical manifestations tend to abate around puberty, when great hormonal changes occur. Patients' exaggerated hyperreactivity to nonspecific stimuli (such as heat, sun, and wind) during active and nonactive phases of the disease indicates neural involvement.16,17 Furthermore, increased plasma levels of nerve growth factor (NGF) have been found,18 and its high-affinity receptor, TrKA, which is normally expressed in the conjunctival epithe-
Junctival epithelium and stroma of healthy and inflamed conjunctiva of VKC patients. Moreover, elevated levels of substance P, a neuropeptide released during allergen challenge and a basic mediator of neurogenic inflammation, was found in tears and plasma of patients affected by VKC. Neuronal stimulation participates in the symptoms of allergic diseases (sneezing, coughing, itching, and ocular irritation), and allergic inflammation activates local neuronal activity. Anatomical and pharmacological communication occurs between peripheral nerves and immune cells (mast cells, eosinophils, and lymphocytes), inducing various important functions, such as secretion of cytokines and liberation of neuropeptides and growth factors. Healthy conjunctiva also expresses some adrenergic and muscarinic receptors along its epithelium layer. Healthy conjunctiva also expresses some adrenergic and muscarinic receptors along its epithelium layer.

This study aimed to identify the presence of various neurotransmitter receptors, a neuropeptide, and a neurotransphin in the healthy conjunctiva of healthy subjects and in the inflamed conjunctiva of VKC patients. Expression of the protein gene product (PGP) 9.5, a neuron-specific ubiquitin C-terminal hydrolase, was used as a marker of nerve fibers. The M1, M2, and M3 cholinergic muscarinic receptors; the β1-adrenergic receptor (β1-AR); the neuropeptide vasoactive intestinal peptide (VIP); and a neurotransphin (NGF) were identified in the conjunctival epithelium and stroma of healthy and inflamed conjunctiva.

METHODS

PATIENTS AND HEALTHY SUBJECTS

Research followed the tenets of the Declaration of Helsinki and was approved by an institutional review board. Informed consent was obtained from patients or parents in the case of minors. Eight patients (7 males and 1 female) affected by the tarsal form of VKC, diagnosed by clinical history, signs, and symptoms, participated in this study. They were between the ages of 7 and 16 years. Five patients were positive for VKC according to specific IgE in serum and/or to the prick test result for at least 1 allergen, most to Graminaceae and/or Dermatophagoides. Four patients were affected by a second allergic pathological feature (asthma, rhinitis, or eczema). Personal and family history and allergic test results of the remaining 3 patients were completely negative for extraocular allergic diseases.

Under local anesthesia, giant papillae characteristic of the tarsal form of VKC were removed from the upper tarsal conjunctiva of VKC patients. These samples were polygonal formations with a central core of blood vessels, from 1 to 8 mm wide. For control subjects, bulbar conjunctival specimens were obtained from otherwise healthy individuals at cataract surgery. These subjects had no sign of inflammation, had no clinical history of allergic conjunctivitis or atopic disorders, were not wearing contact lenses, and were not taking topical medications. Although bulbar conjunctiva is known to differ some-what from tarsal conjunctiva regarding the number of epithelial layers and the density of goblet cells, tarsal biopsy specimens from healthy age-matched subjects were impossible to procure because of ethical considerations. Nevertheless, these discrepancies were not critical because the differences between inflamed and healthy tissues were evident.

A histological examination was performed on 6-µm cryosections from control and patient conjunctival tissues. Sections were fixed in 4% buffered paraformaldehyde for 7 minutes, washed in tap water thoroughly, and hydrated in distilled water for 5 minutes. Hematoxylin-eosin (to better identify eosinophils) and Giemsa (to better identify neutrophils and mast cells) stains were used. Indirect immunohistochemistry was performed using the following rabbit polyclonal primary antibodies: anti–muscarinic receptors M1, M2, and M3, 1:6000 (R & D Antibodies, Benicia, Calif); anti–β1-AR, 1:500 (Santa Cruz Biotechnol-ogy, Inc, Santa Cruz, Calif); anti-VIP, 1:400 (DiaSorin, Stillwater, Minn); anti-NGF, 1:200 (Santa Cruz Biotechnology, Inc); and anti–PGP 9.5, 1:40 (Novocastra Laboratories Ltd, Newcastle upon Tyne, England). Biotinylated anti–rabbit secondary antibody (BioGenex, San Ramon, Calif) was used for all primary antibodies, with the exception of the PGP 9.5 primary antibody, for which the anti–mouse secondary antibody (BioGenex) was used.

Sections were fixed in 4% buffered paraformaldehyde for 7 minutes, then hydrated in phosphate-buffered saline (PBS) and treated with 3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidase activity. Subsequently, to block nonspecific binding, sections were treated with buffer containing 1% bovine serum albumin, 4% fetal bovine serum, and 0.3% Triton X-100 for 30 minutes at 37°C, and then incubated with primary antibodies diluted in fetal bovine serum at 4°C overnight.

After incubation, slides were carefully washed with PBS and treated with the secondary antibody for 20 minutes. After a second wash with PBS, slides were treated with peroxidase-conjugated streptavidin complex for 20 minutes and washed again with PBS. Last, a solution of chromogen diaminobenzidine and hydrogen peroxide was added to yield the colored end product. Sections were washed in PBS, stained with aqueous hematoxylin, washed in water, dehydrated with alcohol, cleared in xylene, and mounted.

Healthy conjunctiva not incubated with primary antibodies was used as a negative control for verification of immunostaining. Several tissues (brain, heart, bladder, and adrenal tissues) from 2 rats were used for positive controls. The intensity of staining was subjectively evaluated using a scoring system (0 indicates absent; 1, slight; 2, intense; and 3, very intense) with light microscopy, considering separately the epithelial layers and the density of goblet cells. These discrepancies were not critical because the differences between inflamed and healthy tissues were evident.

Tissues were embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek Europe BV, Zoeterwoude, the Netherlands) and immediately frozen and stored at −80°C. Biopsy specimens were sectioned by cryostat into 6- to 8-µm sections, placed on poly-L-lysine slides, and stored at −80°C.

IMMUNOHISTOCHEMISTRY

STATISTICAL ANALYSIS

Numerical staining scores for the VKC and control groups were analyzed using the Mann-Whitney test (nonparametric analysis). Results were considered significant at P < .05.

RESULTS

In healthy bulbar conjunctiva, stratified cylindrical epithelium with nongoblet epithelial cells, goblet cells, and
a healthy stroma were observed. The histological structure of inflamed giant papillae in VKC specimens appeared quite disorganized compared with healthy conjunctival tissue (Figure 1). Changes made it difficult to distinguish different layers of epithelium and a clear border between the epithelium and stroma. Tarsal conjunctiva acquired a pseudoglandular conformation: the epithelium dipped into the stroma, creating numerous digital indentations filled with mucus. Hyperplastic zones and thinned areas coexisted in the epithelium, with significant edema and intense inflammatory infiltration, also involving the stroma and completely altering the histological characteristics of the conjunctiva. Increased numbers of lymphocytes, eosinophils, and mast cells were noted in VKC samples compared with healthy samples (data not shown). In addition, the stroma showed signs of remodeling, such as hyperproduction of collagens, overgrowth of connective tissue, and proliferation of capillaries.

The \( \beta_1 \)-AR immunoreactivity was intense or very intense in all layers; however, there were no significant differences between healthy and inflamed tissues (Figure 2A and B).

Staining of M\(_1\) muscarinic receptors was observed over the entire thickness of healthy epithelium. In contrast, staining of M\(_1\) muscarinic receptors was negative or slightly positive in localized areas of pathological specimens (Figure 2C and D).

The expression of M\(_2\) muscarinic receptors was similar to that of M\(_1\) muscarinic receptors in healthy samples. In VKC biopsy specimens, staining was variable: intense in some areas and absent in others. The staining of M\(_2\) muscarinic receptors was also clearly evident in rare single cells of the epithelium (Figure 2E and F).

The staining of M\(_3\) muscarinic receptors was of a different pattern: immunoreactivity was clearly limited to the epithelial basal layer in healthy tissue. In comparison, immunostaining of M\(_3\) muscarinic receptors involved the whole thickness of the epithelium in VKC tissues (Figure 2G and H).

In the healthy control specimens, PGP 9.5 expression was intense and diffuse in the superficial epithelial layers and slight in the stroma, while in pathological conjunctiva, it was clearly decreased in the epithelium and unchanged or slightly increased in the stroma (Figure 3A and B).

Nerve growth factor staining was highly positive in all layers of the healthy epithelium and scattered in the stroma, whereas in VKC patients it was decreased in conjunctival epithelium and irregularly increased in the conjunctival stroma. Vascular endothelium showed moderate NGF staining only in VKC tissues (Figure 3C and D).

Vasoactive intestinal peptide immunoreactivity was restricted to isolated limited areas of the epithelium in healthy and pathological biopsy specimens (Figure 3E and F).

Except in scattered cells, conjunctival stroma of healthy samples did not express specific immunoreactivity for the M\(_1\), M\(_2\), and M\(_3\) muscarinic receptor, \( \beta_1 \)-AR, and VIP antibodies. In inflamed conjunctival stroma, increased immunostaining for the muscarinic receptors (M\(_1\), M\(_2\), and M\(_3\)), for \( \beta_1 \)-AR, and for VIP was remarkable (Figure 2 and Figure 3).

Statistical analyses (Table) demonstrated significantly reduced staining of PGP 9.5 (\( P = .04 \)), the M\(_1\) muscarinic receptor (\( P = .04 \)), and NGF (\( P = .05 \)) in the epithelium of VKC vs healthy biopsy specimens. The immunoreactivity of the other antibodies tested was not statistically significantly different between inflamed and healthy conjunctival epithelium. In VKC conjunctival stroma, the increased staining of the M\(_1\), M\(_2\), and M\(_3\) muscarinic receptors (\( P = .01 \) for all 3) and of VIP (\( P = .04 \)) was statistically significant compared with healthy tissues. Moreover, in pathological stromal tissue, PGP 9.5 (\( P = .04 \)) and NGF (\( P = .01 \)) expression was significantly increased, but it was not localized on specific cells.

**COMMENT**

Muscarinic and adrenergic receptors have been identified in healthy conjunctiva, where the corresponding autonomic, parasympathetic, and sympathetic pathways

![Figure 1. Histological features of healthy conjunctival tissues (A) and conjunctival tissues affected by vernal keratoconjunctivitis (VKC) (B) (Giemsa, original magnification ×40). The histological structure of inflamed giant papillae in VKC specimens appeared quite disorganized and filled with inflammatory cells compared with the healthy conjunctival tissue.](https://jamanetwork.com/doi/10.1001/archophthalmol.2005.350)
Figure 2. Immunohistochemical distribution of β1-adrenergic receptor (β1-AR) (A and B) and the M1 (C and D), M2 (E and F), and M3 (G and H) muscarinic receptors in healthy conjunctival tissues (A, C, E, and G) and conjunctival tissues affected by vernal keratoconjunctivitis (VKC) (B, D, F, and H). The expression of M1 muscarinic receptors was reduced in VKC epithelium, and M2 and M3 muscarinic receptors and β1-AR are irregularly distributed in VKC epithelium compared with healthy tissue. All 3 muscarinic receptors are highly expressed in VKC conjunctival stroma compared with healthy tissue.
regulate mucous and fluid secretion.\textsuperscript{10,23} We were not able to find any studies in the literature regarding the expression of neuroreceptors in inflamed conjunctiva of humans or animals.

The present study demonstrated a significantly reduced immunostaining for the M\textsubscript{1} muscarinic receptor in the conjunctival epithelium of VKC subjects compared with healthy tissues, while the M\textsubscript{2} and M\textsubscript{3} muscarinic receptors and $\beta_{1}$-AR showed, in VKC patients, an irregular, at times very intense, staining pattern in all epithelial layers. Stimulation of the M\textsubscript{2} and M\textsubscript{3} muscarinic receptors and, to a lesser extent, the M\textsubscript{1} muscarinic receptors activates mucous production by goblet cells.\textsuperscript{10} In the lung, the M\textsubscript{3} muscarinic receptors are known to be involved in the control of mucous production and in disorders characterized by mucous hypersecretion, such as asthma, chronic bronchitis, and rhinitis.\textsuperscript{26,27} The M\textsubscript{1} muscarinic receptors seem to play a role more in water out-
put control than in mucous regulation in healthy conjunctiva. The reduction in the M2 muscarinic receptor and the irregular distribution of the M3 and M5 muscarinic receptors observed in VKC conjunctival epithelium may lead to the production of mucus with a low water content and an increased stickiness, which is one of the typical clinical signs of this disease.

Pathological samples were characterized by a scattered and abnormal distribution and by an overall reduction of VIP and NGF staining compared with healthy tissues. Similarly, NGF messenger RNA expression was decreased in the nasal epithelium of patients affected by allergic rhinitis compared with healthy subjects, whereas there was an increased serum level of NGF. A down-regulation of epithelial NGF production may occur due to increased secretions of NGF from other sources. The PGP 9.5 distribution was also decreased in VKC conjunctival epithelium, indicating fewer nerve fibers. A reduction of epithelial cell–nerve communication in this tissue may lead to alterations in epithelial growth and goblet cell secretion.

In the conjunctival stroma of VKC tissues, numerous cells were positive for all antibodies tested: the M1, M2, and M3 muscarinic receptors and β2-AR. All cells were similar in appearance, larger than epithelial cells, and sparsely distributed or assembled in small clusters; they were not observed in the stroma of healthy conjunctiva. While only double staining would have identified them without a doubt, it is likely that they were immune cells, because they were present only in inflamed conjunctiva, which usually displays signs of cellular infiltration, mostly of eosinophils, mast cells, and helper T cell 2 lymphocytes.

Muscarinic receptors have been described on mast cells, which play a key role in the development of allergic diseases such as VKC. Their activation by muscarinic agonists seems to cause a decreased histamine release by mucosal mast cells but an increased histamine release by connective tissue mast cells, the latter of which are prevalent in VKC conjunctiva. Thus, muscarinic agonists may induce an increase in histamine release in VKC patients.

Peripheral blood lymphocytes also express muscarinic receptors and, in addition, can produce acetylcholine. In fact, increased expression of the M2 through M5 muscarinic receptors on lymphocytes has been correlated with the intensity of bronchial hyperresponsiveness in asthmatic subjects.

Not only neuromediators, but also inflammatory mediators, are known to act on neurotransmitter receptors. For example, activated eosinophils release eosinophil major basic protein, which is an endogenous antagonist for M2 muscarinic receptors. The M2 muscarinic receptors on parasympathetic nerves in the lungs normally inhibit release of acetylcholine. When the M2 muscarinic receptors are blocked by major basic protein, acetylcholine release is increased, resulting in bronchial hyperresponsiveness. A similar mechanism may occur in the VKC conjunctiva, where increased eosinophil products, such as major basic protein, may bind to the M2 muscarinic receptors on nerve fibers and epithelial and inflammatory cells, increasing acetylcholine release and regulating cell functions.

Vasoactive intestinal peptide is a neuropeptide present in parasympathetic nerves that innervate the conjunctiva. Vasoactive intestinal peptide is not only produced in the central and peripheral nervous systems but also in endocrine and immune cells, acting as a potent immunomodulator, with several effects on T lymphocytes. Vasoactive intestinal peptide’s role in VKC is still ambiguous, because it could stimulate the local helper T cell 2 response or, conversely, down-regulate inflammatory activity. Further studies of VIP’s effects on conjunctival cells are required for greater understanding of its role in allergy and inflammation.

In contrast to the epithelium, NGF staining in the conjunctival stroma was increased in pathological tissues compared with controls: immunoreactivity was diffuse and not clearly localized in cells. Interestingly, NGF staining was also present on vascular endothelial cells; these cells had an enhanced production of NGF in the presence of interleukin 1β, a proinflammatory cytokine highly expressed in VKC tissues. Nerve growth factor is not only a neurotrophine essential for the survival, differentiation, and function of neurons but also an immunomodulator that can be produced by nerves, immune cells, epithelial cells, and fibroblasts. Its increased expression and localization in inflamed substantia pro-

Table. Immunohistochemical Staining in Conjunctival Epithelium and Stroma From Healthy Subjects and Patients Affected by VKC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Healthy Group</th>
<th>VKC Group</th>
<th>Healthy Group</th>
<th>VKC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5</td>
<td>2</td>
<td>1 (0.5-1.5)†</td>
<td>0</td>
<td>1.5 (0-2)†</td>
</tr>
<tr>
<td>Muscarinic receptor</td>
<td>M1</td>
<td>2</td>
<td>1 (0-2)†</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>2</td>
<td>1.5 (1-3)†</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M3</td>
<td>1</td>
<td>2 (1-3)†</td>
<td>1</td>
</tr>
<tr>
<td>β2-Adrenergic receptor</td>
<td>2</td>
<td>2 (1-3)</td>
<td>2</td>
<td>2 (2-3)</td>
</tr>
<tr>
<td>VIP</td>
<td>1</td>
<td>1 (0-2)†</td>
<td>1</td>
<td>2 (1-3)†</td>
</tr>
<tr>
<td>NGF</td>
<td>3</td>
<td>2 (1-3)†</td>
<td>0</td>
<td>2 (0-2)†</td>
</tr>
</tbody>
</table>

Abbreviations: NGF, nerve growth factor; PGP, protein gene product; VIP, vasoactive intestinal peptide; VKC, vernal keratoconjunctivitis.

*Data are given as median score and median score (range). The intensity of staining was subjectively evaluated using a scoring system from 0 to 3 (0 indicates absent; 1, slight; 2, intense; and 3, very intense) considering separately the epithelium and the stroma.

†P < .05 (nonparametric Mann-Whitney test).
pria may be related to the tissue repair, fibrosis, and neovascularization typical of VKC.

It is still unclear if the changes observed in neuroreceptor and neuromediator expression in pathological inflamed conjunctiva are specific to VKC. It is possible that they indicate only an epiphenomenon response to a non-specific inflammation. In fact, recently, expression of muscarinic and α-adrenergic receptors was shown to be up-regulated in conjunctival epithelial cell cultures when treated with the proinflammatory cytokines interferon γ and/or tumor necrosis factor α. Future studies on other allergic and nonallergic conjunctival tissues and on conjunctival cells in vitro may clarify if the neural component of conjunctival inflammation plays a relevant role in the development of VKC and/or other conjunctival diseases.

In conclusion, the role of neuroendocrine factors in the development of VKC is still unclear. The altered expression and distribution of neuroreceptors, neuromediators, and nerve fibers in inflamed conjunctiva of VKC patients substantiates the importance of neurogenic mediation in this disease, and may indicate a new opportunity for potential therapeutic modalities.

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