**Background:** There is growing evidence that autonomic innervation is involved in the pathogenesis of mucus 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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muscarinic receptors; the flamed conjunctiva.  

In healthy conjunctiva, stratified cylindrical epithelium with nongoblet epithelial cells, goblet cells, and less, these discrepancies were not critical because the differences between inflamed and healthy tissue were evident.  

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 β1-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 M1 muscarinic receptors was observed over the entire thickness of healthy epithelium. In contrast, staining of M1 muscarinic receptors was negative or slightly positive in localized areas of pathological specimens (Figure 2C and D).

The expression of M2 muscarinic receptors was similar to that of M1 muscarinic receptors in healthy samples. In VKC biopsy specimens, staining was variable: intense in some areas and absent in others. The staining of M2 muscarinic receptors was also clearly evident in rare single cells of the epithelium (Figure 2E and F).

The staining of M3 muscarinic receptors was of a different pattern: immunoreactivity was clearly limited to the epithelial basal layer in healthy tissue. In comparison, immunostaining of M3 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 M1, M2, and M3 muscarinic receptor, β1-AR, and VIP antibodies. In inflamed conjunctival stroma, increased immunostaining for the muscarinic receptors (M1, M2, and M3), for β1-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 M1 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 M1, M2, and M3 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 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 β\textsubscript{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 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-

\textbf{Figure 3.} Immunohistochemical distribution of protein gene product (PGP) 9.5 (A and B), nerve growth factor (NGF) (C and D), and vasoactive intestinal peptide (E and F) in healthy conjunctival tissues (A, C, and E) and conjunctival tissues affected by vernal keratoconjunctivitis (VKC) (B, D, and F). The staining of PGP 9.5 and NGF was reduced in VKC epithelium compared with healthy tissues. Conversely, all 3 were highly expressed in VKC stroma compared with healthy conjunctiva.
put control than in mucous regulation in healthy conjunctiva. The reduction in the M₁ muscarinic receptor and the irregular distribution of the M₂ and M₃ 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 secretion 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 M₃ 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 neurotrophe 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-

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**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></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₁</td>
<td>2</td>
<td>1 (0-2)†</td>
<td>1</td>
<td>2 (3-3)†</td>
</tr>
<tr>
<td>M₂</td>
<td>2</td>
<td>1.5 (1-3)†</td>
<td>1</td>
<td>2 (3-3)†</td>
</tr>
<tr>
<td>M₃</td>
<td>1</td>
<td>2 (1-3)</td>
<td>1</td>
<td>3 (3-3)†</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>2 (0-2)</td>
<td>2</td>
<td>2 (2-3)†</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 neo-
vascularization typical of VKC.

It is still unclear if the changes observed in neuroreceptor and neuropediatri expression in pathological in-
flamed 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 mus-
caricin 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 tor
necrotosis factor [α]. Future studies on other allergic and nonallergic conjunctival tissues and on conjunctival cells in vitro may clarify if the neural component of conjuncti-
val inflammation plays a relevant role in the develop-
ment of VKC and/or other conjunctival diseases.

In conclusion, the role of neuroendocrine factors in the development of VKC is still unclear. The altered ex-
pression and distribution of neuroreceptors, neurome-
diators, and nerve fibers in inflamed conjunctiva of VKC
patients substantiates the importance of neurogenic me-
diation in this disease, and may indicate a new opportu-
nity for potential therapeutic modalities.

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