

Histamine H₄ receptors in normal conjunctiva and in vernal keratoconjunctivitis

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Abstract

Background: While it is known that histamine is the primary mediator of ocular allergy, the presence and distribution of histamine receptors are not well documented in the human eye. Our aim was to evaluate histamine receptor expression in normal and vernal keratoconjunctivitis conjunctiva.

Methods: Mucosal biopsies were obtained from conjunctiva of healthy donors and from tarsal conjunctiva of vernal patients. Immunostaining and semi-quantitative reverse-transcriptase polymerase chain reaction for H₁, H₂, H₃, and H₄ receptors were performed. Histamine receptor expression was also evaluated in conjunctival cell cultures exposed to histamine, interleukin-4, interleukin-5, interferon- γ and tumor necrosis factor- α .

Results: Immunostaining for H₁ and H₂ receptors was slightly positive in normal and over-expressed in vernal tissues. H₃ receptors were rarely present in normal and inflamed conjunctiva. In striking contrast to control tissues, H₄ receptors were highly expressed in all inflamed tissues, particularly by stromal inflammatory cells. Semi-quantitative reverse-transcriptase polymerase chain reaction demonstrated an over-expression of H₁, H₂, and H₄ receptors in vernal vs control tissues. Notably, H₄ receptors were five times more expressed in vernal vs control tissues. In cell cultures, H₂ receptor expression was stimulated eight times the normal levels by interleukin-4 and three times by histamine, but the H₄ receptor was only slightly affected by stimulation with these mediators.

Conclusions: Increased expression of H₁, and particularly of H₂ and H₄ receptors in vernal keratoconjunctival tissues indicate their important role in the pathogenesis of this disease. H₄ receptors may be a target in the treatment of allergic inflammation.

Ocular itching, hyperemia, tearing, and lid swelling are the classic ocular manifestations related to histamine release from mast cells (1). By challenging patients with allergens, an early-phase conjunctival reaction is induced, tear histamine levels are significantly increased from baseline (2), and signs and symptoms reflect those observed in a clinical allergic environmental response. High tear levels of histamine can be found in patients with active vernal keratoconjunctivitis (VKC) (3, 4), a chronic ocular allergic disease characterized by lymphocyte Th2 and eosinophil infiltration, and conjunctival remodeling (5, 6).

Histamine is known to increase epithelial permeability and to stimulate the expression of adhesion molecules and pro-

inflammatory cytokines. In recent years, research has aimed to define these secondary histamine-driven effects and has led to a deepening appreciation of its critical role in the pathogenesis of ocular allergy (7–9).

Four distinct histamine receptors (HR) have been characterized to date (10). It is generally accepted that the H₁R plays the greatest role in allergic disease and H₁R antagonists have a long history of clinical efficacy in a variety of allergic disorders. H₂R and H₃R play critical roles in a variety of tissues including the central and peripheral nervous systems, gastrointestinal tract, and heart (11). The H₄R is the most recently discovered of the HRs (12, 13), and because of its distribution on immune cells and its proven role in inflamma-

tory functions, it might be a potential therapeutic target for the treatment of a variety of immune disorders (14), including allergy (13).

In the conjunctiva, histamine appears to immediately act through the H₁R and H₂R to induce hyperemia, cytokine secretion, and increases in microvascular cell permeability (15). The role of histamine and its effects on the eye via its receptor subtypes, H₁R, H₂R, and H₃R, have been explored to various extents in human as well as other animal models (15); however, no data regarding the involvement of H₄R in human ocular allergy are available to date. Recently, functionality of the H₄R has been shown in the rat eye with high selectivity of the antagonist JNJ7777120 suggesting an H₄R-mediated effect on conjunctival histamine (16).

The purpose of this study was to evaluate the anatomic localization and mRNA expression of HR in normal conjunctival tissue and in patients with VKC. Additionally, expression of HRs in conjunctival cell cultures at baseline and after stimulation with histamine and cytokines was evaluated.

Methods

Subjects and tissue specimens

Nine patients with tarsal VKC (six boys and three girls, mean age 11.2 ± 4.9), in an active disease phase, free of topical mast cell stabilizers and/or antihistamines for at least 3 days and free of topical corticosteroids for at least 7 days, were included. Diagnosis of VKC was based on the typical clinical history and evaluation of signs and symptoms (17). Of the nine patients with VKC, five were positive to serum specific IgE for at least one aeroallergen, such as bluegrass, mites, *parietariae*, *compositae*, and/or tree pollens.

The control group included six subjects (five boys and one girl, mean age 13 ± 5) who underwent lid surgery. None of these subjects used contact lenses or had any inflammatory signs and symptoms or history of allergy. Upper tarsal conjunctival biopsies were performed under local anesthesia. All tissue specimens were snap frozen with OCT compound (Tissue-Tek) in liquid nitrogen and maintained at -70°C for immunohistochemistry. This research was approved by the Institutional Review Board. A written informed consent was obtained from all subjects or their parents before obtaining tissue specimens.

Immunohistochemistry (IHC)

Serial 5-mm-thick cryosections were cut, mounted on gelatin-covered slides, fixed in acetone, and processed for IHC. The following anti-human antibodies were used: anti-H₁R (sc-19767), anti-H₂R (sc-50314), anti-H₃R (sc-17921), and anti-H₄R (sc-50312) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). Briefly, samples were treated with avidin and biotin blocking solutions (Vector, Burlingame, CA, USA) and monoclonal or polyclonal antibodies at the appropriate dilution in Tris-buffered saline (TBS: 10 mM Tris, 150 mM NaCl, pH 7.4). Slides were then washed in TBS, incubated

with biotinylated secondary antibodies (Vector), treated with the alkaline phosphatase ABC kit (Vectastain, Burlingame, CA, USA) and fast red staining (Sigma, St Louis, MO, USA). Light microscopic analysis was performed at a magnification of 400 $\times$. The positive red reaction was evaluated in the epithelium and sub-epithelial stroma of conjunctival tissues and classified as very intense (3+), intense (2+), slight (1+) or absent (0). All the slides were evaluated by three masked investigators.

Quantification of histamine receptor mRNA levels in VKC biopsies

Quantification of HR mRNA was performed in six of the patients with VKC and in the six controls. Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (74804; Qiagen, Milan, Italy) from 30- μm -thick cryostat sections of conjunctival tissues. Five nanograms of total RNA were used for cDNA synthesis, utilizing the Enhanced Avian HS RT-PCR Kit (HSRT100-1KT; Sigma, Milan, Italy). Primer pairs for H₁R variant (v)1, H₁R v3, H₂R v1, H₂R v2, H₃R, and H₄R (Invitrogen, Milan, Italy) are shown in Table 1. Primers were obtained using the Primer3 Pick primers from a DNA sequence program. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using the Enhanced Avian HS RT-PCR Kit (Sigma) following the manufacturer's protocol and a thermo cycler Master cycler personal (Eppendorf). The TOTALLAB TL120 program was used for quantifying the density of amplified bands. Relative levels of mRNAs were expressed as the ratio of optical density (OD) of the gene of interest to the OD of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Cell cultures

Fibroblast cultures were obtained from upper tarsal conjunctival biopsies of two of the normal subjects. Briefly, biopsies were cut in small pieces and seeded in Nunclon Multidishes (NUNC, Roskilde, Denmark) containing 100 μl of Ham's F12 medium (Sigma) supplemented with 10% fetal calf serum (FCS: Sigma) and antibiotics. Samples were incubated at

Table 1 Primer pairs for histamine receptor (HR) variants (v)

HR	Forward primer	Reverse primer
H ₁ R v1 (NM_001098213)	ccagcgtctaaggaacaagg	tccgagcagttctctcaatct
H ₁ R v3 (NM_001098211)	atgtgccaggctttgtgttt	tggggctgacattttctgtatc
H ₂ R v1 (NM_001131055)	catattccctctgtccttgc	ccagctgcctcctgaataag
H ₂ R v2 (NM_022304)	cgggtaccaacagctcttct	accagcaccacctaaacagc
H ₃ R (NM_007232)	tgcttttccattctgggtgt	cctgggtgagcaaacagagt
H ₄ R (NM_02624)	cttttgagatgggggtcttg	accaaagcgtgtaagaactgc

37°C in 5% carbon dioxide in a humidified atmospheric chamber. Fibroblasts were subcultured with 0.05% trypsin and replated into 24-well plates (>95% vitality). Third- to fifth-passages fibroblasts were used for experiments.

Wong-Kilbourne derived Chang conjunctival (ChWK) epithelial cells were obtained from ATCC (clone 1-5c-4) and were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and antibiotics. Cultures were incubated in a humidified incubator under standard conditions.

Real-time polymerase chain reaction (PCR) in conjunctival cell cultures

Quantification of HR mRNA was performed in conjunctival fibroblasts and conjunctival epithelial cell cultures. Total RNA was extracted using the RNA spin Mini Kit (GE Healthcare, Milan, Italy). All the remaining steps were identical to those described for semi-quantitative RT-PCR in VKC. To evaluate the effects of mediators on HRs mRNA expression in fibroblast and epithelial cells, cell cultures were similarly treated. Briefly, 5000 cells/well were seeded on multi-well chamber slides (NUNC). After 24 h, the medium was replaced with 200 µl of serum-free medium. After another 24 h, the medium was replaced with fresh medium supplemented with 0, 1, 10, 20 ng/ml of: histamine (Sigma) and human recombinant IL-4, IL-5, IFN-γ, and TNF-α (Peprotech, London, UK). After 4, 6, 24, and 48 h of exposure, total RNA was extracted. Preliminary data showed that the 24-hour exposure time period with 10 ng/ml cytokines yielded the best results for the purposes of this study; therefore, these experimental conditions were considered for all subsequent experiments. Three culture wells were used for each treatment, and the experiments were repeated twice.

Statistical analysis

Differences in histological staining scores and the comparison of expression in VKC and normal samples were analyzed using the nonparametric Mann-Whitney *U*-test. The nonparametric Kruskal-Wallis one-way analysis of variance test by rank was used to analyze the multiple cytokine/mediator effects on cell cultures, and thus, the data are expressed as mean ranks. For statistical significance, the assigned *P* value was ≤0.05.

Results

IHC in conjunctival tissues

H₁R immunoreactivity was weak in epithelial cells of normal subjects, while H₁R were over-expressed in VKC tissues (Table 2), particularly in vascular endothelial cells (Fig. 1). H₂R and H₄R were slightly present in normal tissues, but both were clearly expressed in the epithelium, in the subepithelial stroma, and around the blood vessels in VKC (Table 2 and Fig. 1). H₄R staining was especially notable in stromal inflammatory cells in VKC. As expected, the H₃R was rarely

Table 2 Immunohistochemical expression of histamine receptors in normal (*N* = 6) and in vernal keratoconjunctivitis conjunctival tissues (*N* = 9)

	Normal conjunctiva	VKC conjunctiva	<i>P</i> value
H ₁ R	0.5 ± 0.1	1.8 ± 0.2	0.0004*
H ₂ R	0.3 ± 0.1	1.8 ± 0.1	0.0001*
H ₃ R	0.2 ± 0.1	0.3 ± 0.1	0.5747
H ₄ R	0.3 ± 0.1	2.0 ± 0.1	0.0001*

The values expressed are mean scores on a scale of 0–3 plus or minus the standard deviation from the mean. *P* values of ≤0.05 resulting from the Mann-Whitney nonparametric analysis were considered significant.

*Statistically significant.

VKC, vernal keratoconjunctivitis.

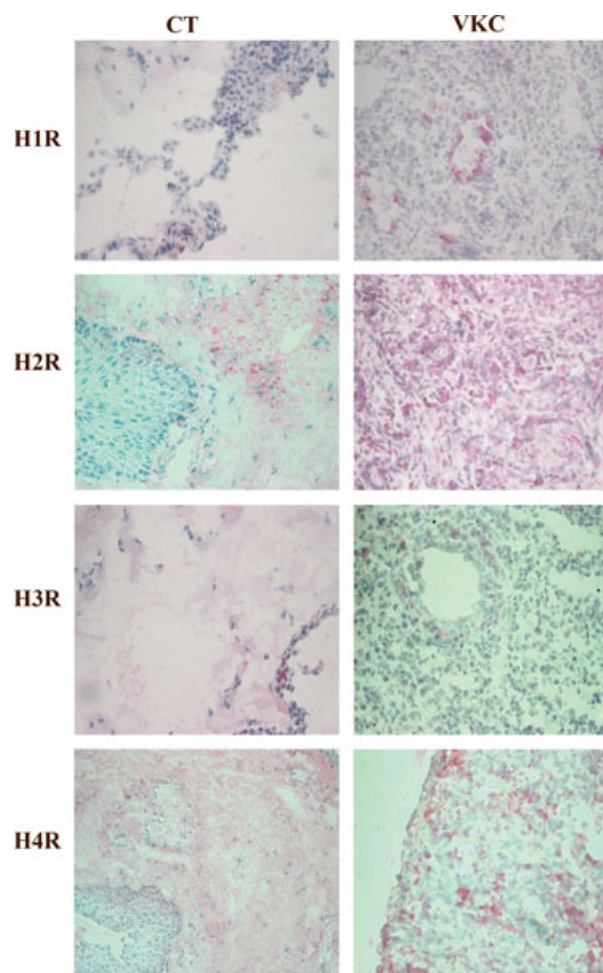
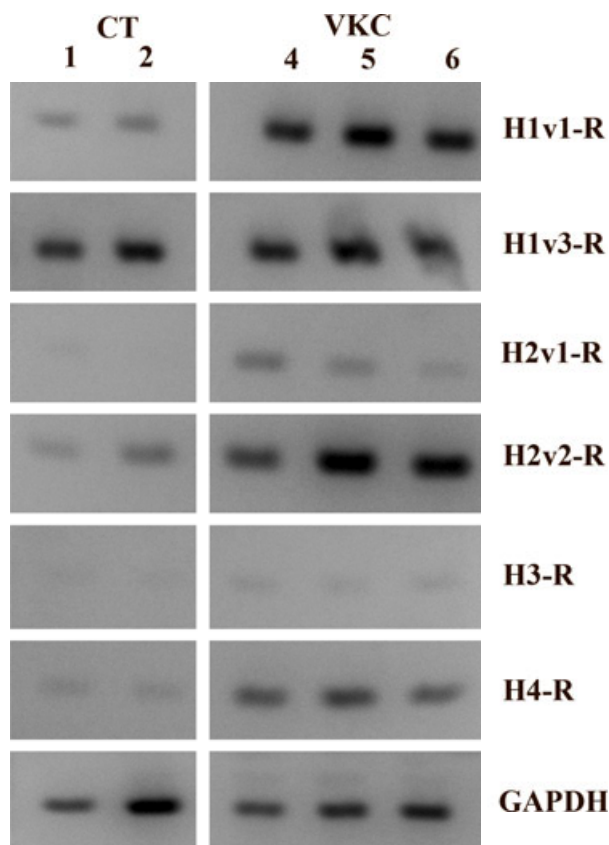


Figure 1 Immunohistochemical expression of histamine receptors (HR) in the normal conjunctiva (CT) and in VKC conjunctiva (VKC). H₁R and H₂R were highly expressed in VKC conjunctiva, particularly on endothelial cells and around blood vessels. H₃R was only slightly expressed in VKC tissues. Immunostaining for H₄R was particularly evident in VKC stroma (original magnification 200×).

Table 3 Qualitative polymerase chain reaction (PCR) analyses of histamine receptor expression on normal and vernal keratoconjunctivitis (VKC) conjunctival tissues

		Normal conjunctiva	VKC conjunctiva	Mann-Whitney <i>P</i> value
H ₁ R var1 mRNA	Median	0.3	0.9	0.03
	Range	0.0–1.3	0.7–1.5	
H ₁ R var3 mRNA	Median	0.8	1.0	0.04
	Range	0.63–1.27	0.8–1.7	
H ₂ R var1 mRNA	Median	0.02	0.1	0.02
	Range	0.0–0.1	0.0–0.4	
H ₂ R var2 mRNA	Median	0.2	0.8	0.008
	Range	0.04–0.6	0.2–1.2	
H ₃ R mRNA	Median	0.05	0.16	0.12
	Range	0.0–0.6	0.0–0.6	
H ₄ R mRNA	Median	0.1	0.7	0.008
	Range	0.0–0.7	0.5–0.9	

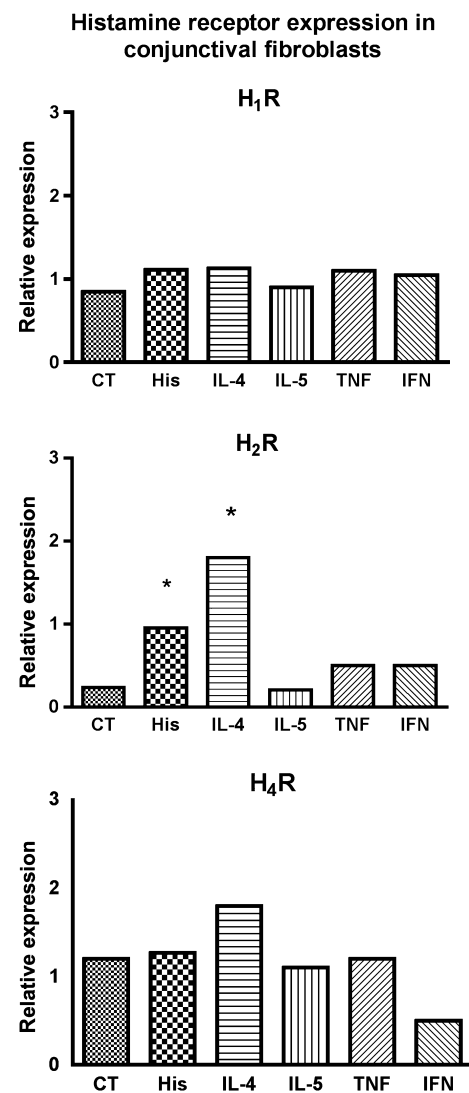
HR, histamine receptor; var, variant.

**Figure 2** Histamine receptor mRNA levels in vernal keratoconjunctivitis (VKC) biopsies. Increases in H₁R, H₂R, and H₄R were observed in VKC tissues. Vernal keratoconjunctivitis subjects 4, 5, and 6 and control subjects 1 and 2 are illustrated. H₁R, especially its variant (v)3, was of greater density in inflamed tissues. H₂R-v2 and H₄R were over-expressed in VKC tissues, while H₃R was only slightly increased in VKC.

found in normal or inflamed conjunctiva. Although not statistically significantly increased, some expression of H₃R was evident in the conjunctival peri-vascular regions of VKC. The staining scores for H₁R, H₂R, and H₄R were statistical significantly greater in VKC vs control tissues (Table 2).

Quantification of histamine receptor mRNA levels in VKC biopsies

The ratio of mRNA/GAPDH was significantly increased for H₁R, H₂R and particularly for H₄R in VKC compared with normal subjects. In fact, normal conjunctiva expressed both variants of H₁R, H₂R-v2 and H₄R. H₂R-v1 and H₃R were only faintly expressed (Table 3 and Fig. 2). All HR mRNA

**Figure 3** The relative expression of H₁R, H₂R, and H₄R mRNA by conjunctival fibroblasts exposed for 24 h to 10 ng/ml histamine, IL-4, IL-5, TNFα and IFNγ. Findings showed that the relative expression of H₂R was significantly increased by histamine and IL-4 (*P* < 0.05), while H₄R expression was slightly increased only by IL-4.

except H₃R were over-expressed in VKC samples. In particular, H₁R-v1, H₁R-v3, and H₂R-v2 were highly expressed in VKC compared with normal tissues (Table 3 and Fig. 2). Interestingly, H₂R-v2 and H₄R in VKC were five times more expressed than normal tissues ($P = 0.008$). Although H₃R was three times more expressed in VKC than normal tissues, this difference was not statistically significant.

Real-time polymerase chain reaction (PCR) in conjunctival cell cultures

In untreated fibroblast cultures, H₁R-v1, H₂R-v2, and H₄R, but not H₃R, were slightly expressed. After treatment with histamine or cytokines, H₁R-v1 expression did not change compared with untreated cells. In contrast, H₂R-v2 expression was increased eight times by IL-4 and three times by histamine compared with controls ($P < 0.05$). H₄R mRNA was only slightly increased by IL-4 compared with controls (Fig. 3). IL-5, IFN γ and TNF α did not have an effect on mRNA expression.

In baseline Chang epithelial cell cultures, H₂R-v2 and H₄R mRNA were minimally expressed. H₃R expression was absent. Only TNF α and IFN γ significantly increased both H₁R-v1 and H₂R-v2 expression by two times ($P < 0.05$). H₄R was not expressed, neither at baseline nor after exposure to histamine, IL-4, IL-5, IFN γ , or TNF α .

Discussion

Histamine exerts its activities by binding to HR, which are important therapeutic targets for various diseases such as asthma, heart disease, peptic ulcer, and Alzheimer's disease (14). The H₄R is found in many organs of the human body; its ubiquity indicates an important physiological and pathological role in humans (18).

In ocular allergy, topical selective H₁R antagonists have been considered to be the first line in the treatment of allergic conjunctivitis. Vernal keratoconjunctivitis is a severe ocular allergic disease mediated by both IgE and non-IgE-mechanisms, characterized by an intense inflammatory cellular infiltration, corneal complications, tissue remodeling (5) and profound changes in tear composition (19). High levels of tear histamine and a reduced tear histaminase activity have been shown in VKC (4), suggesting that prolonged high levels of histamine on the ocular surface contribute to chronic inflammation and conjunctival remodeling (20). Although the relevance of histamine is well established, the expression of HRs in the conjunctiva has been evaluated only through the inhibitory effects of H₁ and H₂ antagonists (15, 21).

The present study shows that H₁R and H₂R are expressed in the conjunctiva by endothelial blood vessels and the epithelium. This is not surprising as conjunctival histamine challenge induces an immediate vascular-driven allergic reaction in the eye (22, 23) and conjunctival epithelial cells *in vitro* have been shown to produce pro-inflammatory mediators after histamine exposure (8, 24). Conjunctival fibroblasts have been shown to increase expression of H₂R after exposure to histamine and IL-4. Similarly, previous studies have

shown that conjunctival fibroblasts respond to histamine by raising their production of procollagen and proinflammatory cytokines (9, 20, 25). In lung fibroblasts, histamine increases the expression of H₁R, H₂R and connective tissue growth factor (26, 27) indicating that histamine plays a role in the inception of airway remodeling.

At the mRNA level, H₁R and H₂R have multiple alternatively spliced variants, encoding the same protein. However, functionally, no differences were noted among these different variants of H₁R and H₂R. In the present study, H₁R and H₂R were expressed by normal conjunctival epithelium and by epithelial cells *in vitro*. Their expression was increased in culture with stimulation by TNF α and IFN γ . Thus, under extracellular stimulation by histamine (8) or pro-inflammatory cytokines (28), conjunctival epithelial cells play an active role in propagating ocular surface inflammation (28).

H₄R was weakly expressed in normal conjunctiva, and significantly increased in VKC tissues, particularly in the stroma, where inflammatory cells were abundantly present. The cell infiltrate in VKC has been characterized to be a combination of eosinophils, Th2 lymphocytes, dendritic cells, and NK cells (5, 29, 30). The H₄R has been identified on cells involved in inflammation, allergy, and immune response (12, 31), as well as on cells of the innate immune system, including NK cells and dendritic cells (32). Although a double staining technique was not performed in the present study, the over-expression of H₄R was clearly seen in association with the inflammatory infiltrate in VKC tissues.

In the present study, Chang conjunctival cells showed little mRNA expression of H₄R and only a slightly increased by IL-4 compared with controls. In a previous study in human dermal fibroblast cultures, H₄R expression was unaffected by histamine stimulation (33). Expression of the H₁R, H₂R, and H₃R, but not H₄R was demonstrated in the human bronchial epithelial cell line (34). All these data suggest that the H₄R is probably not critical in the regulatory effects of histamine on fibroblast and epithelial cell activation in allergic inflammation.

In a mouse model, JNJ7777120, an H₄R antagonist, significantly reduced both histamine- and substance P-induced scratching (12, 13). Similarly, in a mouse skin inflammation model, the dual effects of H₄R antagonists on pruritus and Th2-cell-mediated inflammation indicate their therapeutic potential in the treatment of Th2-mediated skin disorders (35) and possibly H₁R antagonist-resistant pruritus.

Unfortunately, there are no animal models that reproduce the complex spectrum of clinical and immunological features of VKC. In a 48/80 rat conjunctival mast cell degranulation model (16), instillation of JNJ7777120 had no protective effect on mast cell histamine release and failed to reverse the protective effect of DSCG, suggesting that H₄R are not involved in the mast cell-dependent conjunctival early phase reaction. Vernal keratoconjunctivitis is known to be much more than simply mast cell degranulation, but rather a constellation of cell-mediated, self-propagating chronic inflammatory events. Because the H₄R was over-expressed in VKC tissues and because the cells involved in VKC, such as mast cells, eosinophils and Th2-

cells (5), express this receptor, it is natural to speculate that these cells are the source of the over-expression observed, and that certainly some of the effects of histamine in this disease are because of H₄R activation. Therapeutic treatment with an H₄R antagonist has been shown to inhibit the Th2-driven allergic inflammation (35). Thus, dual H₁R/H₄R antagonism may be an ideal glucocorticoid-sparing treatment of VKC.

In conclusion, the increased expression of HR, and particularly of the H₄R in VKC, in conjunction with the previously reported direct effects of H₄R perturbation on mast cell and eosinophil function, point to a possible role of H₄R antagonists as novel therapeutics in ocular allergic diseases.

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Conflict of interest

The Authors have no commercial associations that might pose a conflict of interest. Each Author contributed in the production of the present work.

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