Objective: To evaluate the effects of topical ocular drugs with histamine H1-antagonist activity on histamine-stimulated phosphatidylinositol turnover and interleukin (IL) 6 and IL-8 secretion from human conjunctival epithelial cells.

Methods: Primary human conjunctival epithelial cell cultures were stimulated with histamine in the presence or absence of test drugs. Phosphatidylinositol turnover was quantified by ion exchange chromatography and cytokine content of supernatants by enzyme-linked immunosorbent assay.

Results: Antazoline hydrochloride, emedastine difumarate, levocabastine hydrochloride, olopatadine hydrochloride, and pheniramine maleate attenuated histamine-stimulated phosphatidylinositol turnover and IL-6 and IL-8 secretion. Emedastine was the most potent in ligand binding, phosphatidylinositol turnover, and IL-6 secretion, with dissociation constant and 50% inhibitory concentrations of 1-3 nmol/L. Olopatadine, antazoline, and pheniramine exhibited similar H1-binding affinities (32-39 nmol/L). However, olopatadine was approximately 10-fold more potent as an inhibitor of cytokine secretion (50% inhibitory concentration, 1.7-5.5 nmol/L) than predicted from binding data, while antazoline and pheniramine were far less potent (20- to 140-fold) in functional assays. Levocabastine (dissociation constant, 52.6 nmol/L) exhibited greater functional activity (50% inhibitory concentration, 8-25 nmol/L) than either antazoline or pheniramine.

Conclusions: Histamine-stimulated phosphatidylinositol turnover and cytokine secretion by human conjunctival epithelial cells are attenuated by compounds with H1-antagonist activity. However, antihistaminic potency alone does not predict anti-inflammatory potential. Olopatadine, emedastine, and levocabastine were notably more potent than pheniramine and antazoline.

Clinical Relevance: Selected topical ocular drugs with antihistaminic activity may offer therapeutic advantages to patients with allergic conjunctivitis by inhibiting proinflammatory cytokine secretion from human conjunctival epithelial cells.

MATERIALS AND METHODS

CELL CULTURES

Methods detailing the preparation of primary epithelial cell cultures and cytokine release studies with the use of these cells have been described. Briefly, cultures of HCEs were initiated from donor tissues obtained by various eye banks within 8 hours post mortem. The tissues were enzymatically digested overnight. Epithelial cells were gently scraped from the tissue surface, dissociated into a single cell suspension, and cultured in Clonetics keratinocyte growth medium (Biowhittaker Corp, Walkersville, Md). Cells were used only through passage 6. Cultures were maintained in a preconfluent state to prevent differentiation. Cells were identified as epithelial by positive keratin staining, as described previously.

CYTOKINE ASSAYS

Several compounds with histamine H1-antagonist activity were evaluated for their ability to inhibit secretion of cytokines (IL-6 and IL-8) from cultured HCEs in response to histamine stimulation. Cells were plated at 2 x 10⁴ cells per well and cultured overnight in 5% carbon dioxide at 37°C. The following day, keratinocyte growth medium containing test compound was added directly to wells and the cells were incubated for 30 minutes before 24-hour stimulation with histamine (30 µmol/L). Three culture wells were used for each treatment group. At harvest, cell monolayers were examined microscopically to confirm viability and supernatants were collected, centrifuged at 200 g, and stored at −20°C. Samples were analyzed for IL-6 and IL-8 by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn) as directed by the manufacturer. The sensitivities of each enzyme-linked immunosorbent assay are 0.7 pg/mL for IL-6 and 3.0 pg/mL for IL-8.

HISTAMINE-INDUCED PHOSPHATIDYLINOSITOL TURNOVER

The determination of phosphatidylinositol (Pi) turnover induced by stimulation of phospholipase C in HCEs was performed as previously described with minor modifications. The HCEs were incubated with tritiated ([3H]myoinositol) from cultured HCEs in response to histamine stimulation. Cells were plated at 2 x 10⁴ cells per well and cultured overnight in 5% carbon dioxide at 37°C. The following day, keratinocyte growth medium containing test compound was added directly to wells and the cells were incubated for 30 minutes before 24-hour stimulation with histamine (30 µmol/L). Three culture wells were used for each treatment group. At harvest, cell monolayers were examined microscopically to confirm viability and supernatants were collected, centrifuged at 200 g, and stored at −20°C. Samples were analyzed for IL-6 and IL-8 by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn) as directed by the manufacturer. The sensitivities of each enzyme-linked immunosorbent assay are 0.7 pg/mL for IL-6 and 3.0 pg/mL for IL-8.

The antagonist potency (IC₅₀) was defined as the concentration of the drug required to produce 50% inhibition of the agonist-stimulated functional response. Data obtained from the cytokine assays were calculated as mean and SEM values that represent the variability among identically treated culture wells. The dose-dependent effect of pharmacological agents and IC₅₀s were determined by linear regression. Data obtained in the PI turnover assays were analyzed by means of a nonlinear, iterative curve fitting program as previously described. Data are expressed as mean ± SEM from 3 to 5 independent experiments.

TEST COMPOUNDS

Compounds were obtained as follows: antazoline hydrochloride and pheniramine maleate (Sigma-Aldrich Corp, St Louis, Mo); emedastine difumarate (Kanebo Ltd, Osaka, Japan); olopatadine hydrochloride (Kyowa Hakko Kogyo Co Ltd, Tokyo, Japan); and levocabastine hydrochloride (Livostin, Ciba Vision Ophthalmics, Atlanta, Ga). Histamine dihydrochloride was obtained from Research Biochemicals International, Natick, Mass.

HCEs. These receptors were coupled to inositol phosphate generation, which mobilized intracellular calcium. Calcium mobilization peaked within 10 seconds and was sustained for 20 minutes after stimulation with histamine. Pharmacological studies indicated that histamine H1-receptor antagonists potently antagonized these effects. Recently, Weimer et al demonstrated that histamine stimulation of HCEs induced the secretion of IL-6, IL-8, and GM-CSF in a concentration- and time-dependent manner. They also presented evidence that the potent H1-antagonist emedastine inhibited cytokine secretion at concentrations consistent with its published affinity for the H1 receptor.

The current experiments were conducted to compare the inhibitory effects of compounds with H1-antagonist activity currently available for topical ocular use on histamine-stimulated activation of the second messenger system and cytokine release from HCEs.
RESULTS

Exposure of HCEs to 100-µmol/L histamine maximally stimulated PI turnover (2.54 ± 0.16-fold above basal levels). Similarly, exposure of these cells to 30-µmol/L histamine increased IL-6 and IL-8 secretion 1.59 ± 0.19- and 1.80 ± 0.28-fold above basal levels, respectively. (Basal levels of the cytokines were 7667 ± 2110 pg/10⁶ cells [n = 4] for IL-6 and 9857 ± 2386 pg/10⁶ cells [n = 6] for IL-8.)

Treatment of HCEs with drugs possessing antihistaminic activity and available for topical ocular administration before histamine exposure resulted in concentration-dependent inhibition of PI turnover, IL-6 secretion, and IL-8 secretion. All 5 compounds tested produced concentration-dependent inhibition of the histamine-stimulated cell functional responses (Figure 1, Figure 2, and Figure 3).

Emedastine was the most potent compound tested (Table). The potency of emedastine in intact cells was consistent with its activity determined in receptor binding assays with the use of tissue homogenates. Its IC₅₀ values for histamine-induced PI turnover and IL-6 and IL-8 secretion were 1.54, 2.5, and 4.0 nmol/L, respectively. Levocabastine and olopatadine were also potent inhibitors of these histamine-stimulated responses. Levocabastine inhibited the PI turnover and IL-6 and IL-8 secretion (IC₅₀s, 8.32, 25.1, and 11.9 nmol/L, respectively). Olopatadine was more potent than predicted from its published histamine H₁-receptor binding affinity (36 nmol/L),²² with IC₅₀s of 10.03, 5.5, and 1.7 nmol/L for PI turnover and IL-6 and IL-8 secretion, respectively. In fact, olopatadine inhibited histamine-stimulated secretion of IL-8 at a concentration (IC₅₀, 1.7 nmol/L) lower than emedastine’s efficacious concentration in the same assay system.

Antazoline and pheniramine, 2 first-generation topical ocular antihistamines, were dramatically less potent inhibitors of these histamine-stimulated cell-based responses (PI turnover, IL-6 and IL-8 secretion) than predicted from their histamine H₁-receptor binding affinities (Table). The calculated IC₅₀ values for these compounds on the parameters listed above ranged from

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**Figure 1.** Effect of histamine H₁-antagonists on histamine-induced phosphoinositide (PI) turnover in human conjunctival epithelial cells. Tritiated ([³H]myoinositol–labeled cells were exposed to drug for 20 minutes before stimulation with histamine for 60 minutes. [³H]inositol phosphates were quantified by ion exchange chromatography followed by liquid scintillation spectrometry.

**Figure 2.** Effect of histamine H₁-antagonists on histamine-induced interleukin 6 secretion from human conjunctival epithelial cells. Cells were incubated with test compound for 30 minutes before stimulation with histamine (30 µmol/L) for 24 hours. Supernatants were analyzed for cytokines by specific enzyme-linked immunosorbent assay.

**Figure 3.** Effect of histamine H₁-antagonists on histamine-stimulated interleukin 8 secretion from human conjunctival epithelial cells. Assay and data collection procedures are as noted in Figure 2.
Histamine H₁ Antagonists: Inhibition of IL-6 and IL-8 Secretion and PI Turnover in Human Conjunctival Epithelial Cells and H₁ Receptor Binding Affinities

<table>
<thead>
<tr>
<th>H₁ Antagonist</th>
<th>IC₅₀, nmol/L</th>
<th>IC₅₀, nmol/L</th>
<th>PI Turnover, nmol/L</th>
<th>Binding, Kᵣ, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emedastine difumarate</td>
<td>2.5</td>
<td>4.0</td>
<td>1.54</td>
<td>1.22*</td>
</tr>
<tr>
<td>Olopatadine hydrochloride</td>
<td>5.5</td>
<td>1.7</td>
<td>10.03</td>
<td>36.00*</td>
</tr>
<tr>
<td>Levocabastine hydrochloride</td>
<td>25.1</td>
<td>11.9</td>
<td>8.32</td>
<td>52.60*</td>
</tr>
<tr>
<td>Antazoline hydrochloride</td>
<td>1014.0</td>
<td>652.0</td>
<td>4200.00</td>
<td>38.40†</td>
</tr>
<tr>
<td>Pheniramine maleate</td>
<td>4826.0</td>
<td>1216.0</td>
<td>4500.00</td>
<td>33.90†</td>
</tr>
</tbody>
</table>

*IL indicates interleukin; PI, phosphoinositide; IC₅₀, 50% inhibitory concentration; and Kᵣ, the concentration of the drug required at equilibrium to inhibit the receptor binding by 50%.
†From Sharif et al. 20
‡From Yanni et al. 22

Histamine is recognized as a primary mediator of allergic disease. Its acute vascular effects lead to erythema and edema, and its pruritogenic effects are responsible for the itch characteristic of allergic conjunctivitis. 23 Additional biological effects of histamine have been reported. The most interesting of these relative to its role as a mediator of allergic diseases is its ability to stimulate or up-regulate proinflammatory cytokine synthesis and/or secretion.

Delneste et al. 24 investigated the effect of histamine on adhesion molecule expression and IL-6 production by human vascular endothelial cells. The authors reported that histamine at concentrations ranging from 10 nmol/L to 1 mmol/L increased IL-6 synthesis from these cells. These authors also reported that IL-8 messenger RNA expression and secretion were enhanced by exposure of endothelial cells to histamine in concentrations greater than 1 μmol/L. 25 Similar findings by Tommel et al. 26 with the use of human umbilical vein endothelial cells indicated that histamine H₁ and H₂ receptors play a role in cytokine secretion.

Histamine has also been reported to stimulate cytokine secretion from epithelial cells. Secretion of IL-6, IL-8, and GM-CSF by bronchial epithelial cells has been demonstrated. 3 Noah et al. 27 suggested that a correlation exists between calcium influx and IL-6 secretion in a bronchial epithelial cell line in response to stimulation with histamine (100 μmol/L). However, using the antihistamines loratadine and cetirizine hydrochloride, Ansellén et al. 28 failed to demonstrate an inhibitory effect on cytokine secretion from bronchial epithelial cells. These investigators therefore suggested that histamine does not play a role in cytokine production. Further experimentation has shown that human tracheal epithelial cells do produce GM-CSF after exposure to histamine. 9 Recently, Weimer et al. 30 demonstrated that histamine induces a concentration- and time-dependent secretion of IL-6, IL-8, and GM-CSF from HCEs. These authors presented evidence that the histamine-stimulated cytokine response was the result of histamine H₁-receptor activation in these cells. These findings are supported by data showing that the effects of histamine on PI turnover and intracellular Ca²⁺ concentration in HCEs were not significantly blocked by H₂ and H₃ antagonists but were dramatically reduced by H₁ antagonists. 31

Our data confirm histamine’s ability to stimulate PI turnover and cytokine secretion from HCEs. The concentrations of the agonist used in the present studies (30–100 μmol/L) are consistent with previous cytokine-stimulating concentrations of the biogenic amine. 2,3,19,24,25

Current data demonstrate that compounds capable of antagonizing histamine H₁ receptors prevent histamine-stimulated IL-6 and IL-8 secretion. These data confirm emedastine’s ability to prevent cytokine secretion 20 and characterize the effects of other compounds that possess antihistaminic activity. First-generation topical ocular antihistamines antazoline and pheniramine have reported affinities for the histamine H₁ receptor of 38.4 and 33.9 nmol/L, respectively. 25 The binding paradigms used to generate these values used cell membranes as the receptor source. In the present studies, which used living, whole cells as the test systems, the 2 compounds were surprisingly less potent. This was true not only for cytokine secretion but also for histamine-stimulated PI turnover. These physiological effects are linked via increased intracellular Ca²⁺ concentrations, which are known to facilitate secretory events. Supporting data obtained with calcium ionophore A₂₃₁₈₇ exposure of HCEs have shown a stimulation of cytokine secretion. 1 The IC₅₀ values for antazoline and pheniramine ranged from 652 to 4200 nmol/L and from 1216 to 4826 nmol/L, respectively. 30 These data suggest that the first-generation antihistamines have limited ability to interact with intact cells of the human conjunctiva.

First-generation topical ocular antihistamines, levocabastine and emedastine, inhibited histamine-stimulated PI turnover and cytokine secretion. The potency of these molecules in these assays was consistent with their affinities for the H₁ receptor (52.6 nmol/L for levocabastine and 1.22 nmol/L for emedastine). 20 Levocabastine’s IC₅₀ values ranged from 8.3 to 25.1 nmol/L. Emedastine has been reported to be the most potent antihistamine available for topical ocular use. 20,29 The drug’s histamine H₁-receptor affinity of 1.22 nmol/L is reflected in the data obtained in the present experiments. Emedastine’s potency in preventing cytokine secretion by HCE may partially explain the advantages noted with this compound during clinical comparative trials with levocabastine. 30

Interesting results were obtained with olopatadine. Olopatadine is marketed as Patanol for topical ocular use. The compound is a human conjunctival mast cell degranulation inhibitor and antihistamine. 22,31,32 Published reports demonstrate the compound’s antiallergic activity in vivo. 33 Results presented herein indicate that the compound is more potent as an inhibitor of cytokine secretion than would have been predicted from its
H1-receptor affinity (36 nmol/L). Olopatadine potently inhibited secretion of IL-6 and IL-8 from HCEs. The IC50 values for the drug were 5.5 and 1.7 nmol/L, respectively. These IC50 values were also approximately 2-fold and 10-fold lower, respectively, than those predicted from the functional second messenger data. Olopatadine was reported to be more potent as an inhibitor of histamine-enhanced tumor necrosis factor α–stimulated adhesion molecule expression than predicted from the drug’s receptor binding affinity. A possible explanation for this increased efficacy is a non-specific antisecretory effect. However, this does not appear to be the case. A report by Ikemura et al15 presented data showing that olopatadine (1-100 μmol/L) did not inhibit the release of β-glucuronidase from human polymorphonuclear neutrophils after stimulation with the calcium ionophore A23187.

The results presented herein confirm histamine’s ability to stimulate PI turnover and cytokine secretion from HCEs. First-generation antihistamines pheniramine and antazoline are dramatically less potent in the whole cell assays used compared with their receptor affinities, possibly providing some insight into their limited clinical utility. The current generation of topical ocular antihistamines, emedastine and levocabastine, exhibit potencies consistent with their receptor affinities (deviation approximately 2-fold). Importantly, olopatadine is 10 times more potent as an inhibitor of histamine-stimulated cytokine secretion than predicted from its histamine H1-receptor affinity. These data suggest that olopatadine may offer additional therapeutic benefits that complement the mast cell stabilization and antihistaminic activities observed in the clinic.

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Reprints: John M. Yanni, PhD, Allergy/Inflammation Research, Alcon Laboratories Inc, 6201 South Freeway, Fort Worth, TX 76134-2099 (e-mail: John.Yanni@AlconLabs.com).

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