

ORIGINAL PAPER

Ocular anti-allergic compounds selectively inhibit human mast cell cytokines *in vitro* and conjunctival cell infiltration *in vivo*

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Summary

Background Conjunctival mast cells (MCs) are important effector cells in seasonal allergic conjunctivitis, via histamine and cytokine secretion. Several new anti-allergic eye drops stabilize MCs and block histamine receptors, but their anti-inflammatory effects are unclear. **Objective** Anti-allergic drugs were compared for their anti-inflammatory effects in an *in vitro* model of human MC activation and in an experimental murine model of allergic conjunctivitis.

Methods Human cord blood stem cell-derived (CBMC) and conjunctival biopsy-derived MCs were stimulated via FcεRI, degranulation and histamine release were assayed at 1 h and cytokine secretion at 24 h using multiplex arrays. Mice sensitized to short ragweed pollen were given anti-allergics topically before allergen challenge, and conjunctival immunostaining was performed at 24 h.

Results After a 1 h stimulation, 80% of the CBMC had degranulated and secreted histamine (27.9 ± 4.7 ng/10⁶ cells; $P < 0.05$). Pre-treatment by all drugs significantly reduced histamine and TNF-α, whereas IL-5, IL-8, IL-10 and TNF-β profiles were differentially decreased. For conjunctival biopsy-derived cultures ($n = 11$), FcεR1 stimulation increased histamine, TNF-α, TNF-β, IL-5 and IL-8 levels and the production of IL-5, IL-6 ($P < 0.05$), histamine and IL-8 ($P < 0.01$) was inhibited by epinastine. *In vivo*, epinastine and olopatadine pre-treatment significantly reduced the clinical scores and eosinophil numbers ($n = 6$; $P < 0.05$) while epinastine also reduced neutrophils ($P < 0.02$).

Conclusion Differential effects on MC cytokine inhibition were observed, with epinastine inhibiting MC secretion of IL-5, IL-8, IL-10 and conjunctival neutrophil infiltration. The anti-allergic drugs have anti-histamine and mast-cell stabilizing properties but might differ in clinical improvement depending on the individual and the cytokines involved.

Keywords cytokines, human mast cells, seasonal allergic conjunctivitis

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Introduction

Mast cells (MCs) are important effector cells in the conjunctiva. During seasonal-allergic conjunctivitis (SAC), MCs are activated following allergen cross-linking of surface IgE receptors (FcεR1), resulting in degranulation and release of histamine, leukotrienes, proteases, prostaglandins and cytokines. Several drugs used clinically for treating SAC (azelastine, epinastine, ketotifen, olopatadine) combine anti-histamine, MC stabilization and anti-inflammatory roles, do not penetrate the blood-brain barrier and exert effects on other cells. Azelastine (Optivar™ 0.05%, Vetter Pharma Fertigung GmbH & Co., KG, Ravensburg, Germany), an H1 antagonist, inhibits the

release of tryptase [1], IL-6, IL-8 and TNF-α from human cord blood stem cell-derived MC (CBMC) [2]. Epinastine (Elestat™ 0.05%, Allergan Inc., Irvine, CA, USA), an H1 and H2 antagonist [3], has no effect on membrane fluidity, and yet inhibits calcium uptake and release from intracellular stores and dose dependently suppresses calmodulin in guinea-pig MC [4]. Ketotifen (Zaditor™ 0.025%, Novartis, Duluth, GA, USA), an H1 and H2 antagonist, inhibits eosinophil oxidative metabolism [5] and eosinophil chemotaxis [6]. Olopatadine (Patanol® 0.1%, Alcon Laboratories Inc., Ft Worth, TX, USA), an H1 antagonist [7], inhibits the release of TNF-α from human MC [8], resulting in the down-regulation of ICAM-1 expression in conjunctival epithelial cells [9], and has inhibitory effects

on eosinophils and neutrophils in tears. Although the anti-allergic roles of these drugs are well characterized, their anti-inflammatory properties are less well defined.

Using CBMC and, where possible, conjunctival biopsy-derived MC, the aim of this study was to compare the anti-inflammatory effects of these drugs on CBMC cytokine secretion in response to IgE cross-linking *in vitro*, and *in vivo* using an experimental mouse model of SAC.

Methods

Subjects

Biopsies of the upper tarsal conjunctiva were obtained as described previously [10] from unaffected sites from patients ($n = 11$; six males; mean age 46.5) undergoing pterygium surgery at Moorfields Eye Hospital. Two patients had a history of non-ocular atopic disease. None were receiving any therapy at the time of surgery. All studies involving human subjects were conducted according to the tenets of the Declaration of Helsinki. The protocol had ethics committee approval, and informed consent was given by all donors.

Conjunctival mast cells

Biopsy tissues were finely chopped with a scalpel and incubated in three to five wells of a 96-well plate in StemsanTM (StemCell Technologies, Vancouver, BC, Canada) containing SCF (100 ng/mL) and 10% fetal calf serum (FCS). After 5 days, there was a predominance of single cells in the cultures, at which point cells were used for experiments. The cell yield varied between biopsies with approximately 5×10^4 cells per well assayed, and cells within biopsies were plated at similar densities per well for relative comparisons. The mean level of CD117⁺ MC in the cultures was 40%, with a range of 7–70% ($n = 5$).

Human cord blood mast cells

CD34⁺ stem cells from anonymous donors ($n = 4$; 10^5 cells per donor, Cambrex, Wokingham, UK) were cultured in StemsanTM (StemCell Technologies) serum-free medium containing SCF (100 ng/mL) and IL-6 (50 ng/mL), with IL-3 (1 ng/mL) added during the first 14 days (all Peprotech, London, UK) [11]. Cells were fed weekly, with 10% FCS (Labtech International, Ringmer, UK) added at week 9. Cells were used for experiments between 11 and 18 weeks of culture.

Mast cell activation

CBMC and conjunctival MC were activated by cross-linking surface IgE with an anti-IgE antibody in fresh medium but without IL-6. Cells were incubated overnight

with azide-free IgE (4 µg/mL) to pre-sensitize, before adding anti-IgE antibody (25 µg/mL) (Serotec, Oxford, UK). Cell-free supernatants were taken at 1 h to measure histamine release and 24 h for cytokine production. Aliquots were stored at -70°C .

Drugs

Preservative-free azelastine, epinastine and olopatadine were synthesized for Inspire by custom vendors and solubilized in dimethyl sulphoxide (DMSO) (azelastine) or phosphate-buffered saline (PBS) (epinastine, olopatadine). Ketotifen fumarate (Sigma-Aldrich, Poole, UK) was solubilized in methanol. A commercial preparation of nedocromil sodium 2% (RapitolTM, Aventis, West Malling, UK; Alocril[®], Allergan Inc.) was used, containing 0.01% benzalkonium chloride preservative. All the drugs were used at final concentrations of 0–10 µg/mL, and added to cells 30 min' before anti-IgE cross-linking. DMSO, PBS, methanol and benzalkonium chloride were titrated into CBMC in a range of concentrations and were found to have no effects above controls at the equivalent concentrations. Benzalkonium chloride was toxic at higher concentrations.

Cytokine assays

The levels of cytokine secretion were determined by multiplex bead arrays (Bender Medsystems, Vienna, Austria) according to the manufacturer's instructions, using 25 µL culture supernatants in triplicate wells to detect simultaneously the secretion of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α, TNF-β and IFN-γ, with a minimum level of detection of 5 pg/mL.

Histamine detection

Histamine release was measured in triplicate by ELISA (IBL, Hamburg, Germany) according to the manufacturer's instructions, with a minimum detectable level of 100 pg/mL. Total histamine in the unstimulated MCs was determined by lysis and the amount released after stimulation represented $23.9 \pm 2.2\%$ of the total.

Immunophenotyping and intracellular cytokine expression

For expression of c-kit and FcεR1 after 11 weeks, cells were incubated with optimized concentrations of anti-CD117^{FITC} (Chemicon International, Temecula, CA, USA) and anti-FcεR1^{PE} (eBioscience, San Diego, CA, USA) for 30 min at 4°C , washed once with PBS before acquiring.

To detect intracellular IL-8, cells were stimulated for 24 h as above, with or without the drugs. Brefeldin A (10 µg/mL; Sigma-Aldrich) was added for the final 16 h. Cells were fixed and permeabilized [Cytofix/Cytoperm,

Becton, Dickinson (BD), Oxford, UK] before intracellular staining with IL-8^{PE} (BD). Epithelial cell cytokine expression was visualized by staining for cytokeratin (pan cytokeratin^{FITC}, Sigma-Aldrich) and IL-8^{PE} after fixation and permeabilization as above. All acquisition was performed using a FacScan flow cytometer (BD) with a minimum of 5000 events acquired. All flow cytometry was analysed using CellQuest (BD) or WinList (Verity Software, Topsham, ME, USA).

Immunocytochemistry

Cytospins of 10³–10⁵ CBMC or cultured conjunctival MC were prepared on PolysineTM slides (VWR, Lutterworth, UK) and stained for tryptase (AA1, DakoCytomation, Ely, UK) or chymase (CC1, Serotec, UK) at previously optimized concentrations. All primary staining used amplification with F(ab')₂-biotin and streptavidin HRP. Staining was visualized using AEC (DakoCytomation). A minimum of 100 cells was counted from at least three cytopins by two independent observers, and each experiment was repeated three times.

Animals

Animal studies were approved by the Allergan Animal Care and Use Committee. Animals were housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals, National Institutes of Health and the Association for Research in Vision and Ophthalmology statement about the Use of Animals in Ophthalmic and Vision Research. Female BALB/c (H-2d; 5–9 weeks old) were obtained from Taconic (Germantown, NY, USA) or The Jackson Laboratory (Bar Harbor, ME, USA).

Induction of atopic conjunctivitis

BALB/c mice were immunized with 50 µg of short ragweed (SRW) pollen (from *Ambrosia artemisiifolia*; International Biologicals, Piedmont, OK, USA) in 5 mg alum (Imject; Pierce Chemical, Rockford, IL, USA) by footpad injection on day 0. At day 10, AC was induced by a topical challenge method in which immunized mice were given 1.5 mg application of SRW pollen in 10 µL PBS to the right eye once [12]. Animals were examined clinically for signs of immediate hypersensitivity responses 20 min after topical challenge with SRW pollen. A clinical scoring scheme, similar to that described [13], was used to evaluate conjunctival redness, lid oedema and tearing. Each parameter was graded on a scale ranging from 0 to 4+. Eyes were removed 24 h following the secondary challenge with the lid and conjunctiva intact and immediately fixed in 4% paraformaldehyde for a minimum of 24 h before processing. The tissue was embedded in

paraffin, serially sectioned (5 µm thick) and stained with haematoxylin and eosin.

Eosinophil and neutrophil cell numbers were counted by two masked observers in five different high-powered fields of the conjunctival forniceal area. To compare the effects of epinastine (Relestat, 0.05%), olopatadine (Patanol, 0.1%) and nedocromil (Alocril, 2%), mice (*n* = 6 per group) received drops in both eyes three times daily for 7 days before primary SRW injection with the drug. Drug treatment continued throughout the entire study. Experiments were performed at least three times.

Statistical analysis

For CBMC data, comparisons of means were performed using Student's *t*-test, with *P* values < 0.05 considered to be significant. Correlations between cytokines were identified where the Pearson coefficient values were 0.90 < *r* < 1.0. Data from conjunctival biopsy-derived cells, which were not normally distributed, were compared using the Wilcoxon signed rank test, and correlations of cytokines were identified using the Spearman coefficient.

Results

Validation of human cord blood stem cell-derived cultures

At week 12, unstimulated CBMC were immunophenotyped as 100% c-kit⁺, >90% FcεR1⁺ (Figs 1a and b), >95% tryptase⁺ and >90% chymase⁺ (data not shown). AA1⁺ (tryptase) cells were further divided into those with and without vacuolation, denoting degranulation (Fig. 1c). Comparing unstimulated (Fig. 1e) with FcεR1-stimulated CBMC (Fig. 1f), a significant increase in degranulation was observed (*P* < 0.001), which was inhibited by pre-treatment with either epinastine (Figs 1c and g) or olopatadine (Fig. 1c).

Effects on histamine and cytokines

Following FcεR1 stimulation, histamine secretion was increased (27.9 ± 4.7 ng/10⁶ cells; *P* < 0.05), with basal levels varying between assays. Histamine secretion by stimulated CBMC was differentially inhibited by all drugs, which was concentration dependent (Fig. 2), with nedocromil being the most effective CBMC stabilizer. In addition to histamine, IL-5, TNF-α and TNF-β were detected in unstimulated CBMC and all except IL-5 were up-regulated upon stimulation. All drugs significantly reduced TNF-α and, with the exception of olopatadine, all drugs decreased the levels of IL-5, despite high basal levels. IL-8 was significantly inhibited by epinastine and azelastine, IL-10 by epinastine and olopatadine and TNF-β by azelastine and olopatadine. Neither IL-1β nor IFN-γ were detectable. IL-2 was up-regulated upon stimulation but

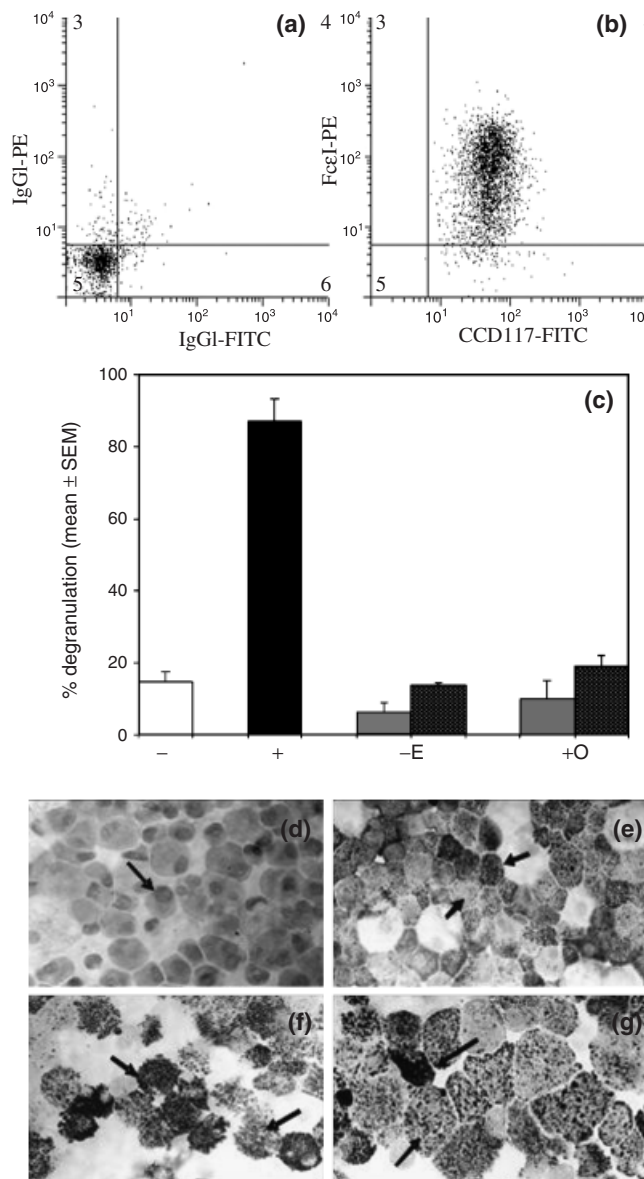


Fig. 1. Flow cytometric histograms showing CD117-expressing human cord blood stem cell-derived (CBMC) (b) and isotype-matched controls (a). CBMC degranulation in response to anti-IgE monoclonal antibody (mAb) stimulation in the presence or absence of epinastine and olopatadine (c) and direct immunostaining with AA1 mAb (d–g). The arrows indicate effects on individual cells. FITC, fluorescein isothiocyanate.

was unaffected by drugs. IL-4 levels were highly variable and IL-6 was present at high levels under all conditions and was unaffected by stimulation or the addition of drugs (data not shown). Following CBMC stimulation, positive correlations were found for IL-8 with histamine ($P < 0.05$) and IL-4 with IL-5 ($P < 0.05$).

Effects of anti-allergic drugs on conjunctival mast cells

A total of five donors' biopsy specimens were large enough to divide into four separate wells, allowing a

direct comparison of the effects of epinastine and olopatadine. In unstimulated cultures, histamine and cytokines were detectable and, in response to FcεR1 cross-linking, the levels increased relative to controls but this did not reach significance due to varying levels between biopsy specimens (Figs 3a–c). In the presence of epinastine or olopatadine, histamine release was decreased and IL-8 secretion was reduced although the effect did not reach significance, whereas TNF-β secretion was unaffected by the drugs. For four separate biopsy-derived cultures, approximately 20–30% of the CD117⁺ MCs (Fig. 3d) were IL-8 positive, with 10–30% of the cytokeratin-positive cells expressing IL-8.

A total of 11 donors' biopsy-derived cultures were obtained with sufficient cells to compare unstimulated with stimulated cells alone, and following pre-treatment with epinastine. Comparing paired mean values within biopsy cultures (Table 1), histamine was detected in nine of 11 biopsies (mean 987.5 ± 178.2 pg/mL) before stimulation. Significant increases in histamine were found in response to stimulation in five out of 11 biopsies, and four of these responded to epinastine by decreasing levels of histamine. In a total of six of 11 biopsies, there was a significant decrease in histamine in the presence of epinastine. TNF-β secretion was significantly up-regulated in response to stimulation in 10 of 11 conjunctival cultures and unaffected by epinastine. IL-6, like histamine, was present in unstimulated cultures (mean 1776.1 ± 592.1 pg/mL) and was not up-regulated in response to stimulation but was down-regulated by epinastine. The levels of both IL-5 and IL-8 were increased following FcεR1 cross-linking and decreased following epinastine. Upon stimulation of the biopsy-derived cultures, increased levels of IL-8 correlated with IL-5 and IL-6 ($P < 0.05$; $r = 0.90$) and IL-4 correlated with IL-5 ($P < 0.01$; $r = 0.94$) and TNF-β ($P < 0.05$; $r = 0.90$).

Effects of anti-allergic drugs on conjunctival cell infiltration in vivo

Using an established model of AC (11), the effects of epinastine, olopatadine and nedocromil were investigated. Tearing was significantly reduced by both nedocromil and epinastine ($P < 0.03$) while olopatadine had no effect (data not shown). Topical treatment with epinastine, olopatadine and nedocromil significantly reduced redness ($P < 0.05$), whereas only epinastine reduced lid oedema ($P < 0.05$) in comparison with controls (Fig. 4a). Cell counts were performed histologically at 24 h and there was a dense infiltration of cells around the lid fornix at this time-point (Fig. 4c) in comparison with normal tissue (Fig. 4b). Epinastine treatment reduced the cellular infiltration (Fig. 4d) and olopatadine treatment had less of an effect on infiltrating cells (Fig. 4e). Forniceal cell counts were performed and eosinophil numbers were

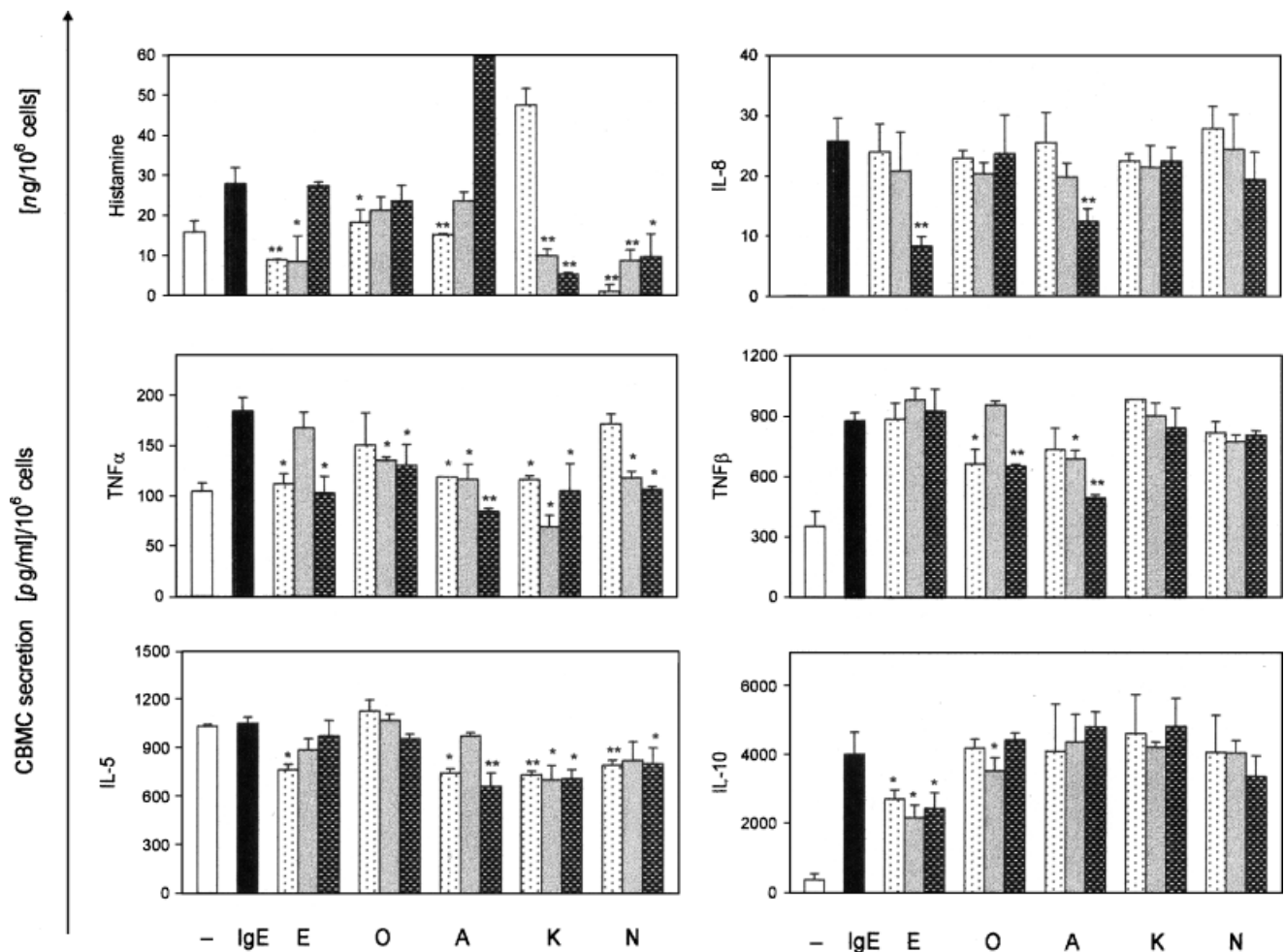


Fig. 2. Levels of histamine and IL-8 (ng/mL), TNF- α , TNF- β , IL-6 and IL-10 (pg/mL) in supernatants from unstimulated human cord blood stem cell-derived (CBMC), stimulated alone or with epinastine (+E), olopatadine (+O), azelastine (+A), ketotifen (+K) or nedocromil (+N) at 0.1, 1.0 and 10 μ g/mL shown in light, medium and dark shading, respectively. Data, pooled from three separate experiments, are expressed as means $\pm$ SEM. * P < 0.05; ** P < 0.01.

significantly reduced following treatment with all three drugs (P < 0.03), whereas neutrophil counts were selectively decreased by epinastine (P < 0.02; Fig. 4f). A summary of the effects of the drugs in each model is shown in Table 2.

Discussion

Studies of human conjunctival MC are severely hampered by the low numbers of cells recoverable from biopsy specimens. For optimizing the assays used in this study, human CBMC were used and were found to share many features of their conjunctival counterparts, including co-expression of tryptase and chymase [14], degranulation upon Fc ϵ R1 cross-linking and expression of CD117 (c-kit) [15]. CBMC were therefore used as a model for MC responses to the anti-allergic drugs, and conjunctival MC-containing cultures were assayed in parallel where possible. Before stimulation, both *in vitro* MC models

contained > 90% preformed IL-4 (data not shown), and secreted detectable levels of histamine, TNF- α , TNF- β , IL-5 and IL-6, and no IL-2, IFN- γ or IL-10. The only difference between the two cell models was in IL-8, which was readily detected in unstimulated biopsy-derived cultures but absent or minimally detected in unstimulated CBMC. One explanation for this discrepancy is the presence of other IL-8-producing cells because the biopsy-derived cultures were maintained for at least 5 days before stimulation, when the likeliest contaminating cells are fibroblasts and epithelial cells. The fact that the IL-8 levels did not increase in response to IgE cross-linking in six out of 11 biopsies supports the hypothesis that IL-8 was being produced by cells other than MC in those cultures. In addition, because cultures were maintained in SCF, the conjunctival MC could potentially become activated via c-kit, although this was not found in CBMC [16], and would not explain why IL-8 alone was different between the two models. Another possibility is that the

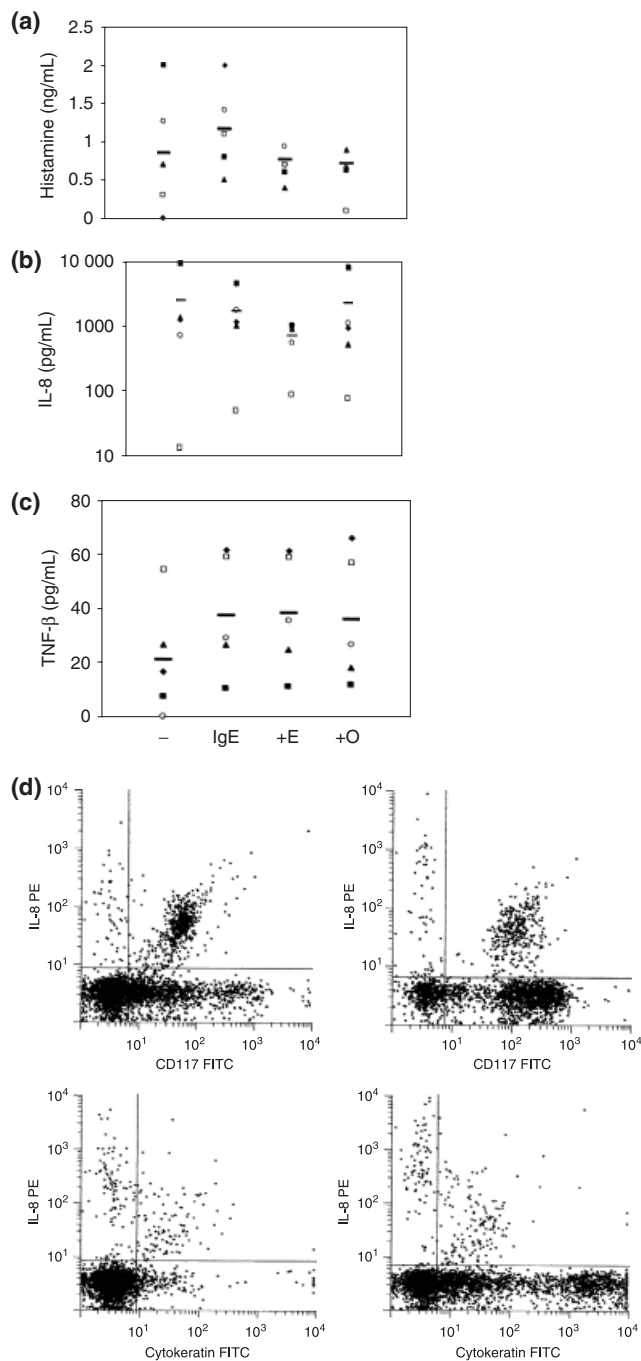


Fig. 3. Conjunctival biopsy-derived cultures were analysed for histamine (a), IL-8 (b) and TNF- β (c). Cells were unstimulated (-), stimulated alone (+) or with epinastine (+E) or olopatadine (+O). Bars indicate mean levels. Intracellular expression in four biopsies of IL-8, on CD117-positive mast cells (d) and on cytokeratin-positive epithelial cells. FITC, fluorescein isothiocyanate.

conjunctival MC were already activated *in vivo* before culturing because the biopsies were taken from pterygium patients, although great care was taken to remove tissue from unaffected areas where the MC have been shown to be phenotypically similar to those from normal

Table 1. Numbers of responding conjunctival biopsy cultures responding (total of 11) and comparison of their mean levels of histamine and cytokines

Response	Increase in response to Fc ϵ RI stimulation <i>n</i> (<i>P</i> value <)	Decrease in response to epinastine (0.1 μ g/mL) <i>n</i> (<i>P</i> value <)
Histamine	5 (0.05)	6 (0.01)
TNF- α	4 (0.05)	NS
TNF- β	10 (0.001)	NS
IL-5	8 (0.01)	5 (0.05)
IL-6	NS	5 (0.05)
IL-8	5 (0.05)	7 (0.01)

n = No. of biopsies.

NS, not significant.

conjunctival tissue [17]. Given the potential variation in the initial activation status, all cytokine responses were compared within individual biopsies to obtain a relative effect of the drugs within each specimen.

The anti-allergics in this study were used within the range of concentrations found in tears minutes after topical application, and were thus considered to be physiologically relevant [18]. For some drugs (azelastine and ketotifen), there was an increase in histamine secretion at specific concentrations, suggesting an antagonistic effect, which has been reported previously [19]. Because both the drugs have been approved for clinical use, one could speculate that their ability to prevent histamine binding to its H1 receptor effectively neutralizes any effects of an increase in histamine production. In initial experiments, epinastine and olopatadine were added at the time of MC stimulation, and a partial inhibition of degranulation was observed with reduced levels of histamine and cytokine production. When added to the cells 30 min before stimulation, however, the drugs were more inhibitory, although the cytokine profiles were identical (data not shown) [5]. Although this allowed the drugs more time to access their targets, the mechanisms involved are unknown. Preliminary data in our lab suggest a drug-specific inhibition of activation-induced nuclear translocation of NF- κ B and c-jun, but not NFAT, and we are currently investigating these mechanisms (unpublished data).

Nedocromil is a gold standard MC stabilizer that has been used for many years as a therapy for SAC [20]. It was found to be effective in preventing histamine, TNF- α and IL-5 release, which is probably a non-specific effect of stabilization because these mediators were also secreted by unstimulated cells. For those cytokines in which there was *de novo* synthesis and secretion after stimulation (IL-8, IL-10, TNF- β), nedocromil and ketotifen were minimally inhibitory whereas the other drugs were able to inhibit the secretion of these latter cytokines selectively.

Intracellularly, CBMC were found to express high levels of IL-4 before stimulation, as reported previously in

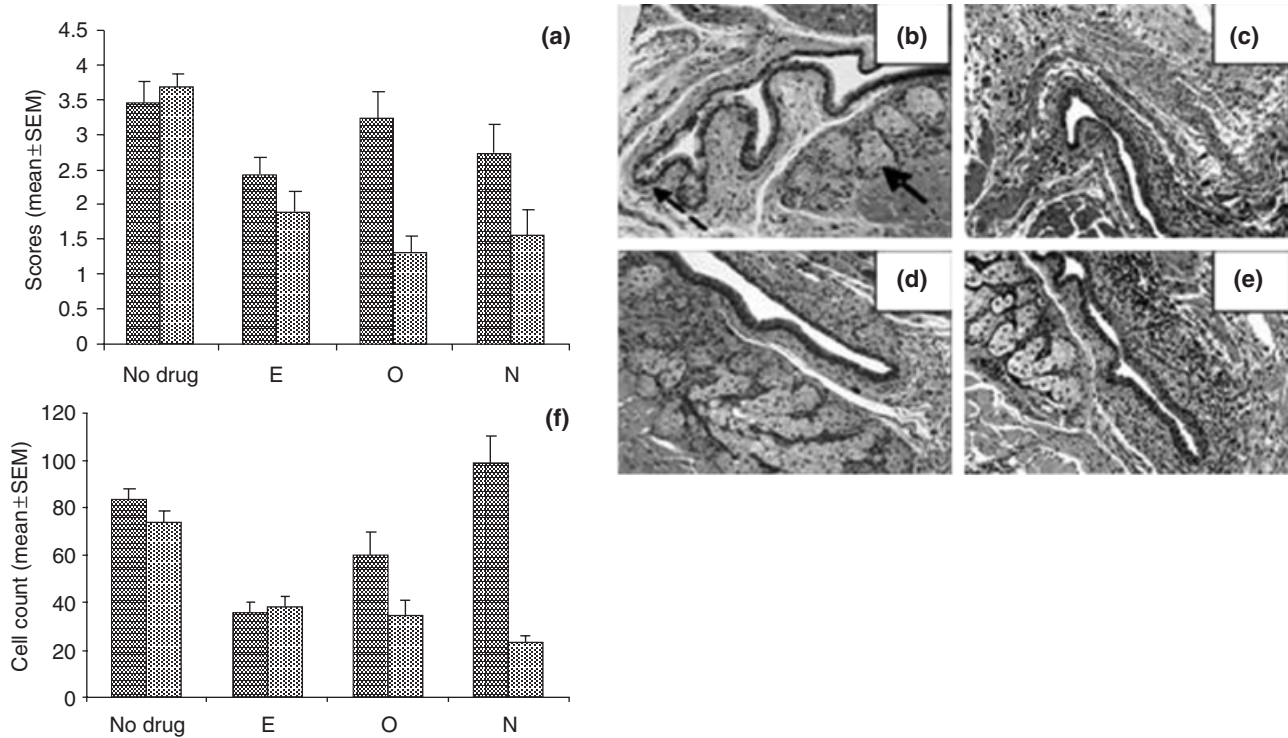


Fig. 4. Scores for lid oedema (filled) and redness (shaded) (a); sections (magnification 20×) from a control (b), treated with vehicle (c), epinastine (d) or olopatadine (e), showing the fornix (hatched arrow) and the meibomian gland (solid arrow). Forniceal cell counts for neutrophils (filled) and eosinophils (shaded) (f). E, epinastine; O, olopatadine; N, nedocromil.

Table 2. Summary of effects of anti-allergic drugs on CBMC, human conjunctival biopsy cultures and mouse AC

<i>In vitro</i>	IgE	Epinastine	Olopatadine	Azelastine	Ketotifen	Nedocromil
CBMC						
IL-5	–	↓	–	↓↓	↓↓	↓↓
IL-8	↑↑	↓↓	–	↓↓	–	–
IL-10	↑↑	↓	↓	–	–	–
TNF- α	↑↑	↓	↓	↓↓	↓	↓
TNF- β	↑↑	–	↓↓	↓↓	–	–
Histamine	↑↑	↓↓	↓	↓↓	↓↓	↓↓
Conj MC						
IL-5	↑↑	↓	ND	ND	ND	ND
IL-6	–	↓	ND	ND	ND	ND
IL-8	↑	↓↓	ND	ND	ND	ND
TNF- α	↑	–	ND	ND	ND	ND
TNF- β	↑↑	–	ND	ND	ND	ND
Histamine	↑	↓↓	ND	ND	ND	ND
<i>In vivo</i>						
Edema	↑↑	↓	–	ND	ND	–
Tearing	↑↑	↓	–	ND	ND	↓
Redness	↑↑	↓	↓	ND	ND	↓
Neutrophils	↑↑	↓	–	ND	ND	–
Eosinophils	↑↑	↓	↓	ND	ND	↓

↑, significant increase ($P < 0.05$); ↑↑, highly significant increase ($P < 0.01$); ↓, significant decrease ($P < 0.05$); ↓↓, highly significant decrease ($P < 0.01$); –, no effect; ND, not done.

CBMC, human cord blood stem cell-derived; MC, mast cell; AC, atopic conjunctivitis.

human basophils [21] and conjunctival MC [22], which was reduced following stimulation with or without brefeldin A. Because brefeldin inhibits protein transport through the golgi to the cell membrane for exocytosis, if a protein continues to be secreted by a cell in the presence of brefeldin, it must involve a mechanism that excludes the golgi. In MCs, soluble factors can be stored preformed in vesicles and, while the golgi is required for their processing it is not for exocytosis of preformed vesicle contents, suggesting that the release of IL-4 by CBMC in this study bypassed the golgi. In contrast, IL-8 and IL-13 were not expressed in unstimulated cells but were detected intracellularly after stimulation and only in the presence of brefeldin A (data not shown), confirming that these CBMC-derived cytokines required *de novo* synthesis.

The drugs could be causing indirect cytokine- and histamine-induced effects on non-MC present by directly inhibiting epithelial cell activation via H1 receptor blockade, because it has been shown that epithelial cells secrete IL-6 and IL-8 through stimulation of H1 receptors [23, 24]. Epinastine has also been shown to affect other cell types: it inhibited chemotaxis of IL-5-pre-treated eosinophils in response to eotaxin [25] and dose-dependently down-regulated expression of CD11b in activated eosinophils; it inhibited IL-8 release from eosinophils [26]; it suppressed TARC and IL-4 from CD3/28-stimulated CD4⁺ T cells [27]; and in IL-4-stimulated CD4⁺ T cells, it down-regulated IL-5, IL-6, IL-13 and NF- κ B [28]. Similarly, olopatadine indirectly inhibited the release of neurotoxin from eosinophils via its inhibition of MC although the adhesion of eosinophils to epithelial cells was unaffected [8, 9]; it inhibited calcium influx, which correlated with the degree of degranulation and inhibited IL-4 expression in the RBL-2H3 basophil cell line [29].

In vivo, the resultant effects of nedocromil, epinastine and olopatadine on reducing redness were comparable, although probably via different mechanisms. Based on histamine release, nedocromil was a more effective MC stabilizer *in vitro* than the other two drugs; yet, all three decreased redness to the same extent. Because epinastine and olopatadine are also anti-histamines, they will inhibit histamine-mediated vasodilation via H1 (and H2) receptor antagonism.

Upon binding to its receptors (H1 and H2), histamine induces vascular leakage, resulting in further cellular infiltration from the blood, including eosinophils and neutrophils, leading to chemosis. Following vascular leakage and cellular infiltration, oedema was detected at 24 h post-challenge and was inhibited by epinastine in this study, probably due to its known H2 receptor antagonism. All three drugs significantly decreased eosinophil infiltration, as predicted from the literature; yet, only epinastine reduced neutrophil infiltration, possibly due to its inhibition of MC-derived IL-8, a potent chemokine for neutrophils. In conclusion, the drugs included in this

study had pleiotropic effects on MC responses, involving different cytokines. One prediction from these studies would be that SAC therapy might be improved by combining or alternating the different anti-allergic drugs for each patient to ensure that the full range of inflammatory cytokines is inhibited.

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