Conjunctival Eosinophil Infiltration Evoked by Histamine and Immediate Hypersensitivity

Modification by $H_1$- and $H_2$-Receptor Blockade


The present histological studies have demonstrated that histamine causes dose-dependent eosinophil infiltration into the conjunctiva. A highly directional movement toward the conjunctival epithelium was observed, and the presence of large numbers of degranulating eosinophils appeared to result in epithelial cell damage and goblet cell discharge. Blockade of $H_1$-receptors by systemic cimetidine pretreatment significantly inhibited the eosinophil infiltration elicited by an intermediate histamine dose, whereas the $H_2$-receptor blockade produced by systemic pyrilamine pretreatment markedly reduced the response to all histamine doses. The pyrilamine-insensitive residual eosinophil infiltrate was not affected by administering a cimetidine-pyrilamine combination. In animals presensitized to ovalbumin, antigen challenge evoked extensive and directional emigration of eosinophils toward the conjunctival epithelium with resultant exfoliation and depletion of goblet cell populations. In conjunctival immediate hypersensitivity, neither cimetidine nor pyrilamine alone produced an inhibitory effect, but a cimetidine-pyrilamine combination caused a significant reduction in the number of infiltrating eosinophils and prevented epithelial damage and goblet cell depletion. These results suggest that histamine may participate in the recruitment of eosinophils during immediate hypersensitivity reactions. The differential effect of pyrilamine on the eosinophil infiltration evoked by histamine or immediate hypersensitivity may, perhaps, reflect the importance of increased microvascular permeability in facilitating eosinophil emigration.


The presence of intact eosinophils, or the free granules and proteins that result from eosinophil fragmentation, is a characteristic feature of conjunctival immediate hypersensitivity. A variety of mediators are capable of affecting eosinophil motility; these include histamine, ECF-A tetrapeptides, lipooxygenase and cyclooxygenase metabolites of arachidonic acid, and lymphokines. However, the activity of these substances as eosinophil chemotactants in the conjunctiva and their relative importance in conjunctival allergy remain to be fully elucidated.

Histamine has long been recognized as an important mediator of immediate hypersensitivity. In the conjunctiva, tear histamine levels are elevated in vernal conjunctivitis, and application of histamine to the ocular surface reproduces the vascular and pruritic symptoms associated with conjunctival mast cell discharge. Since histamine is capable of stimulating eosinophil motility and oxidative metabolism, it also has the potential to participate in the local recruitment and activation of eosinophils typically associated with episodes of conjunctival allergy. Thus, the microscopic studies described herein were undertaken to further define histamine effects on the conjunctiva, with particular emphasis on eosinophil infiltration. Initial studies involved determining the time course of histamine-induced eosinophil emigration and characterizing associated morphological changes in the conjunctiva. The dose-response relationship for histamine-induced eosinophil emigration was then established at an appropriate time point, and susceptibility to $H_1$- and $H_2$-receptor blockade was quantitatively evaluated. Finally, the extent to which histamine may contribute to eosinophil infiltration associated with conjunctival immediate hypersensitivity was investigated by examining the inhibitory activity afforded by $H_1$- and $H_2$-receptor antagonist pretreatment.

Materials and Methods

Animals, Sensitization, and Drug Treatment Regimens

Albino, Hartley strain guinea pigs of either sex were employed. Animals weighing 300–350 g were selected...
for sensitization to chicken ovalbumin (Sigma Chemical Co., St. Louis, MO) by a previously described method. A 4-week period was allowed for sensitization to develop, by which time the animals achieved the 400-500 g weight range employed for the histamine studies. In order to prevent ocular scratching, the hind legs of the animals were restrained in a polyethylene cone.

Cimetidine (Smith, Kline, and French, Welwyn, England), pyrilamine maleate (Hexagon, Bronx, NY), histamine (Sigma Chemical Co., St. Louis, MO) and chicken ovalbumin solutions were prepared as previously described. Histamine and chicken ovalbumin % w:v (weight:volume) solutions were administered to the left eye in a 20 μl volume over a 0.05-5% concentration range (10-1000 μg/eye) for histamine and at a single 0.5% concentration (100 μg/eye) for ovalbumin. The contralateral eye received 20 μl saline as a control. Cimetidine (120 mg/kg) and pyrilamine (2 mg/kg) were administered subcutaneously into the scruff of the neck 30 min before topical histamine application. In the case of the immediate hypersensitivity studies, cimetidine and pyrilamine were also administered at 3 hr post-antigen challenge to ensure specific H1- and H2-receptor blockade throughout the experimental time course. Animals were sacrificed by intracardiac injection of T61 euthanasia solution (Hoechst, Somerville, NJ) as previously described.

Microscopic Studies

Immediately after the death of the animal, 10% neutral-buffered formalin was administered to the ocular surface. The globe and the attached eyelid ring were then surgically excised intact and fixed in 10% neutral-buffered formalin for 24 hr at room temperature. Tissues were embedded in paraffin and two 6 μm sections were obtained per eye in such a manner as to prevent the same cell population from being counted in both sections. The sections were stained by Luna's technique for eosinophil granules. Counting of eosinophil polymorphonuclear leukocytes was at 400× magnification with the aid of a calibrated (10 × 10 lattice) ocular grid according to set, predetermined rules that allowed counting of epithelial and subepithelial eosinophils and goblet cells to be achieved without moving the grid. Free eosinophil granules that were dissociated from leukocytes were not counted. Therefore, in some instances, the number of emigrated eosinophils may have been underestimated. In order to facilitate counting, the conjunctival areas were defined according to general morphological features that are easily discernable under the microscope. These areas were the limbal, fornix, and tarsal regions. Counting fields were selected randomly by maintaining an unfocused image; this prevented observation of eosinophils but allowed general topographical alignment of the grid and prevented overlapping fields. Each field was brought into sharp focus for counting. Five 250 μm² high power fields per section per conjunctival region were counted for each section. Mean epithelial and subepithelial cell counts were obtained for each eye (epithelial counts also included eosinophils adjacent to the epithelial basement membrane). The difference in these values between treated and control eyes was determined in order to calculate the mean epithelial and subepithelial cell counts ± SEM per high power field for each treatment group.

The investigations described conform to the ARVO Resolution on the Use of Animals in Research.

Statistical Analyses

Potency ratios were determined by the parallel line assay method of Finney and are given with 95% confidence limits in parentheses. Additional statistical analyses employed analysis of variance.

Results

The time course of eosinophil infiltration into the conjunctiva evoked by topical application of 5% histamine is depicted in Figure 1. At 15 min post-histamine application, eosinophils were virtually absent in both epithelial and subepithelial areas, but by 1 hr modest numbers were observed in some instances. Marked eosinophil infiltration was observed at 6 hr with accumulation in the epithelium as the apparent result of directional motility. The morphological changes typically associated with eosinophil emigration and activation are depicted photomicrographically in Figure 2. The absence of an eosinophil infiltrate and associated tissue damage at 15 min post-5% histamine
application is revealed by comparing Figure 2a with the saline-treated control tissue in Figure 2b. The marked cellular infiltrate at 6 hr post-histamine application is illustrated in Figures 2c and 2d. Although eosinophils in the subepithelial area appeared intact, those associated with the epithelium were frequently fragmented, and free granules were a common occurrence. Severe conjunctival epithelial damage was coincident with the presence of degranulating eosinophils. The epithelial surface was pitted and roughened with epithelial cells sloughing off. A marked decrease in goblet cell numbers, apparently due to the expulsion of their contents, was also observed. The apparent directional diapedesis of eosinophils and the morphological events that occur in the conjunctival epithelium are illustrated diagramatically in Figure 3. By 24 hr,
Fig. 3. Diagrammatic representation of eosinophil emigration and action in the guinea pig conjunctiva, based upon our observations. Eosinophils in the peripheral circulation (A) marginate and adhere to the microvascular endothelium (B). Eosinophils eventually migrate through to the perivascular space, where they briefly gather before rapidly emigrating through the substantia propria (C) toward the epithelial layer. Eosinophils gather at the lamina propria (D) before working their way into the epithelium. Eosinophil degranulation and fragmentation takes place in and immediately adjacent to the epithelium, with consequent epithelial exfoliation and depletion of goblet cell populations (E) (no evidence of degranulated or fragmented eosinophils was ever observed in subepithelial or vascular regions).

The tissue had substantially recovered; eosinophil numbers were markedly less than at 6 hr (Fig. 1), the goblet cell population was largely restored, and the epithelium had regenerated to the extent that it approached a normal appearance. A modest, diffuse neutrophil infiltrate was also observed at 6 hr and 24 hr.

The effect of pyrilamine (H₁-receptor antagonist) and cimetidine (H₂-receptor antagonist), alone and in combination, on eosinophil infiltration into the conjunctiva evoked by graded concentrations of histamine is shown in Figure 4. Cimetidine significantly displaced the eosinophil infiltration dose-response curve in both the epithelial and subepithelial regions, as indicated by the respective potency ratio values: epithelial potency ratio = 0.251 (0.051–0.827), and subepithelial potency ratio = 0.456 (0.204–0.962). Since comparison of the dose-response relationships between groups that received pyrilamine and the control group did not provide parallel lines, the determination of potency ratio values was not possible. This necessitated performing an analysis of variance for each histamine dose (Table 1). Pyrilamine consistently produced a significant decrease in eosinophil numbers in both epithelial and subepithelial regions relative to the control group, but not to the cimetidine-treated group. The activity of the cimetidine-pyrilamine combination was essentially indistinguishable from pyrilamine alone. The modest
neutrophil infiltration associated with higher doses of histamine also appeared to be reduced by pyrilamine and the cimetidine-pyrilamine combination. Goblet cell discharge and epithelial cell damage seemed to be related to the number of eosinophils that achieved the conjunctival epithelium. Severe damage was only associated with a relatively dense eosinophil infiltrate, and treatments which reduced the marked eosinophil emigration evoked by high doses of histamine afforded protection to the conjunctival epithelium.

The effect of cimetidine and pyrilamine, alone and in combination, on the eosinophil infiltrate associated with conjunctival immediate hypersensitivity is shown in Figure 5; a statistical comparison of treatment groups is provided in Table 2. Neither cimetidine nor pyrilamine alone altered eosinophil infiltration into the conjunctiva. A significant inhibition was, however, achieved with the cimetidine-pyrilamine combination relative to all other treatment groups in both epithelial and subepithelial regions. A combination of cimetidine and pyrilamine also prevented goblet cell discharge and epithelial damage, as illustrated in Figure 6. Figure 6a shows the conjunctiva from a presensitized guinea pig that received the ovalbumin challenge. Eosinophils are present in the subepithelial and epithelial regions, and marked damage to the epithelial surface is apparent. A higher power view of the epithelium in Figure 6b shows more clearly the damaged surface and the virtual absence of goblet cells. Numerous eosinophils and free eosinophil granules were associated with the damaged conjunctival epithelium. The effect of the cimetidine-pyrilamine combination on conjunctival eosinophil infiltration and associated conjunctival epithelial damage is illustrated in Figures 6c and 6d. A reduction in eosinophil numbers in the subepithelial region and, notably, in the epithelium was apparent, although a distinct residual infiltrate remained. Goblet cells were largely intact, and epithelial exfoliation was not observed.

**Discussion**

The vascular and sensory effects of histamine on the ocular surface have been previously described. The present studies indicate that histamine may also elicit
epithelium. Although eosinophils in the subepithelium were often fragmented and degranulating. Severe epithelial damage and goblet cell expulsion were coincident with the presence of numerous degranulating eosinophils in the immediate vicinity. The association between degranulating eosinophils and epithelial damage suggests that such changes in epithelial morphology may possibly be mediated indirectly by histamine-induced recruitment and activation of eosinophils. Eosinophils may cause tissue destruction by virtue of their cytotoxic granular proteins; notably, major basic protein (MBP). MBP damages many types of mammalian cells, and the marked disruption and damage to the airway epithelium\textsuperscript{24,25} that occurs in response to MBP resembles the conjunctival epithelial changes evoked by histamine or antigenic challenge.

A direct effect of histamine on the conjunctiva provides a possible alternative explanation for epithelial damage. However, the absence of eosinophils and epithelial damage at earlier time intervals, during which substantial plasma exudation occurs\textsuperscript{26} does not lend support to this suggestion. Furthermore, at 24 hr post-histamine administration, epithelial eosinophils were absent and the conjunctival epithelium had substantially regenerated. Goblet cell numbers were also returning to normal by this time.

The increase in conjunctival eosinophil numbers associated with graded doses of histamine was reduced by pretreatment with specific doses of both cimetidine (H\textsubscript{2}-receptor antagonist) and pyrilamine (H\textsubscript{1}-receptor antagonist).\textsuperscript{19,27} The ability of both cimetidine and pyrilamine to attenuate the response indicates that the inhibitory activity may involve functionally different H\textsubscript{1} and H\textsubscript{2}-receptors. The activity profile whereby both cimetidine and pyrilamine can markedly reduce the same pathobiological phenomenon is distinctly different from situations where histamine elicits a single response by stimulating both receptor subtypes; in the latter case, H\textsubscript{1}- or H\textsubscript{2}-antagonists alone exert only a modest effect, whereas combined H\textsubscript{1}- and H\textsubscript{2}-antagonist pretreatment produces substantial blockade.\textsuperscript{19,28}

In terms of eosinophil motility, in vitro studies have demonstrated that neither pyrilamine nor metiamide alone altered histamine-induced eosinophil chemotaxis.\textsuperscript{7,29} It has, therefore, been presumed that both H\textsubscript{1}- and H\textsubscript{2}-receptor stimulation increase eosinophil locomotor activity,\textsuperscript{30} but direct evidence for this hypothesis remains to be supplied. However, since the profile of histamine antagonist effects in vitro appears to differ from that obtained in vivo in the conjunctiva, other influences on leukocyte emigration must be considered.

The histamine dose-response characteristics for eosinophil chemotaxis in vitro are complex. Histamine acts as a chemoattractant for eosinophils at low doses, whereas high concentrations may arrest eosinophil chemotaxis\textsuperscript{5,7} and thereby cause localized trapping. H\textsubscript{2}-receptors appear to mediate the eosinophil immobilization associated with high concentrations of histamine.\textsuperscript{7,29} In addition to chemotactic influences on the eosinophil, histamine could possibly promote adhesion of eosinophils to the microvascular endothelium. This provides yet a further potential point for drug intervention. Increased microvascular permeability may also greatly assist in the local recruitment of leukocytes,\textsuperscript{31} and the abolition of the conjunctival microvascular permeability response to histamine by H\textsubscript{1}-receptor blockade could account for the inhibitory activity of pyrilamine on eosinophil infiltration. The combination of cimetidine and pyrilamine does not abolish histamine-induced eosinophil infiltration. This may be due to an inability of cimetidine to effectively antagonize the response to higher doses of histamine. Alternatively, the residual eosinophil infiltration may be due to imidazole acetic acid, a histamine metabolite which is reported to be a chemoattractant for eosinophils.\textsuperscript{32}

The degree of damage to the conjunctival epithelium was associated with the local presence of degranulating eosinophils and any pretreatment that reduced eosinophil infiltration into the epithelium abrogated epithelial destruction. Histamine has been reported to activate eosinophils in vitro, with the possible involvement of predominantly H\textsubscript{1} but also H\textsubscript{2}-receptors.\textsuperscript{18} It is not readily apparent from the histological studies described herein whether antihistamines are preventing activation, since any pretreatment which prevented large numbers of eosinophils from achieving the conjunctival epithelium also prevented eosinophil degranulation, epithelial desquamation, and goblet cell expulsion.

### Table 2. Effect of cimetidine (C), pyrilamine (P), and a cimetidine-pyrilamine combination (C-P) on eosinophil infiltration associated with immediate hypersensitivity in the epithelial and subepithelial regions of the conjunctiva

<table>
<thead>
<tr>
<th>Treatment Comparison</th>
<th>Epithelium</th>
<th>Subepithelium</th>
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<tbody>
<tr>
<td>V vs C</td>
<td>NS</td>
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<tr>
<td>V vs C-P</td>
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<tr>
<td>P vs C</td>
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<td>P vs C-P</td>
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<td>C vs C-P</td>
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\* V = saline-treated control group.
\* P < 0.05.
\* NS = nonsignificant according to analysis of variance.
Conjunctival immediate hypersensitivity exhibited a pattern of eosinophil infiltration, goblet cell discharge, and epithelial damage similar to that produced by histamine. The only discernible difference between the responses evoked by immediate hypersensitivity and histamine was that, in conjunctival anaphylaxis, bands of directional eosinophil migration were apparent which resulted in eosinophils reaching discrete, intermittent regions of the conjunctival epithelium. Consequently, some areas of the epithelium contained abundant eosinophils and were damaged, whereas other areas were free of eosinophils and cytological damage. Inhibition by $H_1$- and $H_2$-receptor blockade was entirely different with respect to the morphological
events associated with histamine and immediate hypersensitivity. Although cimetidine and pyrilamine alone were capable of reducing histamine-induced eosinophil infiltration, neither antagonist alone inhibited the conjunctival eosinophil accumulation associated with immediate hypersensitivity. The combination of cimetidine and pyrilamine provided the only treatment that significantly reduced the eosinophil infiltration associated with immediate hypersensitivity. The cimetidine-pyrilamine combination reduced, but did not abolish, the eosinophil infiltrate; some eosinophils remained in both the subepithelial and epithelial regions. However, the inhibitory activity of the cimetidine-pyrilamine combination was sufficient to considerably improve the condition of the conjunctival epithelium of eyes subjected to antigen challenge. Eosinophils in the conjunctiva appeared intact, little damage to the epithelium was noted, and goblet cell populations appeared normal.

In attempting to provide an explanation for the differential effects of H1- and H2-receptor blockade on conjunctival inflammation evoked by histamine and immediate hypersensitivity, it may be worthwhile to consider other disparities concerning the conjunctival response to histamine and immediate hypersensitivity. A distinct difference has been shown with respect to microvascular permeability. Histamine-induced increases in conjunctival microvascular permeability are virtually abolished by H1-receptor blockade, whereas, in immediate hypersensitivity, a substantial nonhistaminergic, residual response remains after pyrilamine pretreatment. A similar nonhistaminergic microvascular permeability response has also been identified in cutaneous anaphylaxis. Thus, if pyrilamine inhibits histamine-induced eosinophil infiltration by preventing increased conjunctival microvascular permeability, the lack of effect of pyrilamine against eosinophil infiltration associated with immediate hypersensitivity may possibly be due to the continued presence of a microvascular permeability response.

The present studies confirm previous investigations that also demonstrated eosinophil infiltrates as a characteristic feature of conjunctival reactions involving mast cell degranulation. The previously reported directional movement of eosinophils invading conjunctival tissues was also observed. In contrast, it has been claimed that H1-receptor antagonists are capable of markedly reducing the eosinophil infiltrate associated with conjunctival immediate hypersensitivity. The reason for this discrepancy may lie in the techniques employed for examining leukocyte infiltration. The previous study employed brush smears at 6 hr post-challenge, and even conjunctival scappings may fail to demonstrate eosinophil presence, even in eyes where the epithelium is known to be infiltrated with eosinophils. The lack of effect of H1-receptor blockade on eosinophil infiltration is consistent with studies on systemic and cutaneous anaphylaxis. Although a previous study has claimed that a single dose of histamine produces a predominantly neutrophilic infiltrate with few eosinophils, the present studies involving a wide dose-range of histamine indicate that the major effect of histamine was to cause a dose-dependent and directional eosinophil infiltration. Nevertheless, a relatively modest, diffuse subepithelial neutrophil infiltrate did accompany eosinophil emigration elicited by the highest histamine doses, and treatment regimens that reduced eosinophil infiltration also seemed to decrease the number of neutrophils present in the subepithelium.

Several substances may alter eosinophil motility, but the relative importance of such putative mediators in eosinophil infiltration associated with immediate hypersensitivity reactions in the conjunctiva has remained unclear. This study suggests that histamine plays a significant role in recruiting eosinophils into the conjunctiva, but that blockade of both H1- and H2-receptors is needed to reduce the number of infiltrating eosinophils and the coincident damage to the conjunctival epithelium.

Key words: histamine, immediate hypersensitivity, conjunctiva, eosinophils, pyrilamine, cimetidine, guinea pigs

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References

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