Conjunctival Basophil Hypersensitivity Lesions in Guinea Pigs
Analysis of Upper Tarsal Epithelium

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The upper tarsal conjunctival epithelium was analyzed for inflammatory cell profile and accompanying morphological changes in a guinea pig system with histopathology resembling two human ocular diseases: vernal conjunctivitis and contact lens-associated giant papillary conjunctivitis (GPC). Female Hartley strain guinea pigs were immunized intradermally on day 0 with 200 μg keyhole limpet hemocyanin (KLH) and challenged on day 6 with varying doses of KLH by injection beneath the conjunctival epithelium of one lid and phosphate-buffered saline in the contralateral lid. Tissues containing the reaction site were examined by light microscopy. The 50 μg dose of KLH elicited the maximal accumulation of basophils and eosinophils. These values were significantly higher than in the PBS-injected control. Injection of KLH, PBS, or insertion of a sterile needle into unsensitized animals, and uninjected tissue served as additional controls. Neutrophils were significantly higher in the epithelium of the traumatized tissue (repeated needle insertions) than in the uninjected control. Basophils and mast cells were rarely found in the epithelium of unsensitized animals. Epithelial thickening, quantified by a Zeiss Videoplan2 Image Analysis system (Zeiss, West Germany), was greatest in the traumatized tissue, followed by the KLH-challenged tissue of sensitized animals. These values were significantly greater than that of the PBS-injected lid or of naive animals, un.injected or KLH-injected. These results indicate that epithelial changes can be induced by both antigen and trauma. Such epithelial changes may have a role in both vernal conjunctivitis and giant papillary conjunctivitis. Invest Ophthalmol Vis Sci 27:1255-1260, 1986

The study of two ocular disease states, vernal conjunctivitis and contact lens-associated giant papillary conjunctivitis (GPC), has been the primary focus in our laboratory over the past years. These disorders, which afflict a number of children and contact lens wearers, have three common features: both diseases 1) involve the formation of large papillae on the upper tarsal conjunctiva as well as thickening of the conjunctival epithelium, 2) present a delayed-type hypersensitivity reaction, as evident by the accumulation of basophils and eosinophils in the substantia propria and epithelium of the conjunctiva, and 3) threaten corneal integrity and may lead to severe visual impairment. Though the symptoms of GPC can usually be interrupted by discontinuing contact lens wear, this is not a viable alternative for patients who must wear contact lenses, such as those with keratoconus.

Despite the clinical importance of these conditions, the mechanisms underlying their pathology are not well understood. Recently, we have developed a system in female guinea pigs which mimics the histopathology found in vernal conjunctivitis and GPC. Administration of defined antigens to the conjunctiva of sensitized animals resulted in the accumulation of basophils and eosinophils in the substantia propria. Furthermore, this antigenic challenge produced an inflammatory reaction common to delayed-type hypersensitivity reactions, including increased vascular permeability as evidenced by the presence of compacted red blood cells in blood vessels and red blood cell extravasation (unpublished observations).

In the present study, we analyzed the epithelium of the upper tarsal conjunctiva of our guinea pig system for inflammatory cell profile as well as epithelial thickness. Our results suggest that the histopathology exhibited in the epithelium of this system mimics that seen in the two human ocular disease states.
Materials and Methods

All investigations involving animals that are described in this manuscript conform to the ARVO Resolution on the Use of Animals in Research.

Immunization

Outbred, Hartley strain female guinea pigs (Elm Hill Farms, Chelmsford) weighing 250–360 g were immunized on day 0 with keyhole limpet hemocyanin (KLH) (Calbiochem-Behring, San Diego). Briefly, 200 μg KLH dissolved in 0.1 ml phosphate-buffered saline (PBS) was injected intradermally into the shaved flank. On day 6, animals were anesthetized with pentobarbital (5 mg pentobarbital per 100 g body weight) (Abbott Laboratories, Chicago). A 4-0 silk suture was inserted through the lid margin to aid in everting the lid, exposing the conjunctival epithelium. Animals (n = 5 per group) were injected with KLH (0.1, 1.0, 10.0, 25.0, 50.0, or 100.0 μg per 5 μl PBS). Tissue was viewed with a portable stereoscopic microscope and the cell layers of the upper tarsal conjunctival epithelium were lifted using a 30-gauge needle on a Hamilton (Reno, NV) syringe allowing antigen injections. Stromal tissue was not penetrated prior to injection of antigen. PBS was injected similarly into the lid of the contralateral eye. Control and experimental lids were alternated between the right and left eyes of each group.

Two groups of animals (n = 5 per group) were used as additional controls. One group received a similar conjunctival injection of 5 ng or 100 ng KLH in the lid of one eye and PBS in the contralateral eye. The other group received 3–5 insertions of a sterile needle (to produce trauma), and the contralateral eye was left untouched.

Tissue Processing

Guinea pigs were sacrificed by CO₂ inhalation 24 hr after antigen challenge. The conjunctival injection site was excised and cut into 2X2 mm pieces. The tissue was then placed in primary fixative (one part 3% glutaraldehyde in 0.1 M morphoethanesulfonic acid buffer [MES] at pH 7.4 [Eastman Kodak, Rochester] and one part Bouin’s fixative containing formalin, acetic acid, ethanol, and picric acid)³ for 3 hr. Tissues were rinsed twice for 15 min in 0.1 MES, postfixed in 0.5% osmium tetroxide in 0.1 M MES for 2 hr, dehydrated in a graded ethanol series (50, 70, 85, 95, 100%), placed in propylene oxide for two 15-min periods, and embedded in Spurr’s resin⁹ (Electron Microscopy Sciences, Fort Washington). Longitudinal sections (1 μm thick) were stained in alkaline Giemsa (Fisher Scientific, Springfield) at 60°C for 1.5 hr. Slides were rinsed in running distilled water, dehydrated in alcohol, cleared in xylene, and mounted.

Cell Counts

Cell types were identified at 1000× magnification according to the criteria of Askenase and Atwood.¹⁰ Basophils, eosinophils, neutrophils, and mast cells were counted in ten adjacent fields of the conjunctival epithelium above the stromal reaction site. All cell counts were masked.

Image Analysis

Overall epithelial thickness was measured with a Zeiss Videoplan2 Image Analysis system (Zeiss, West Germany). Twenty random sites were measured along the reaction site for each animal at 1000× (100 total measurements). A line perpendicular to the outermost surface of the epithelium into the basal layer of the epithelium was drawn and measurements were taken along this line.

Statistics

The Mann-Whitney U test, one-tailed, was used to determine the statistical significance of differences in the numbers of inflammatory cells. The student’s t-test was used to analyze image analysis data of epithelia. All statistical operations were performed using the RS1 program on the VAX 11/780 computer.

Results

Inflammatory Cells in the Conjunctival Epithelium of Sensitized Guinea Pigs

The 50 μg/5 μl challenge dose of KLH elicited maximal recruitment of inflammatory cells into the conjunctival epithelium (Fig. 1). Only basophils and eosinophils were significantly greater in the antigen-challenged eye than in the PBS-injected eye (median, 20 vs. 1 and 23 vs. 2, respectively, P < 0.05). Few basophils and eosinophils were seen following challenge with PBS (Fig. 2).

Inflammatory Cells in the Conjunctival Epithelium of Naive Guinea Pigs

Basophils and mast cells were found only rarely in the epithelium of naive animals, regardless of the treatment. There was no significant difference in the number of eosinophils in uninjected controls and traumatized animals, or between traumatized animals and PBS-injected animals. However, when uninjected controls were compared with PBS- and KLH-injected tissues in naive animals, the number of eosinophils was significantly lower in uninjected tissue (P < 0.05; Fig. 3).

Neutrophils were significantly higher in the epithelium of naive traumatized and PBS-injected guinea pigs than in untreated (uninjected) controls (P < 0.05; Fig.
Fig. 1. Mean number of inflammatory cells recruited into the conjunctival epithelium at various challenge doses. Asterisk indicates individual value off the scale of the graph: (A) basophils, (B) mast cells, (C) neutrophils, and (D) eosinophils. The 50 μg/ml challenge dose of KLH elicited a significant (P < 0.05), recruitment of eosinophils and basophils into the conjunctival epithelium when compared to the PBS challenged contralateral eyelids.

3). Though there were more neutrophils in the conjunctival epithelium of KLH-treated lids of naive animals than in uninjected controls, this difference did not reach statistical significance.

Conjunctival Epithelial Thickness

We compared the overall conjunctival epithelial thickness of uninjected, KLH-injected, and traumatized naive animals with sensitized animals which had been challenged with either PBS or KLH (50 μg dose). The value was greatest in traumatized naive animals followed by KLH-challenged sensitized animals (Table 1). These values were significantly greater (P < 0.05) than those of the epithelia from naive animals, both non-injected and KLH-injected, and PBS-challenged controls. All control values had the same approximate thickness (Table 1; Fig. 3).

Discussion

The recruitment of eosinophils and basophils into the conjunctival epithelium in our guinea pig system agrees well with the epithelial inflammatory profile of the human diseases, vernal conjunctivitis, and contact lens-associated giant papillary conjunctivitis. We have described elsewhere that the histopathology of vernal conjunctivitis and giant papillary conjunctivitis tends to be less basophilic than reported for guinea pig tissue.7 The reason for this may be the reduced amount of basophils in human tissue1 or the low levels of antigen encountered in the human disease state. The accumulation of basophils at the 50 μg dose was statistically significant when compared to the PBS-injected control. This dose elicited a maximal basophil accumulation in the stroma at 24 hr after challenge.7 The recruitment of basophils appears to be antigen-specific, since none...
were found in the conjunctival epithelium of naive animals and were rarely found in PBS-injected controls. Possible effects that these cells may have upon the conjunctival epithelium and the corneal surface is being investigated.

Furthermore, the number of eosinophils recruited in sensitized animals following challenge with KLH (50 µg dose) was significantly higher than those in uninjected animals or those injected with PBS. However, in naive animals there was a significantly higher number of eosinophils following PBS or KLH injection. This evidence suggests that the accumulation of eosinophils was in response to non-specific injury, since these animals had no prior antigen sensitization. This effect was masked in the results obtained from sensitized animals.

Eosinophils have been shown to be associated with delayed hypersensitivity reactions (DH), as well as cutaneous basophil hypersensitivity reactions, especially those involving the eye. Udell et al report two eosinophil-derived proteins, major basic protein (MBP) and Charcot-Leyden crystal protein, in the tears of patients with vernal conjunctivitis. In the guinea pig, MBP comprises more than 50% of the granule protein. This protein has been shown to be toxic to a variety of mammalian cells. Recent studies on MBP from human eosinophil granules have shown that MBP stimulates histamine secretion from basophils. This would provide an additional mechanism by which eosinophils could amplify inflammatory reactions. However, the presence of eosinophils does not predict the presence of MBP in the tissue. Further studies will determine if this protein is present in the conjunctiva of guinea pigs exhibiting conjunctival basophil hypersensitivity.

Findings in naive guinea pigs suggest that neutrophils are recruited into the conjunctival epithelium in response to trauma. The number of these cells were significantly higher following multiple needle insertion when compared to KLH- or PBS-injected tissue. This is of particular interest in light of recent reports that neutrophils and neutrophil lysate delay corneal wound healing in vitro. Possible damage to the corneal surface of eosinophils may be augmented by the delay in healing. The number of eosinophils was also significantly higher in the conjunctival epithelium of PBS-
and KLH-injected tissue when compared with the uninjected control, indicating that these cells might be recruited in response to non-specific injury.

Further evidence of the involvement of non-specific injury was the finding that the epithelium of traumatized tissue was approximately twice as thick as the epithelium of KLH-challenged tissue. This thickening is not due to increases in cell layers (data not shown), since the number of epithelial cell layers is within the normal range for this conjunctival area. Resolution of the epithelial cell junctions at the electron microscope level will show if this apparent epithelial thickening is due to increased intrajunctional distances or to increased cell length.

Our results which show that neutrophils and eosinophils accumulate in response to trauma support the conclusions made from numerous cases in which patients developed giant papillae in response to nylon sutures received after cataract surgery or keratoplasty. Tissue samples from these patients contained neutrophils and eosinophils. The presence of inflammatory cells in the conjunctiva of these patients can best be attributed to mechanical injury, since nylon has been demonstrated to be, on the whole, biologically inert. This can be contrasted to the findings of Srinivasan et al who report the development of GPC in patients wearing ocular prostheses. Biopsies of the papillae show neutrophils, basophils, and mast cells, suggesting a hypersensitivity response in the conjunctiva in addition to mechanical trauma which may be induced on the conjunctiva by the prostheses. This latter situation probably more closely approximates that of the contact lens.

We have previously reported a cutaneous basophil hypersensitivity lesion in the conjunctival stroma of KLH-sensitized and challenged guinea pigs which approximated the histopathology of vernal conjunctivitis and contact lens-associated giant papillary conjunctivitis. The presence of basophils in the conjunctiva of guinea pigs points to a definite delayed-type hypersensitivity response. Our methodology, which uses a defined soluble antigen to produce a conjunctival basophil hypersensitivity response, also produces a cutaneous basophil hypersensitivity reaction in guinea pig flank.

Khatami and associates report an animal model for vernal conjunctivitis using guinea pigs sensitized topically with fluoresceinyl ovalbumin which elicits a type 1 hypersensitivity response causing a dense eosinophil infiltration. They postulate that these infiltrates may be the direct cause of the pathological changes seen in vernal conjunctivitis. This is interesting in light of our results which show that maximal accumulation of eosinophils and basophils occurs at the 50 μg dose. Isolation and analysis of inflammatory cell products will determine if biochemical evidence supports this idea.

Morphological studies are important in order to understand what cell types are involved in a given cellular immune reaction, thus allowing for its characterization. However, the information derived from such studies is limited. The temporal relationship between inflammatory cells in the conjunctival epithelium and their interaction on the corneal surface, as well as biochemical analysis of their products, will allow for further understanding of the role of inflammatory cells in the evolution of conjunctival basophil hypersensitivity lesions. This should better our understanding of conjunctival basophil hypersensitivity and its possible involvement in the non-proliferative aspects of the two human disease states: vernal conjunctivitis and giant papillary conjunctivitis.

**Key words:** eosinophil, basophil, delayed-type hypersensitivity, giant papillary conjunctivitis, vernal conjunctivitis

**Table 1.** Changes in epithelial thickness of the upper tarsal conjunctiva

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thickness (in μm)</th>
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<tbody>
<tr>
<td>None</td>
<td>46.91 ± 15.39</td>
</tr>
<tr>
<td>Needle-insertion (naive)</td>
<td>128.35 ± 46.50</td>
</tr>
<tr>
<td>KLH-injection (naive)</td>
<td>47.22 ± 16.14</td>
</tr>
<tr>
<td>PBS-injection (sensitized)</td>
<td>48.98 ± 15.16</td>
</tr>
<tr>
<td>KLH-injection (sensitized)</td>
<td>64.69 ± 19.40</td>
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</tbody>
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* Words in parenthesis indicate immunological state of the animal.

**References**