Analysis of Human Corneal IgG by Isoelectric Focusing

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Parameters which regulate the localization and retention of IgG within the corneal stroma are complex and poorly understood. Although multiple factors are involved, electrostatic interactions between IgG and anionic corneal tissue components, i.e., proteoglycans (PG) and glycosaminoglycans (GAG) may regulate the distribution of antibodies within the corneal stroma. Isoelectric focusing (IEF) and blotting analysis of IgG revealed a restricted pI profile for both central and peripheral regions of the normal cornea. Similar analysis of pathological corneas from keratoplasty specimens in Fuchs' dystrophy and keratoconus reveal a variable IEF profile. In the majority of keratoplasty specimens from patients with corneal edema or graft rejection, there was generally little or no IgG detectable. These results suggest that in edematous corneas where there is altered PG/GAG in the stroma and modified fluid dynamics, there is a concomitant loss of IgG. These findings may have implications for immunologic surveillance and protection of the avascular cornea. Invest Ophthalmol Vis Sci 29:1538-1543, 1988

The humoral immune system plays an important role in mediating immunologic surveillance and protection of the avascular cornea. Immunoglobulin G (IgG) is the major component of this humoral network which is responsible for diverse functional and biological properties within the eye. The source of corneal IgG is thought to be diffusion from the limbal vessels. Factors that regulate the diffusion of IgG into the corneal stroma are extremely important since there is normally no IgG produced locally within the quiescent eye. The localization and retention of IgG in the tissues, including the corneal stroma, is dependent, in part, on the plasma IgG concentration, catabolic rate, and diffusion coefficient. Interactions between IgG and tissue components also regulate the tissue distribution of circulating IgG. Electrostatic factors may be paramount in regulating the corneal distribution of plasma proteins. This is suggested by studies demonstrating the differential distribution of positively charged IgG and negatively charged albumin within the corneal stroma. In the corneal stroma, the acidic proteoglycans (PG) and the glycosaminoglycans (GAG) may function as fixed, anionically charged tissue elements which indirectly regulate the distribution of the soluble plasma proteins through ionic interactions. The PGs and GAGs have long been known to serve important physiological functions within the cornea regulating the collagen fiber organization, pH, and swelling properties. Borcherding et al have demonstrated that the concentration of acidic GAGs differs from the central to the limbal regions of the cornea. Since the different GAGs vary with respect to their numbers of negatively charged sulfate groups, a subsequent electrostatic gradient within the corneal stroma might, therefore, indirectly determine which species of IgG gain access to the central and peripheral regions. Experiments were designed to test this hypothesis through isoelectric focusing and analysis of IgG isolated from normal human corneas obtained at autopsy and diseased corneas removed at keratoplasty.

Materials and Methods

Ocular Tissues

Normal human globes and corneas were obtained within 24 h of death from the Georgia Lions Eye Bank at Emory University Eye Center (Atlanta, GA) and the Lions Eyes of Texas Eye Bank at the Cullen Eye Institute of Baylor College of Medicine (Houston, TX). The corneas and aqueous humor were removed and frozen at −80°C prior to analysis. Keratoplasty specimens, aqueous humor and serum were obtained at the time of surgery and were frozen within three hours. Just prior to analysis, 8 mm normal corneal buttons were thawed and the central 4 mm was separated from the remaining peripheral 4 mm ring using a trephine. Keratoplasty specimens consisted of one-half of an 8 mm button which was
trephined into a central 4 mm hemicircle and a peripheral portion.

Isoelectric Focusing (IEF)

Gels were prepared for IEF as described by Hoffman and Jump, on GelBond Agarose Film (FMC Corporation, Rockland, ME) by an open casting technique. Using pyrogen-free water, 0.64 ml of pl 3–10 ampholytes (FMC) was added to 7 ml of 60–65°C melted FMC isogel agarose (1% W/v final concentration) containing 1.0 gram sorbitol and 1.0 ml of glycerol. Some gels were supplemented with pI 8.0–10.5 ampholytes to increase resolution of basic proteins. The final volume was adjusted to 10 ml, and the gels were poured under a heatlamp onto prewarmed, GelBond film on a level casting platform. After solidification, the gels were cured for a minimum of 1 hr at 4°C in a humidified chamber. Samples were added using applicator wicks (FMC pl 3–10 standards, serum, or aqueous humor) or corneal pieces were applied directly to the gel for direct tissue isoelectric focusing. A pH gradient was generated across the gel using a 1.0 N NaOH catholyte solution (pH 13.0) and a 0.5 N acetic acid anolyte solution (pH 2.6). An initial current of 1.0 W was used for 10 min to establish the pH gradient. Isoelectric focusing was initiated for 30 min and the sample applicators and corneal pieces were removed from the gel surface. Isoelectric focusing was continued for an additional 30 min (600 volt/hours). Water accumulation on the gel surface was removed by periodic blotting with filter paper. The isoelectric point (pI) or the pH at which there is no net mobility of a protein in an electric field was determined using defined pl standards.

Blotting

The IEF gels were rinsed for 60 seconds in distilled water with agitation. The IEF gels were then blotted by direct application of pieces of nitrocellulose equilibrated in 0.05 M Tris-Saline, pH 7.6. A wet piece of filter paper was placed onto the surface of the nitrocellulose, and the gel was then inverted onto ten layers of absorbent paper towels. A glass sheet was then placed against the back of the GelBond surface and a 1 kg weight applied for 10 min. Transfer of the focussed proteins occurred by diffusion. The nitrocellulose blot was then removed and cut for staining and analysis. The Standards were stained with amido black or india ink.

The blots were analyzed for IgG distribution by the biotin-avidin ABC peroxidase technique. The blots were first blocked for 30 min with a solution of 3% BSA, 0.25% gelatin, 5 mM EDTA, and 0.05% NP-40 (BGEN). Next, the blots were incubated in a 1:100 dilution of mouse monoclonal antibody to human IgG (MAB 066, Chemicon International, El Segundo, CA) for 60 min. The blots were washed for 15 min with three changes of Tris-Saline and incubated with a 1:100 biotin-conjugated horse anti-mouse IgG (Vectastain kit). After washing for 15 min with three changes of Tris-Saline, the blots were incubated in a 1:200 dilution of the biotin-avidin ABC peroxidase complex (Vectastain kit, Vector Laboratories, Burlingame, CA) for 30 min. The blots were then washed for 15 min in Tris-Saline with three changes and developed for 5 min in chloro-1-naphthol (Sigma, St. Louis, MO), 30 mg in 10 ml methanol plus 40 ml 0.05 M Tris Buffer pH 7.6, and 5-µl of 30% H2O2. The developed blots were washed in dH2O and stored in the dark to dry, overnight. All antibody dilutions were made in BGEN. All incubations and washing procedures were carried out on a rocker platform. The pl range of the serum and corneal IgG was determined from the migration distances of the pl standards.

Results

As an extension of previous quantitative and qualitative analyses of immunoglobulins eluted from human corneas, we sought to further characterize corneal IgG using direct tissue isoelectric focusing (IEF) and blotting onto nitrocellulose. Our assays required as little as a 2 × 4 mm piece of corneal tissue for IgG analysis. Initial IEF analysis of normal human corneal IgG revealed that the pl of both central and peripheral corneal IgG is restricted when compared to the pl of normal human serum IgG (Figs. 1, 2). The pls of IgG species within the normal cornea is distributed within a narrower pH range than that of serum. The average lower limit detected for central corneal IgG was 5.78 ± 0.11 and the average for peripheral corneal IgG was 5.60 ± 0.10 (Table 1). These lower limit pl values are significantly different from the corresponding lower serum IgG pl (5.27 ± 0.27) (Table 1). The upper pl limit for central corneal IgG was 8.73 ± 0.14 and for peripheral corneal IgG was 8.95 ± 0.11 (Table 1). These pl values were not significantly different from the corresponding upper serum IgG pl (9.4 ± 0.10) (Table 1). Qualitative IEF profiles of aqueous humor were not obtained since only trace amounts were detectable on the nitrocellulose blots due to extensive fractionation of the IgG within the pH gradient.

IEF and IgG blotting analyses were carried out using pathological human corneas obtained at the time of penetrating keratoplasty. In five patients with Fuchs’ corneal dystrophy, although more variable, the pl of the central and peripheral IgG was not sig-
one of the two edema patients with normal corneal IgG, there was asymmetrical, localized edema surrounded by normal tissue. In the 17 IgG deficient corneas, there was no apparent correlation between IgG content, and duration of edema, severity of edema or pathology.

A similar absence of corneal IgG was detected in one of two patients grafted for alkali burns (Table 1). It is of interest to note that both of these corneas were vascularized. Analysis of aqueous humor from patients with graft rejection, corneal edema or alkali

significantly different from the serum IgG or the normal corneal IgG profile (Table 1). The IEF and IgG blotting analysis of keratoconus corneas also apparently mirrored that of the serum, although the lower pH limit for the serum, central and peripheral cornea tended to be elevated with marked variability (Table 1). The pH of IgG present in keratoconus corneas was not quantitatively different from that of normal corneas or those with Fuchs' dystrophy.

In contrast, corneal specimens from patients with graft failure or corneal edema revealed an entirely different pattern (Fig. 3). Fifty-seven percent (4/7) of failed corneal grafts had little or no detectable IgG within the central cornea, 43% (3/7) had little or no detectable IgG in the peripheral cornea (Table 1). One of the rejected corneas that was vascularized displayed an IEF profile like that of serum. Another was vascularized but had reduced amounts of corneal IgG. Most striking was the finding that there was little or no detectable IgG in 92% (17/19) of central corneas and 84% (16/19) of peripheral corneas from patients who were undergoing penetrating keratoplasty for corneal edema (Table 1).

All of the 17 who had little or no detectable corneal IgG had relatively normal serum IgG profiles, indicating that the absence of corneal IgG was probably due to abnormalities of the cornea rather than a defective humoral immune system in the patients. In

Fig. 1. IEF blot of normal human serum IgG with multiple bands at different isoelectric points within the pH gradient. The pH range for this normal was 5.5–9.5. The gel range was pH 3.0–10.0.

Fig. 2. IEF blot of normal human corneal IgG after direct tissue isoelectric focusing. The pH range of the central (C) cornea was 6.0–9.1; the peripheral (P) cornea was 5.8–9.3; and the limbal (L) cornea was 5.8–9.5. The gel range was pH 3.0–10.5.
Table 1. IEF of IgG in normal and abnormal corneas

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Serum Range of IgG pi values (lower to upper limits) in:</th>
<th>Central cornea</th>
<th>Peripheral cornea</th>
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<tr>
<td>Normal</td>
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<td></td>
<td>5.27 ± 0.27 to 9.40 ± 0.10 (3/3)*</td>
<td>5.78 ± 0.11†</td>
<td>5.60 ± 0.10†</td>
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<td>Fuchs' dystrophy</td>
<td>5.86 ± 0.33 to 9.38 ± 0.24 (5/5)</td>
<td>6.08 ± 0.77 to 9.18 ± 0.22 (5/5)</td>
<td>6.58 ± 0.67 to 9.26 ± 0.19 (5/5)</td>
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<td>Keratoconus</td>
<td>6.13 ± 1.28 to 9.40 ± 0.56 (6/6)</td>
<td>6.10 ± 0.90</td>
<td>6.27 ± 1.00</td>
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<tr>
<td>Graft failure</td>
<td>6.04 ± 0.21 to 9.36 ± 0.26 (7/7)</td>
<td>Deficient (4/7)</td>
<td>Deficient (3/7)</td>
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<tr>
<td>Corneal edema</td>
<td>5.94 ± 0.33 to 9.15 ± 0.21 (19/19)</td>
<td>Deficient (1/19)</td>
<td>4.80-8.60 (1/19)§</td>
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<td>5.15 ± 0.49</td>
<td>5.10 ± 0.42</td>
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<td>to 9.15 ± 0.21 (2/19)</td>
<td>9.05 ± 0.21 (2/19) §</td>
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<td>Deficient (1/2)</td>
<td>Deficient (1/2)</td>
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<tr>
<td>Alkali Burn</td>
<td>6.25 ± 0.21 to 8.95 ± 0.64 (2/2)</td>
<td>6.20-9.30 (1/2)</td>
<td>6.20-9.40 (1/2)</td>
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* Number of specimens/number of patients.
† Different from serum IgG pi at P < 0.01 by student t-test.
‡ Not different from serum IgG pi.
§ 25% of normal serum value.

burns generally revealed only trace or nondetectable amounts of IgG.

Discussion

Immunoglobulins (Ig) with antibody activity are the mediators of the humoral immune system. These bifunctional glycoproteins are capable of binding foreign antigens and initiating a variety of biological activities such as complement fixation, cell receptor interaction and regulation of catabolic processes. In humans, at least five distinct classes of Ig have been identified with different size, charge, amino acid composition and carbohydrate content. Each Ig class is a complex group of heterogeneous molecules. This is clearly demonstrated by the characteristic electrophoretic migration pattern of the IgG extending from the alpha to the gamma fractions of normal serum. Of the Ig classes, IgG is the major Ig in both the serum and the tissues. IgG is extremely heterogeneous with respect to biological activity and exhibits the most diverse electrophoretic migration pattern. Analysis of human serum IgG has demonstrated that there are multiple species with pis from the anionic range of 5.5 to the cathodic range of 9.5.15

The parameters that regulate the egress of IgG from the circulation and its ultimate localization within the tissues are complex and poorly understood. These factors are particularly important within the avascular cornea which normally contains no plasma cells for the local production of IgG. It is generally believed that corneal IgG is derived from the circulation through diffusion from the limbal vessels. The rates at which different IgG molecules traverse from...
the limbal vessels through the corneal stroma is dependent on individual diffusion coefficients. The diffusion coefficient may, however, be partially regulated in the eye by electrostatic interactions. Highly charged, cationic IgG species readily egress from the circulation and deposit within ocular tissues. Additional factors, such as the state of hydration can also regulate the diffusion of molecules into the corneal stroma. The fluid dynamics of the edematous cornea may also play an important role in determining the character of corneal proteins. The flow of fluid from the aqueous humor through the epithelium might alter corneal proteins by "wash out" or by accelerated diffusion. It is, however, unlikely that this mechanism alone could account for the absence of IgG in edematous corneas since there were patients with corneal edema (Fuchs' dystrophy, graft failure and alkali burn) who had detectable IgG within normal range.

Since PG and GAG have been implicated in maintaining the swelling properties of the corneal stroma, it is likely that they also have marked effects on the corneal IgG distribution. In addition, PG and GAG provide fixed anionic sites contributing significantly to the pH of the corneal stroma. It is likely that there is an intimate association between the anionic corneal PG and GAG, fluid dynamics and proteins which indirectly regulates the distribution of IgG within the cornea. Indeed, there is a restricted IgG IEF profile demonstrated in the normal central and peripheral cornea compared to serum. Our results with pathological corneas suggest that the biochemical changes responsible for edema also regulate the retention of IgG within the corneal stroma. Previous observations have demonstrated that there is loss of GAG in edematous corneas. Similar alterations in GAG levels have been reported for rejected corneal transplants. If one considers the corneal GAG as fixed negative charges, somewhat analogous to those on a DEAE anionic exchange resin and capable of binding positively charged proteins, i.e., IgG, then the loss of corneal GAG would likely result in a corresponding loss of corneal IgG. This explanation is consistent with our observations in edematous and rejected corneas.

IgG profiles of pathological corneas from patients with keratoconus and Fuchs' dystrophy were not grossly abnormal and were more difficult to interpret. None of the corneas from keratoconus patients were edematous. Previous studies of PG and GAG in these pathological corneas demonstrated that there are abnormalities, however, these are less pronounced. Tissue culture studies of keratoconus stromal cells demonstrate normal synthesis levels of sulfated GAG in vitro. Analysis of GAG in keratoconus keratoplasty specimens revealed a relative increase in keratan sulfate with a decrease in chondroitin sulfate. The presence of elevated amounts of keratan sulfate in the central cornea might explain our observed IgG profile in keratoconus specimens. The relatively normal IgG profile in Fuchs' dystrophy is more difficult to interpret since some of the patients had mild stroma edema with thickened Descemet's membrane. A possible explanation for the demonstration of IgG in these corneas might be altered corneal biochemistry in which there is an abundance of oxytalan fibers present. These fibers are highly charged, enriched with cysteine, glutamate and aspartate. Thus Fuchs' corneas might maintain different types of fixed negative charges capable of interacting with IgG.

Analysis of corneal IgG has provided insights into the dynamic relationship between corneal physiology, biochemistry and immunology. Our studies suggest that edema causes changes corneal immunoglobulin distribution. Patients with corneal edema may be at increased risk for the development of infectious keratitis because of low levels of intraocular immunoglobulins. The occurrence of ruptured bullae, in combination with the loss of corneal IgG might make these patients more susceptible to certain types of infection. The same might hold true for virtually any episode which alters the PG and GAG and resultant electrostatic interactions either throughout the corneal stroma or within localized regions. Further experiments are in progress to resolve these issues.

Key words: cornea, immunoglobulin gamma (IgG), isoelectric focusing, corneal edema, keratoplasty

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References