Electrophoresis Combined with Immunologic Identification of Human Tear Proteins

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The protein content of normal human tears from five subjects was examined by molecular weight separation using SDS-polyacrylamide gel electrophoresis (PAGE) and by charge separation using agarose isoelectric focusing (IEF) gels. After separation, specific proteins were identified by immunoblot and immunofixation. Tear proteins examined included albumin, IgA, IgG, prealbumin, lactoferrin, lysozyme, secretory component and transferrin. These techniques required 1 to 14 μl of concentrated tears. We found SDS-PAGE superior to agarose IEF to examine total tear protein pattern, and silver stain almost ten-fold more sensitive than Coomassie blue stain. Immunologic staining markedly enhanced protein detection in all tear samples and appeared to offer the definitive method to probe for a specific protein in tears. In this study prealbumin and a portion of the IgG were present in normal tears at higher than expected molecular weight, suggesting they were present in complexed form. Prealbumin and secretory component staining showed marked variability between subjects. These techniques should be applicable to examine tear proteins in a variety of ocular disease states.


The role of the tear film in human health and disease has come under increasing scrutiny.1 Accurate and sensitive characterization of tear components is crucial to interpreting the changes found in ocular surface disorders. Yet the techniques applied in tear studies must be reliable on very small volumes. Although a variety of electrophoretic techniques have been applied to standardize tear proteins, newer and more sensitive immunologic probes such as immunoblot and immunofixation have not. In this study we examined normal human tears for eight protein components using immunologic probes and electrophoresis based on molecular weight and isoelectric point separation.

Materials and Methods

Subjects

Five normal subjects without clinical signs of eye disease were studied. None were on any medications.

Sample Collection

Reflex tears were stimulated with aromatic ammonia and collected using glass capillary tubes gently inserted into the tear pool of the lower lid margin as previously reported.2 Care was taken not to touch or irritate the eye directly. Tears were stimulated until at least 100 μl had been collected; total collection time was 2 to 3 min. Final volumes from the five subjects ranged from 100 to 150 μl. Tears were stored at −20°C or −70°C until use.

Tear Proteins

Total protein concentration was measured on 3 μl of tears using the Bradford method, as previously reported.1 Bovine albumin was used as the protein standard. The following proteins were examined in tears using immunologic identification as outlined below: albumin, IgA, IgG, lactoferrin, lysozyme, prealbumin, secretory component and transferrin.

Tear Electrophoresis

Two different techniques were used to study total tear proteins. To separate proteins by molecular weight, fixed protein concentrations of tears were

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electrophoresed on 12% separating SDS-polyacrylamide gels (PAGE) using 5% stacking gels as previously reported. Because 12% gels will not give reliable resolution of proteins above 160 kilodaltons (kD), 7% separating gels were also used to analyze higher molecular weight tear proteins. Samples were run both reduced and unreduced, and a Tris-HCl with glycine buffer system was used. Commercial molecular weight standards (BRL Inc, Gaithersberg, MD; and Sigma Chemical Co) were obtained as follows: albumin and IgG (Tago, Inc., Burlingame, CA), secretory component (Sigma Chemical Co.), IgA and IgG (Tago, Inc., Santa Barbara, CA), and transferrin (Jackson Immunoresearch, West Grove, PA). Commercial primary antisera (affinity-purified antibodies were used when available) were obtained as follows: goat anti-albumin, goat anti-IgA and goat anti-IgG (Tago, Inc.), rabbit anti-lactoferrin (Jackson Immunoresearch), rabbit anti-lysozyme, rabbit anti-prealbumin and rabbit anti-secretory component (Dako Corp., Santa Barbara, CA), and goat anti-transferrin (Cappel). In preliminary experiments cross-reactivity between the standards and antisera was examined by gel electrophoresis followed by immunoblot. No cross-reactivity was noted except for the prealbumin antisera, which reacted with albumin, lactoferrin, lysozyme and transferrin. Prior to use the prealbumin antisera was preabsorbed with these standards until all cross-reactivity (by immunoblot) was removed.

Immunoblot was used to identify tear proteins separated by SDS-PAGE. Five micrograms of total tear protein were run for all immunoprobes except for IgG, in which case 9 µg were used. Proteins were electrophoretically transferred to a 0.1 µm nitrocellulose membrane (Schleicher & Schuell, Keene, NH) at 100 v for 1 hr. The membrane was blocked by exposure to 3% bovine serum albumin–phosphate-buffered saline for 90 min at 37°C and incubated with an optimal dilution of the primary antisera for 30 min at 37°C and 30 min at room temperature (RT). After several washes in PBS-Tween, the membrane was then incubated with horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG antibodies (depending on the species-specific primary antisera) for 1 hr at RT. After washing the membrane was developed using 0.03% diaminobenzidine and 0.015% H2O2 in deionized water for 10 min at RT, blotted dry, and photographed on Panatomic-X black and white film using a Nikon FE 35 mm camera equipped with a Mikro-Nikkor 55 mm f/2.8 lens and green filter.

Immunofixation was used to identify tear proteins separated by agarose-IEF as previously reported. Gels were overlayed with a cellulose acetate membrane saturated with undiluted primary antisera for 1 hr at RT. Proteins not bound by antibody were then washed out of the gel over a 24 to 48 hr period with 0.15 M NaCl, 5% glycerol. After a final wash with 5% glycerol, the gels were dried and silver-stained as reported above.

Results

Tear Protein Content

Total protein concentration of the five samples fell within a fairly narrow range: 4.6, 6.2 (two subjects), 6.3 and 6.9 mg/ml.
Tear Analysis Using SDS-PAGE

Eight micrograms of total tear proteins were run on SDS-PAGE and stained with CB (Fig. 1A). Seven bands were routinely visualized: a very dense 14.5 kD band, moderately dense bands at 16.5, 17.5 and 68 kD, and faint bands at 55, 74 and 230 to 290 kD. Additional bands were noted in two samples at 31 kD and in one sample at 16 kD. Because of the increased sensitivity of silver staining, tears were run at a total protein concentration of 1 µg (Fig. 1B). Silver staining accentuated the seven bands noted with CB as well as the 31 kD band, which now appeared in four samples. In addition all samples showed faint but discrete bands in the region of 13.5, 14, 39, 44 and 210 kD. Two samples showed an additional faint band at 175 kD. When a 7% separating gel was used (Fig. 2), there was improved resolution at the top of the gel with three discrete bands noted from 280–380 kD. However, bands below 35 kD were lost. Reduced tear samples run on both 7% and 12% separating gels showed less intense staining of bands at the top of the gel, with the appearance of a new dense staining at approximately 80–88 kD (Fig. 2). This would be in the region where lactoferrin would run.

Protein Identification by Immunoblot

Tears were electrophoresed on SDS-PAGE, transferred to nitrocellulose and immunostained for specific proteins. The overall patterns noted are outlined in Table 1. Representative samples are shown in Figure 3 for all proteins separated on 12% gels, and in Figure 4 for higher molecular weight proteins (IgA, IgG, secretory component) separated on 7% gels. There were several unexpected findings. IgG stained at higher than expected weights (310 and 350 kD) in addition to the 150 kD for monomeric immunoglobulin (Fig. 4). This was not an artifact of storage, since fresh tear samples gave identical results. To exclude the possibility that IgG reagents were reacting with IgA, specific immunoblot studies were carried out at several concentrations and documented no cross-reactivity with up to 1 µg of purified IgA standard. Prealbumin showed a marked variability in staining between tear samples. All samples stained faintly at 70 kD, higher than the 13 kD reported for prealbu-
Table 1. Tear proteins identified by SDS-PAGE and immunoblot*

<table>
<thead>
<tr>
<th>Protein examined</th>
<th>Tear volume used (µl)</th>
<th>Protein staining pattern</th>
<th>Protein molecular weight (kD)</th>
<th>Intersubject variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>0.8</td>
<td>one band</td>
<td>55</td>
<td>minimal</td>
</tr>
<tr>
<td>IgA</td>
<td>0.8</td>
<td>one band</td>
<td>260-360</td>
<td>moderate</td>
</tr>
<tr>
<td>IgG</td>
<td>1.5</td>
<td>three bands</td>
<td>150, 310, 350</td>
<td>moderate</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.8</td>
<td>two dense bands</td>
<td>74, 81</td>
<td>minimal</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.8</td>
<td>one dense band; two faint bands</td>
<td>14.5, 23, 24</td>
<td>minimal</td>
</tr>
<tr>
<td>Prealbumin</td>
<td>0.8</td>
<td>two faint bands</td>
<td>31, 70</td>
<td>marked</td>
</tr>
<tr>
<td>Secretory component</td>
<td>0.8</td>
<td>two-three bands</td>
<td>59, 78, 260-360</td>
<td>moderate</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.8</td>
<td>one band</td>
<td>75</td>
<td>minimal</td>
</tr>
</tbody>
</table>

* Tears from five normal subjects were electrophoresed on nonreducing 12% or 7% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted for eight specific proteins. Molecular weight was calculated by co-electrophoresing known molecular weight standards. Intersubject variability was judged to be minimal, moderate or marked depending on the degree of conformity between the five samples.

min subunits or the 21 to 24 kD reported for tear-specific prealbumin. Three samples showed an additional band at 31 kD and in one sample this band was quite intense. Secretory component in the 7% gel stained diffusely at 260-360 kD (bound to IgA), as well as at 78 kD and very faintly at 59 kD (free or precursor forms). Based on comparison of molecular weight and band intensity, it appeared that in the 12% gels the 14.5 kD band in CB- or silver-stained gels represented lysozyme; the 31 kD band represented prealbumin; the 55 kD band represented albumin; the 68 kD band represented lactoferrin and possibly prealbumin; the 74 kD band represented lactoferrin and possibly secretory component; and the 230 to 280 kD band represented IgA and IgG. The other CB- and silver-stained bands were not identified as any of the eight proteins probed for.

Because of the enhanced gel staining noted on reduced tears, an immunoblot for lactoferrin was carried out on the same tear sample nonreduced and reduced. There was a marked increase in lactoferrin staining in the reduced sample (data not shown).

Tear Analysis Using Agarose-IEF

Ten microliters of tears were run on agarose-IEF gels (pH 3-10) and silver-stained for total protein (Fig. 5). Multiple bands were noted in all samples from pH 3.6 to 6.4. Staining was almost homogeneous from 5.0 to 6.0. There were major bands at pH 8.0 and 9.7, and occasional faint bands in the region of pH 7.3 and 8.4.

Protein Identification by Immunofixation

Optimal volumes of unconcentrated tears were electrophoresed on agarose-IEF gels, then immunofixed for specific proteins. The volume for each protein had been determined in preliminary studies as giving optimal gel results. Tear patterns are noted in Table 2 and representative samples shown in Figure 6. Single bands were noted for albumin, lysozyme and transferrin. Lysozyme stained at pH 9.7 in this gel system, which only measured up to pH 10. This slightly underestimated its true pH range, which has been reported as 10 to 11. IgA showed multiple bands of varying intensity ranging from pH 4.4 to 6.1, whereas IgG by this technique showed a limited number of very faint bands in the pH range 4.7 to 8.0. Lactoferrin stained diffusely and very intensely over the pH 3.9 to 5.3 region. In this less sensitive technique tear prealbumin was highly variable: one subject showed no prealbumin, three had a single band at 24 kD.
Fig. 4. Tears were electrophoresed on nonreducing 7% SDS-PAGE, transferred to nitrocellulose and immunostained. Specific proteins examined on this gel were IgA, IgG and secretory component (SC). Representative samples and calibrated molecular weights are shown.

Fig. 5. Ten microliters of normal tears were run on agarose IEF gels, pH 3-10, and silver-stained. Three representative samples are shown. The pH gradient measured is noted at the left.

Discussion

Tears are made up of an aqueous layer covered by a thin lipid film. Their chemical composition is quite complex and includes over sixty proteins and polypeptides as well as electrolytes, enzymes, lipids, metabolites and mucin. Tears are produced by the innervated lacrimal gland along with contributions from minor accessory glands and the conjunctiva. With a baseline production rate of 1 to 2 µl per minute, and only 5 to 10 µl available in the eye at any given time, the absolute volume of tears is quite small. Therefore reflex tears produced by neural stimulation are generally used to provide a workable volume. Even so, the absolute amount obtainable from subjects varies, and this can become a limiting factor in tear studies.

A number of investigators have analyzed tear proteins using a variety of methods. Electrophoretic studies have included disc electrophoresis, electrophoimmunoassay, disassociating and nondissociating PAGE, agar and agarose gel immunoelectrophoresis, and two-dimensional electrophoresis. When immunologic means have been used to identify tear proteins they have not been used in conjunction with techniques that distinguished molecular weight or isoelectric point. To our knowledge, the current study is the first systematic analysis of tear proteins using the newer technology of silver stain, immunoblot and immunofixation. These studies were carried out on unconcentrated tears, avoiding the potential loss of protein. And they required only 1 to 14 µl volumes, compared to prior studies which had to pool samples or use up to 100 µl of tears.

We found separation of tear proteins by weight rather than charge to be more useful and interpretable as a screening examination, because many tear proteins had similar charges. This required two different separating gels (7% and 12%) since proteins over 160 kD were not sufficiently resolved on the 12% gel. As expected, silver staining was much more sensitive than CB. Silver staining permitted almost a 10-fold reduction in sample protein, yet resulted in enhanced detection. However, it was clear that immunologic staining was superior even to silver, and offered the definitive way to probe for specific tear proteins. It is simply not reliable to depend on co-
Table 2. Tear proteins identified by agarose IEF and immunofixation*

<table>
<thead>
<tr>
<th>Protein examined</th>
<th>Tear volume used (μl)</th>
<th>Protein staining pattern</th>
<th>Protein pH range</th>
<th>Intersubject variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>5</td>
<td>one band</td>
<td>4.5</td>
<td>moderate</td>
</tr>
<tr>
<td>IgA</td>
<td>5</td>
<td>multiple bands</td>
<td>4.4-6.1</td>
<td>minimal</td>
</tr>
<tr>
<td>IgG</td>
<td>14</td>
<td>faint bands</td>
<td>4.7-8.0</td>
<td>minimal</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.5</td>
<td>homogeneous dense stain</td>
<td>3.8-5.3</td>
<td>minimal</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>3</td>
<td>one band</td>
<td>9.7</td>
<td>minimal</td>
</tr>
<tr>
<td>Prealbumin</td>
<td>10</td>
<td>one-three faint bands</td>
<td>4.4, 4.6, 4.9</td>
<td>marked</td>
</tr>
<tr>
<td>Secretory component</td>
<td>6</td>
<td>multiple bands</td>
<td>4.13-6.7</td>
<td>marked</td>
</tr>
<tr>
<td>Transferrin</td>
<td>10</td>
<td>one band</td>
<td>5.1</td>
<td>moderate</td>
</tr>
</tbody>
</table>

* Optimal unconcentrated tear volumes from five normal subjects were electrophoresed on agarose IEF gels and immunofixed for eight specific proteins. The pH of stained bands was measured using a microelectrode probe. Intersubject variability between the five samples was judged as noted in Table 1.

electrophoresis to identify proteins, as prior studies have done. The immunologic staining was able to enhance detection of all proteins looked at, even those in low concentration which could not be identified on gels stained for total tear proteins.

Our protein weights and charges were in fairly close agreement with those reported in the literature although there were some differences. We noted tear IgA to stain as a diffuse band from 260 to 360 kD on 7% gels. This is close to its expected weight of 380 kD, since tear IgA is known to be dimeric with an attached joining chain and secretory component. It is likely that some of the IgA entering our SDS-PAGE gels was partially dissociated. IgG is present at low levels in tears and is presumed to be monomeric. However in addition to the 150 kD diffuse band, there were two consistent bands at 310 and 350 kD. They did not appear to be due to aggregation, since the bands ran above an aggregated IgG standard. Tear IgG has been said to originate from serum. It is apparent that a large proportion of tear lactoferrin is complexed, since it was of sufficient weight not to enter a gel until it was reduced. In the current study lactoferrin showed a more anodal charge range than previously reported. We also found differences from previous reports with regard to tear prealbumin, which stained at 31 and 70 kD. Although our antisera was directed against serum prealbumin, in preabsorption studies we removed all nonspecific staining. And the isoelectric point values we obtained for prealbumin were in close agreement with those reported in the literature. Our 31 kD band suggests that "protein G" identified by Gachon may actually be prealbumin. We could not confirm a prealbumin 24 kD band. Our 70 kD band may represent joined subunits, since prealbumin, at least in serum, is known to exist frequently as four joined subunits. Alternatively, it could be that prealbumin is bound to albumin. This has been reported to occur for 3 to 7% of prealbumin isolated from human ocular mucus. To explain our prominent staining in tears, however, the percent bound would have to be much higher.
Incubation, 100 μl normal rabbit serum, 100 μl antirabbit IgG goat serum and 200 μl 15% polyethylene glycol were added to each tube, followed by further incubation at room temperature for 1 hr. After centrifugation at 1600 g for 30 min, radioactivity in the precipitates was measured by a gamma counter. Intra-assay as well as inter-assay variations in RIA, estimated using samples of varied concentrations of standard hEGF, were within acceptable ranges. The minimum detectable dose was 0.1 ng/ml. No cross-reactivity was observed with rat and mouse EGFs. Platelet-derived growth factor, human transforming growth factors-α and -β, human insulin-like growth factor-I, human insulin, porcine glucagon and ACTH also showed no cross-reactivities in this assay system.

Radioreceptor assay (RRA)

A431 cells derived from human epidermoid carcinoma were used as the EGF receptor source. Synthetic hEGF was used as standard and substrate for 125I-hEGF. Standard diluent used for RRA was 50 mM sodium phosphate buffer (pH 7.4) containing 25 mM EDTA, 140 mM NaCl, 0.5% bovine serum albumin and 0.02% sodium azide. A mixture of 200 μl test sample or standard hEGF, 100 μl 125I-labeled hEGF and 200 μl formalin-fixed A431 cell suspension (2 × 10^5 cells) in each assay tube was incubated at 25°C for 16 hr. After incubation, 1 ml ice-cold standard diluent was added to each tube. Following centrifugation at 1600 g for 30 min at 4°C, radioactivity in the precipitates was measured by a gamma counter. The sensitivity of this RRA was 0.4 ng/ml.

Gel Chromatography Procedure for Pooled Human Tears

A Sephadex G-50 Superfine (Pharmacia, Piscataway, NJ) column (1.6 × 37 cm) was equilibrated with 1 M acetic acid. Aliquots of tears and urine were collected from one individual. Two milliliter test sample or standard hEGF was applied to the column and eluted at a flow rate of 66 ml/hr. Fractions (0.9 ml each) were collected and lyophilized; hEGF in each fraction was measured by RIA as described above.

Results

Immunoreactive (IR) hEGF was detected in all human tear samples tested (n = 26). The level of IR-hEGF in reflex tears (n = 15) varied from 0.7 ng/ml to 8.1 ng/ml, with a mean (±SD) value of 3.4 (±2.9) ng/ml (Fig. 1). IR-hEGF was detectable at similar concentrations (1.9 to 9.7 ng/ml, with a mean value of 5.3 (±2.9) ng/ml), in non-reflex tears (n = 11) as well. In contrast, IR-hEGF was undetectable in aqueous humor by the present RIA system.
Figure 2 shows the standard curve of the hEGF RIA and the dilution curves of urine and tear samples obtained from one individual. As demonstrated, the curves for such samples paralleled the standard curve, indicating that the activity in the samples measured by the present RIA system was indistinguishable from that of standard hEGF. The competitive binding curves of the urine and tear samples in the present RRA paralleled that for standard hEGF as well (Fig. 3), demonstrating that tear EGF was biologically active. The concentrations of tear EGF estimated by RIA and RRA were at similar levels: 0.73 ng/ml and 0.68 ng/ml, respectively. Gel filtration study using Sephadex G-50 Superfine revealed that the IR-hEGF in tears was eluated as a single peak at the same position as standard hEGF or urine EGF (Fig. 4).

Discussion

The current study clearly demonstrates that an EGF which is biologically, immunologically and biochemically indistinguishable from urine EGF or standard hEGF is present in human tears. The level of EGF in tears is lower than in urine, saliva and milk, almost comparable to the level in plasma, and higher than in cerebrospinal fluid and pancreatic juice. Human tear EGF was shown by RRA to be biologically active. In contrast to our results, Elliott previously failed to demonstrate EGF immunoactivity in human tear film. This discrepancy may simply be due to the fact that the present RIA system is 10 times more sensitive than that used in Elliott's study.

The above finding is of great importance in view of the fact that the ocular surface epithelium is always in contact with tear fluid. Similarly, vitamin A has been shown to be present in tears as a form of retinol. Thus, tear fluid may constitute an environment in which ocular surface epithelia come into contact with a variety of nutritional and/or growth factors. It has been shown that corneal epithelial cells have receptors for EGF and proliferate upon topical application of EGF. It may be that tear EGF is related to the maintenance or differentiation of surface epithelia at rest, and to the acceleration of epithelial regeneration after surface wounding. The origin and biological significance of EGF in tear fluid awaits further investigation.

IR-hEGF was undetectable in human aqueous humor. Corneal endothelial cells have been shown to possess EGF receptors and to undergo mitosis when
cultured in the presence of EGF. It seems possible, therefore, that corneal endothelial cells might proliferate when EGF is introduced into the anterior chamber, although there might be risk of Schlemm’s canal or trabecular cell responses as well.

Key words: epidermal growth factor (EGF), tear, aqueous humor, human, radioimmunoassay, radioreceptor assay

Acknowledgment
The authors wish to thank Mr. Robert Brady for his editorial assistance.

References