The authors applied an ELISA to measure IgG, IgA, and different antigens and antibodies. Investig Ophthalmol Vis Sci 27:622-625, 1986

Small volumes of tears. It can be modified to test for many could be detected. IgM was present in all samples at a geometric mean level of 5.6 μg/ml. Mean IgA level was 186 μg/ml and mean IgG was 6.7 μg/ml. No correlation was found between tear and serum levels, suggesting that local synthesis was responsible for most of the tear immunoglobulin. This ELISA offers a sensitive and reliable method to analyze very small volumes of tears. It can be modified to test for many different antigens and antibodies. Invest Ophthalmol Vis Sci 27:622-625, 1986

Previous investigators have applied a variety of tear collection methods and immune assays to measure tear immunoglobulins.1-9 In addition to inherent variation between individual subjects, these studies have sometimes produced a wide range of immunoglobulin concentrations in part because of the lack of standardization, relative insensitivity of the assays, and differences in assay and collection techniques. The relatively large volumes of tears which most assays require has made research in this area difficult. We applied a modified ELISA immunoassay to study the concentration of immunoglobulins in normal tears. We found this technique could reliably measure immunoglobulins in smaller volumes of tears with greater sensitivity than has been previously reported using other techniques. The purpose of this study is to present our findings on immunoglobulin concentration in normal tears using a modified ELISA assay.

Materials and Methods. Subjects: Simultaneous tear and serum samples were collected from 20 subjects without eye disease (11 men and 9 women) ranging in age from 21 to 62 yr (mean 35 yr). None was taking any medications that could interfere with tear production. Serum was collected from an additional ten nor-

References

Tear Immunoglobulins Measured by ELISA

P. K. Coyle* and Patrick A. Sibony††

The authors applied an ELISA to measure IgG, IgA, and IgM concentration in tears from 20 normal subjects. This assay was more sensitive than any other previously reported technique to quantitate tear immunoglobulin. Only 2 μl of tears were required and concentrations as small as 1 ng/ml could be detected. IgM was present in all samples at a geometric mean level of 5.6 μg/ml. Mean IgA level was 186 μg/ml and mean IgG was 6.7 μg/ml. No correlation was found between tear and serum levels, suggesting that local synthesis was responsible for most of the tear immunoglobulin. This ELISA offers a sensitive and reliable method to analyze very small volumes of tears. It can be modified to test for many different antigens and antibodies. Invest Ophthalmol Vis Sci 27:622-625, 1986

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of the anterior segment of the eye, such as acid and alkaline burns to the epithelium and stroma of the cornea, are frequent pathological cases. These conditions can be easily produced in animals, particularly in rabbits for experimental studies.7 These three monoclonal antibodies should provide valid means to follow the migration, proliferation, and differentiation of the epithelial cells during wound healing.8,9 In extra-capsular surgery of cataract, lens is removed with its epithelium. However, secondary cataract often occurs when the remaining epithelial cells at equatorial zone of the lens proliferate and eventually cover the posterior capsule.10 The results of this study demonstrate that GB4 reacted with the subcapsular epithelium of the lens, but not with the cells located at the equatorial zone. Thus, GB4 will be particularly useful in studying the posterior capsule epithelialization during the formation of secondary cataract.

Although the biochemical nature and the function of the antigens recognized by GB4, GB9, and GB11 await further studies, these antibodies offer valuable tools for ophthalmologists to investigate the epithelial differentiation in the anterior segment of the rabbit eye.

Key words: monoclonal antibodies, conjunctiva, cornea, ciliary process, lens

From INSERM U210* and Laboratoire de Pharmacologie†† Faculté de Médecine, Avenue de Vallombrose, 06034 Nice-Cedex, France.
mal subjects to compare ELISA to radial immunodiffusion (RID) results. Informed consent was obtained from all individuals prior to entry into the study.

**Tear collection:** Tears were collected with glass capillary tubes (Fisherbrand disposable micropipets, Fisher Scientific Co.; Pittsburgh, PA) applied to the lower lid margin tearfilm meniscus, taking care not to touch the eye. The volume collected ranged from 10 to 120 µl.

**Protein determination:** Protein was measured in tear and serum samples with the BioRad protein assay kit (Bio-Rad; Richmond, CA). Bovine albumin was used as the protein standard at dilutions ranging from 1 to 20 µg/ml. One to 2 µl of tears were used per sample assay.

**Immunoglobulin determination:** Tears and serum were examined for IgG, IgA, and IgM content using ELISA. Affinity-purified goat anti-human IgG, IgA, or IgM (Tago Inc.; Burlingame, CA) was diluted to 1 µg/ml in ELISA coating buffer (0.05 M carbonate-bicarbonate, pH 9.5). One hundred microliters was then added to microtiter wells and incubated overnight at 4°C. Plates were washed three times using PBS-Tween (0.05%) and were then ready for use. Tears were diluted and added to triplicate wells in 25-µl amounts. Known amounts of purified human immunoglobulin (Cappel Lab; Westchester, PA) were run with every assay to generate a standard curve. Samples were incubated for 2 hr at room temperature (RT). Plates were washed three times with PBS-Tween, then 100 µl of the appropriate horseradish peroxidase (HRP)-anti human immunoglobulin antiserum was added to each well.

The conjugate was diluted to an optimal concentration in ELISA buffer (5% chick serum-PBS-0.1% Tween). After 1 hr at RT plates were washed and 100 µl of enzyme substrate added (2.3 mM orthophenylenediamine in 0.24 M citrate, 0.05 M phosphate pH 5 with fresh 0.03% H₂O₂). After a 15- to 30-min incubation in the dark, the reaction was stopped with 50 µl of 1N H₂SO₄ and the plate read at 488 nm. The mean absorbance of the known amounts of immunoglobulin were plotted on semi-log graph paper for each assay and used to generate a standard curve. The test samples
testing, optimal screening dilutions for tear samples were determined to give values falling on the middle portion of the standard curves. Dilutions of 1:1000 and concentrations of IgG, IgA, and IgM were run ranging then the rest of the curve. Consequently test samples to 5 ng/ml consistently showed a less marked slope relationship was noted over the range tested, although 1 immunoglobulin over baseline. A fairly linear rela-
tionship was noted over the range tested, although 1

In specified samples immunoglobulin levels were also measured using RID plates (Kallestad Laborato-
ries; Austin, TX).

**Results.** ELISA standard curve: A representative standard curve is shown (Fig. 1). On each assay plate concentrations of IgG, IgA, and IgM were run ranging from 1 to 500 ng/ml. The assay detected 1 ng/ml of immunoglobulin over baseline. A fairly linear relationship was noted over the range tested, although 1 to 5 ng/ml consistently showed a less marked slope than the rest of the curve. Consequently test samples were not read from this portion of the standard curve.

**Comparison of ELISA to RID:** Immunoglobulin values on thirty normal serum samples were obtained by ELISA and RID. The results from both techniques correlated well (Fig. 2).

**Tear immunoglobulins by ELISA:** In preliminary testing, optimal screening dilutions for tear samples were determined to give values falling on the middle portion of the standard curves. Dilutions of 1:1000 and 1:2500 were chosen for IgG; 1:5000 and 1:10,000 for IgA; and 1:500 and 1:1000 for IgM. Final tear values were obtained by averaging both dilutions, unless a dilution ran off the standard curve. If a tear sample fell on the portion of the standard curve below 5 ng/ml it was repeated at a lower dilution. Results for simultaneous serum and tear measurements on 20 normal controls are shown in Table 1. There was no correlation between serum and tear protein or immunoglobulin levels using Pearson's correlation coefficient.

**Discussion.** ELISA is a sensitive new immunoassay, which can be modified to measure many different antigens and antibodies. We applied it to look at immunoglobulins in normal tears. IgG and IgA were detected at levels consistent with previous investigators who also used capillary tube collection (Table 2). In preliminary studies with the 20 normal controls we eluted tears from Schirmer strips and Week cells and measured immunoglobulins after correcting for dilutions. We found a mean concentration of IgG of 124 µg/ml and IgA of 531 µg/ml compared to 5.6 µg/ml and 186 µg/ml respectively with capillary tube collection. Table 2 shows a similar disparity between studies, with lower concentrations found in capillary tube collections. Our results are consistent with Stuchell and associates who have shown that tears collected with Schirmer strips result in an elevation in albumin, IgG and transferrin compared to tears collected by capillary tubes. Thus capillary collection techniques appear to be more accurate than filter paper collection.

Most authorities state that IgM is not normally found in tears. Little and associates reported no IgM in tears from ten subjects using RID. Bazzi and associates with RID detected no tear IgM in nine subjects. McClellan and associates using RID detected IgM in only 2 of 61 normal tear samples. Sen and associates, also using RID, found IgM which could not be quantitated in only 7 of 220 normal subjects. Mannucci and associates could not detect IgM in any of 17 controls.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Number</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>Collection technique</th>
<th>Assay</th>
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<tbody>
<tr>
<td>Little et al1</td>
<td>1969</td>
<td>10</td>
<td>trace</td>
<td>210</td>
<td>0</td>
<td>Capillary tube with mechanical irritation</td>
<td>RID</td>
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<tr>
<td>Bazzi et al2</td>
<td>1970</td>
<td>9</td>
<td>790</td>
<td>230</td>
<td>0</td>
<td>Unknown</td>
<td>RID</td>
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<tr>
<td>McClellan et al3</td>
<td>1973</td>
<td>74</td>
<td>140</td>
<td>170</td>
<td>0</td>
<td>Week cell (rarely capillary tube or filter paper)</td>
<td>RID</td>
</tr>
<tr>
<td>Chandler et al4</td>
<td>1974</td>
<td>3</td>
<td>0-12</td>
<td>230-300</td>
<td>—</td>
<td>Capillary tube</td>
<td>EID</td>
</tr>
<tr>
<td>Sen et al5</td>
<td>1978</td>
<td>220</td>
<td>&lt;100</td>
<td>370</td>
<td>0</td>
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<td>RID</td>
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<tr>
<td>Donshik and Ballow6</td>
<td>1983</td>
<td>12</td>
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<td>Mannucci et al7</td>
<td>1984</td>
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<td>32</td>
<td>113</td>
<td>0</td>
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<td>RID</td>
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<tr>
<td>McGill et al8</td>
<td>1984</td>
<td>55</td>
<td>7-65</td>
<td>410-630</td>
<td>—</td>
<td>Filter paper</td>
<td>ELISA</td>
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<tr>
<td>Mackie and Seal9</td>
<td>1984</td>
<td>54</td>
<td>400-600</td>
<td>&lt;100</td>
<td>—</td>
<td>Filter paper</td>
<td>ELISA</td>
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<tr>
<td>Present study</td>
<td>1986</td>
<td>20</td>
<td>6.7</td>
<td>186</td>
<td>5.6</td>
<td>Capillary tube</td>
<td>ELISA</td>
</tr>
</tbody>
</table>

* EID = electroimmunodiffusion.
Our study is the first to report IgM consistently in normal tears. The apparent absence of tear IgM in prior studies may be due to differences in assay techniques. For example, RID is a much less sensitive test than ELISA. In the only ELISA study previously reported to probe for IgM, Donshik and Ballow were unable to detect IgM in any of 12 control tears. Their assay differed from ours in that they used alkaline phosphatase as a conjugate, required 200 μl volumes and was less sensitive. The present study, using ELISA, quantified IgM in every normal tear sample.

We found that choosing an optimal dilution of tear sample was critical in the assay, since there appeared to be a prozone phenomenon that gave artificially low values with more concentrated specimens. This may also have contributed to the failure of earlier investigators to detect IgM. Optimum screening dilutions of 1:1000 and 1:2500 for IgG, 1:5000 and 1:10,000 for IgA, and 1:500 and 1:1000 for IgM in the ELISA resulted in a sufficient volume of diluted sample tears (approximately 25 μl) to measure all three classes of immunoglobulin on as little as 2 μl of capillary tube collected tears. Using the screening dilutions 7 IgG values and 8 IgM values fell below 5 ng/ml and were rerun at lower dilutions.

To support the validity of the assay we compared our ELISA to standard RID to measure serum concentrations of IgG, IgM, and IgA and found good correlation between both techniques. Our inability to correlate serum (by RID or ELISA) and tear immunoglobulin levels suggests that tear levels reflect, in large part, local production of immunoglobulin.

In summary, this ELISA assay is the most sensitive technique to date that has been applied to measure tear immunoglobulins. Depending on the availability of antiserum, it can be modified to measure a variety of antigens or antibodies. In future studies our technique should allow readily obtainable volumes of tears to be examined for concentration of specific antigens and antibodies. This should prove particularly useful in studying immune-mediated ocular and systemic disorders.

Key words: ELISA, immunoglobulins, tears

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