Changes in Human Tear Protein Levels With Progressively Increasing Stimulus

Roderick J. Fullard and Denise L. Tucker

The levels of 13 proteins were measured in six tear samples collected atraumatically at progressively increasing flow rate from nonstimulated (< 0.5 µl/min) to highly stimulated (>50 µl/min) in ten subjects. Tears were fractionated initially by size-exclusion high-performance liquid chromatography (SE-HPLC). Enzyme-linked immunosorbent assays and kinetic assays were then applied to relevant SE-HPLC fractions to determine specific protein levels. Nine of the 13 proteins assayed showed significantly higher concentrations in nonstimulated tears than in any other tear sample. Immunoglobulin (Ig) M, secretory IgA, polymeric IgA1, and polymeric IgA2 all decreased progressively in concentration from nonstimulated tears to the higher flow-rate stimulated samples. The level of IgG, albumin, and transferrin showed a large drop in concentration between nonstimulated tears and the first (lowest flow-rate) stimulated sample, with relatively little decrease for any subsequent sample. Levels of lactoferrin, tear-specific prealbumin, lysozyme, and peroxidase were relatively constant throughout the series of tear samples. These results indicate that the mechanisms responsible for changes in concentration of constitutive, serum-derived, and regulated tear proteins with stimulus can be studied successfully using noninvasive methods to collect human tears. They also show that simply distinguishing between nonstimulated and stimulated tears is not sufficient to completely characterize the effect of stimulus conditions on tear protein composition.

It is now well known that stimulus conditions and collection technique can strongly influence the protein profile obtained in a tear sample. In a previous study of the levels of 12 tear proteins,1 significant differences were found between nonstimulated and high flow rate-stimulated tears for most proteins. A combination of size-exclusion high-performance liquid chromatography (SE-HPLC) and enzyme-linked immunosorbent assays (ELISA) or kinetic assays was used to identify individual proteins. Another earlier study2 showed that SE-HPLC profiles of a series of five tear samples collected immediately after the onset of stimulus varied with each successive sample. Changes were most evident in the SE-HPLC fraction containing high molecular weight proteins, this fraction becoming progressively smaller with each successive tear sample. A decrease in total tear protein from 9.1 to 6.0 mg/ml occurred between the first and last sample. In this earlier study, however, specific protein assays were not used, and the changes in individual protein levels with each successive tear sample could not be determined. The current study is a direct extension of the previous two. It was designed to investigate (1) the actual changes that occur in the levels of specific tear proteins (rather than simply quantifying changes in SE-HPLC profiles) with progressively increasing stimulus and (2) how these changes relate to protein levels in nonstimulated tears.

Materials and Methods

Informed consent was obtained from all subjects after the nature of all procedures had been fully explained to them. Ten subjects participated in the study.

Tear Collection

All subjects were experienced in tear collection techniques—most subjects had been involved with tear collection procedures over several years. Tear samples were collected atraumatically using flame-polished borosilicate glass micropipettes. Every effort was made to avoid ocular surface contact during tear collection. Preliminary trials were conducted to:

1. Determine the range of tear flow rates that could be established and reliably maintained by each subject and the optimum stimulus method for dif-

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different flow rates. It was necessary to recruit experienced subjects for this study to ensure adequate control of tear flow at the various rates used. During these preliminary trials, it was found that most subjects could maintain relatively steady tear flow rates in the range of 5–20 μl/min by stimulating the sneeze reflex to varying degrees. For the higher flow rates (40–50 μl/min), nasal irritation with dilute ammonia was required in all but one case. With practice, each subject was able to establish and maintain each of the flow rates listed below with reasonable precision, based on tear collection rates. Transition to each higher flow rate could also be achieved rapidly. During collection, subjects were given continual feedback regarding the appropriateness of their tear flow rate. Based on collection times for each sample and variations in tube-filling rates, it was estimated that tear flow rates did not vary by more than 33% from the required rate for any sample in the stimulated sequence described subsequently.

2. Investigate any changes in protein levels resulting from repeated attempts at collecting a series of tear samples at progressively increasing flow rate and the recovery period required to circumvent any cumulative effects. The time required for recovery of tear protein levels to normal was found to depend on the total volume of stimulated tear fluid collected in a given period. Even after three to four repeats of the tear sampling sequence described subsequently, full recovery to initial levels occurred within a few hours. Therefore, to eliminate any possible contribution of cumulative effects, no tear fluid was collected for at least 24 hr before the actual study.

Initially a 10-μl sample was collected using several 10-μl micropipettes. Because of the long collection time for this sample, it was obtained in 1.5-μl steps, a new tube being used for each 1.5-μl aliquot (a total of seven tubes). Flow rate was monitored during collection by observing the rate of filling of the tube. If at any time the flow rate increased noticeably, the tube was discarded. After collecting each 1.5-μl aliquot, it was frozen immediately. Average collection time for a 10-μl nonstimulated sample (excluding breaks) was 24 min, giving an average collection rate below 0.5 μl/min.

Five stimulated samples were then collected in uninterrupted sequence using nasal stimulation of the sneeze reflex with a cotton-tipped applicator or gentle nasal stimulation with dilute ammonia vapor. The following series of tear samples was collected from each subject, the intensity of stimulus being progressively increased to achieve each new flow rate: (1) 10-μl stimulated sample collected over a 2-min period; (2) 20-μl stimulated sample collected over 2 min; (3) 20-μl stimulated sample collected over 1 min; (4) 40-μl stimulated sample collected over 1 min; and (5) 50-μl stimulated sample collected as rapidly as possible. The tear flow rate doubled between each of the first four stimulated tear collections.

HPLC Tear Fractionation

Tear samples were separated initially into ten fractions on a TSK3000 SW SE column (Toyo Soda, Tokyo, Japan). The HPLC system and SE separation conditions used are described in detail elsewhere.

Specific Protein Assays

Sandwich ELISAs were used to measure the levels of immunoglobulin (Ig) M, secretory IgA (IgA-SC), polymeric IgA1, polymeric IgA2, monomeric IgA, IgG, ceruloplasmin, lactoferrin, albumin, and transferrin. The ELISA procedures were modified from previous designs, and the details are summarized in Table 1. The IgA2 and transferrin ELISAs were redesigned completely as a result of less than optimum performance in the previous study. Substantially higher tear transferrin levels were found with the new transferrin ELISA relative to previously reported values. Therefore, tear transferrin levels were subsequently reassayed in parallel by the original ELISA and the new transferrin ELISA. For the original ELISA, the coating antibody was 3.125 μg/ml sheep anti-human transferrin, and the conjugate was 625 ng/ml sheep anti-human transferrin-HRP (both from Biodesign, Kennebunkport, ME). To ensure parallelism, the same standard (Boehringer Manheim, Indianapolis, IN) was used in both ELISAs.

For each ELISA, a panel of negative controls was tested to determine assay specificity. As described previously, checkerboard titrations were conducted to optimize assay conditions, antisera, sample, and standard working dilutions prior to the study. The general ELISA procedure (eg, buffers and incubation procedures) was also the same as described previously with the exception of the IgA2 assay. This assay was modified to incorporate a phosphate-buffered saline (PBS), pH 7.2, coating buffer instead of carbonate buffer, pH 9.6. For all ELISAs, relevant SE-HPLC fractions of tear samples were used as test samples rather than whole tears. The rationale for using HPLC-fractionated tear samples is described in detail elsewhere.

A computer program, "ELISANALYSIS II" (J. Peterman, Atlanta, GA) was used for all aspects of ELISA data analysis. Immunochemical "validity" and working range of both standard curves and test
sample dose–response curves was determined readily by the slope and fit of first-order log–log curves fitted from the raw ELISA dose–response data. The program also tested the extent of parallelism of standard and test sample curves so that dilutions outside the “immunochemically acceptable working range” could be identified and excluded from the analysis.

Other Assays

Kinetic assays were used to measure the levels of two proteins. Peroxidase was assayed by the 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) assay. Peroxidase elutes from the SE-HPLC column over a relatively broad region (approximately, 15–19.5 min). Therefore peroxidase activity could be measured either in fraction 3, fraction 4, or both. Fraction 3 peroxidase activity was measured, and a conversion factor (based on preliminary trials) was applied to obtain total peroxidase. This allowed fraction 4 to be used for two alternative assays. Lysozyme activity was measured by the Micrococcus lysodeikticus assay as described previously. In the earlier study, lysozyme was also assayed by ELISA. Results of the lysozyme ELISA were more variable and were consistently (although not statistically significantly) higher than the M. lysodeikticus assay. The lysozyme ELISA was therefore not used in the current study.

Tear-specific prealbumin (TSP) levels were measured by the Bradford assay. The SE-HPLC fraction containing TSP was determined previously to lack any other proteins apart from trace contamination by lactoferrin. Bovine serum albumin was used as standard for the Bradford assay. Total tear protein was calculated by measuring total SE-HPLC integration area for each tear sample. This was standardized in preliminary experiments using the Bradford assay by determining the relationship between peak area and total protein content for each HPLC fraction. This allowed a factor to be calculated for each integrated peak to enable conversion to protein content.

Results

SE-HPLC profiles of the six tear samples collected from a representative subject are shown in Figure 1. The nonstimulated tear sample chromatogram (Fig. 1A) also shows the elution positions of the ten fractions collected from each tear sample. As was the case in the previous study, preliminary ELISAs and kinetic assays were conducted to determine which SE-HPLC fractions contained each of the proteins under investigation. Distribution of proteins in SE-HPLC fractions is summarized in Table 2.

Comparison of SE-HPLC profiles of the six tear samples (representative subject) in Figure 1 shows that the greatest change occurring between samples 1 and 6 was in SE-HPLC fraction 2 (high molecular weight fraction). This was also evident for the overall group of subjects and is best illustrated by the percentage of total HPLC peak integration area made up by this fraction for each sample. From samples 1–6 the proportion was: 14.50%, 8.89%, 4.72%, 3.87%, 3.19%, and 4.21 mg/ml, respectively.

Mean total tear protein content for the six tear samples was 9.40, 5.80, 4.65, 4.71, 4.29, and 4.21 mg/ml, respectively.

Results of the specific protein assays for the 13 proteins studied are listed in Table 3. For the two redesigned ELISAs, IgA2 and transferrin, a considerable improvement in assay reliability and sensitivity was noted over previous findings. Protein level variations between subjects are evident from the standard error values in Table 3. However, individual trends in protein levels with increasing stimulus were consistent from subject to subject with few exceptions. Examples of individual trends are shown in Figures 2A–C. Data points are expressed in terms of protein levels relative

Table 1. ELISA designs

<table>
<thead>
<tr>
<th>Antigen/Standard</th>
<th>Coating Ab</th>
<th>Layer 3</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM*</td>
<td>5.0 µg/ml GAH-µ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31 ng/ml MAH-µ-MAb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>125 ng/ml GAM-Ig-HRP&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IgA-SC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.0 µg/ml GAH-α&lt;sup&gt;b&lt;/sup&gt;</td>
<td>280 ng/ml GAH-α-Biot&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0 µg/ml GAH-SC-HRP&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>IgA1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.0 µg/ml MAH-IgA1-MAb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>140 ng/ml GAH-α-Biot</td>
<td>200 ng/ml Strep-HRP</td>
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<tr>
<td>IgA2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.0 µg/ml MAH-IgA2-MAb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14 ng/ml GAH-α-Biot</td>
<td>133 ng/ml Strep-HRP</td>
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<td>IgG&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5.0 µg/ml GAH-γ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>280 ng/ml GAH-γ-Biot&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40 ng/ml Strep-HRP</td>
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<td>Ceruloplasmin&lt;sup&gt;g&lt;/sup&gt;</td>
<td>10 µg/ml RAH-Cerul&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 ng/ml Strep-HRP</td>
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<tr>
<td>Albumin&lt;sup&gt;h&lt;/sup&gt;</td>
<td>5.0 µg/ml GAH-Alb&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25 µg/ml SAH-cerul-HRP&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Lactoferrin&lt;sup&gt;i&lt;/sup&gt;</td>
<td>5 µg/ml RAH-Lact&lt;sup&gt;**&lt;/sup&gt;</td>
<td>250 ng/ml GAH-Alb-HRP&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Transferrin&lt;sup&gt;j&lt;/sup&gt;</td>
<td>5.0 µg/ml GAH-Tran&lt;sup&gt;**&lt;/sup&gt;</td>
<td>250 ng/ml RAH-Lact-HRP&lt;sup&gt;**&lt;/sup&gt;</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>1.25 µg/ml MAH-Tran-MAb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>625 ng/ml GAM-Ig-HRP&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Affinity purified, †purified antibody, §Fab fragment, ¶PBS pH 7.2 coating buffer. Immunoochemical suppliers: 'Chemicon (Temecula, CA), "Southern Biotechnology Associates (Birmingham, AL), Jackson (West Grove, PA), "Nordic (Capistrano Beach, CA), 'Gift from Dr J Mestecky (University of Alabama at Birmingham), 'Tago (Burlingam, CA), 'Pel-Freez (Rogers, AR), 'Dako (Carpenteria, CA), 'Biodesign (Kennebunkport, ME), 'Boehringer Manheim (Indianapolis, IN), 'Calgat (San Francisco, CA), 'Medix (Foster City, CA), 'GAH = goat anti-human; MAH = mouse anti-human; Cerul = ceruloplasmin; Alb = serum albumin; Lact = lactoferrin; Tran = transferrin; Biot = biotin; Strep = streptavidin.
Fig. 1. Size exclusion HPLC profiles of 10 μl nonstimulated tears (elution positions of the ten collected fractions are shown) (A), initial stimulated tear sample (10 μl collected over 2 minutes) (B), second stimulated tear sample (20 μl collected over 2 minutes: cumulative stimulated tear volume 30 μl) (C); third stimulated tear sample (20 μl in 1 minute: cumulative stimulated volume 50 μl) (D); fourth stimulated sample (40 μl in 1 minute: cumulative stimulated volume 90 μl) (E); fifth stimulated tear sample (50 μl collected in <1 minute: cumulative stimulated tear volume 140 μl) (F). Only 40 μl of the 50 μl stimulated sample 5 volume was injected onto the HPLC column. HPLC separation conditions: TSK 3000 SW column, mobile phase 0.5 M NaCl/0.1 M sodium phosphate, pH 5, flow rate 0.5 ml/min. The ordinate scale varies from A to F so that the highest peak always corresponds to approximately 100% of the ordinate range.
though all three assays showed a progressive decline in dominantly serum-derived protein, IgG (Fig. 2B), secretory IgA level in nonstimulated tears (Table 3). All three assays revealed the highest secretory protein, secretory IgA (Fig. 2A) and the predecessor protein, IgA-SC (using an ELISA specific for IgA bound to secretory component) and for IgA subclass distribution. All three assays revealed the highest secretory IgA level in nonstimulated tears for each subject. For the constituent protein, secretory IgA (Fig. 2A) and the predominately serum-derived protein, IgG (Fig. 2B), there was very little variability between individuals. In the case of TSP, data points are more scattered, and no clear trends are evident (Fig. 2C).

IgA-SC in SE-HPLC fraction 2 was assayed both for overall IgA-SC (using an ELISA specific for IgA bound to secretory component) and for IgA subclass distribution. All three assays revealed the highest secretory IgA level in nonstimulated tears (Table 3). Although all three assays showed a progressive decline in IgA-SC levels with each successive stimulated tear sample, the most significant decreases consistently occurred between nonstimulated tears and the first two stimulated samples. Thereafter, the decline was more gradual. The IgA subclass distribution was biased slightly toward IgA1 in nonstimulated tears. With each successive stimulated tear sample (apart from the last), however, the distribution became increasingly skewed toward IgA1.

The IgM levels in SE-HPLC fraction 2 followed a similar pattern to IgA-SC, the highest level occurring through to the second stimulated sample. Thereafter, the decline was more gradual. The IgA subclass distribution was biased slightly toward IgA1 in nonstimulated tears. With each successive stimulated tear sample (apart from the last), however, the distribution became increasingly skewed toward IgA1.

The IgM levels in SE-HPLC fraction 2 followed a similar pattern to IgA-SC, the highest level occurring in nonstimulated tears. The greatest decreases in IgM concentration were evident from nonstimulated tears through to the second stimulated sample. The levels of IgG, albumin, and transferrin were all highest in nonstimulated tears. However, after an

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nonstimulated</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM (2)</td>
<td>18.30 ± 6.46</td>
<td>5.71 ± 1.98</td>
<td>2.31 ± 0.73</td>
<td>1.87 ± 0.48</td>
<td>0.92 ± 0.16</td>
<td>0.73 ± 0.14</td>
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<tr>
<td>IgA1 (2)</td>
<td>875.9 ± 225.7</td>
<td>375.6 ± 81.0</td>
<td>267.5 ± 31.2</td>
<td>194.9 ± 23.2</td>
<td>131.7 ± 26.8</td>
<td>123.7 ± 30.9</td>
</tr>
<tr>
<td>IgA2 (2)</td>
<td>13.21 ± 5.09</td>
<td>3.90 ± 2.44</td>
<td>2.17 ± 0.85</td>
<td>1.68 ± 0.43</td>
<td>1.27 ± 0.42</td>
<td>1.27 ± 0.42</td>
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<tr>
<td>IgA-SC (2)</td>
<td>1930 ± 342</td>
<td>762.5 ± 190.2</td>
<td>283.3 ± 43.2</td>
<td>192.9 ± 27.7</td>
<td>153.2 ± 20.0</td>
<td>106.1 ± 10.6</td>
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<td>Peroxidase (3)</td>
<td>5.82 ± 14.0</td>
<td>4.99 ± 0.83</td>
<td>3.82 ± 0.64</td>
<td>4.49 ± 1.03</td>
<td>6.89 ± 1.23</td>
<td>6.22 ± 0.98</td>
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<tr>
<td>IgA (4)</td>
<td>5.36 ± 0.63</td>
<td>0.65 ± 0.20</td>
<td>0.43 ± 0.16</td>
<td>0.37 ± 0.16</td>
<td>0.24 ± 0.08</td>
<td>0.25 ± 0.08</td>
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<td>Lactoferrin (5)</td>
<td>11.37 ± 2.84</td>
<td>5.63 ± 1.83</td>
<td>3.17 ± 0.62</td>
<td>3.13 ± 0.57</td>
<td>3.24 ± 0.56</td>
<td>3.03 ± 0.66</td>
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<tr>
<td>Albumin (5)</td>
<td>42.01 ± 4.65</td>
<td>8.41 ± 1.12</td>
<td>4.03 ± 0.60</td>
<td>3.86 ± 0.83</td>
<td>3.34 ± 0.56</td>
<td>3.00 ± 0.61</td>
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<td>Transferrin (5)</td>
<td>15.50 ± 2.64</td>
<td>4.08 ± 0.95</td>
<td>2.36 ± 0.47</td>
<td>1.73 ± 0.32</td>
<td>1.59 ± 0.30</td>
<td>1.43 ± 0.38</td>
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<tr>
<td>TSP (7)</td>
<td>1549 ± 140</td>
<td>1244 ± 133</td>
<td>1196 ± 176</td>
<td>1270 ± 197</td>
<td>1148 ± 169</td>
<td>1105 ± 166</td>
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<tr>
<td>Lysozyme (9)</td>
<td>2067 ± 242</td>
<td>1635 ± 224</td>
<td>1356 ± 205</td>
<td>1417 ± 207</td>
<td>1308 ± 190</td>
<td>1324 ± 123</td>
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</tbody>
</table>

* HPLC fraction in parentheses.
† Total (assayed protein) IgA1 & IgA2 levels excluded.
SI–S5 = stimulated tear samples 1 to 5. Differences between total protein levels (Bradford Assay, p. 126) and total assayed protein represents the amount of additional protein in each tear sample for which a specific assay was not conducted.
abrupt drop in concentration between nonstimulated tears and the initial stimulated tear sample, a much more gradual decline ensued for the remaining four stimulated samples (Table 3). Tear ceruloplasmin and monomeric IgA levels showed more subtle decreases in concentration between nonstimulated tears and the first two stimulated tear samples, followed by relatively constant levels thereafter. No significant variation in peroxidase concentration was evident through the sequence of tear samples. This was the only assayed protein that did not show its highest concentration in nonstimulated tears.

Levels of the main lacrimal gland proteins, lactoferrin, TSP, and lysozyme all remained more or less constant throughout the sequence of tear samples (Table 3). Although each showed its highest concentration in nonstimulated tears, the difference was not statistically significant for any of the three proteins.

A summary of the statistically significant differences in protein concentration between the six tear samples (for the overall group of subjects) is given in Table 4. A two-way analysis of variance (variables, subject and tear sample) was computed initially for each protein. Next, Tukey's test for global comparisons among means was applied to compare mean levels between the six tear samples for each protein. Column 2 in Table 4 lists the significant differences found at the 95% confidence level when comparing all six tear samples. Tukey's test indicated that nonstimulated tears contained significantly higher levels of protein than all stimulated samples for IgM, IgA-SC, polymeric IgA 1 and 2, IgG, albumin, and transferrin. In situations where one group mean is considerably higher than all the others (which is true of the nonstimulated samples in many cases), Tukey's limits tend to be excessive for other comparisons among means. This is because Tukey's test sets limits based on the largest difference. For this reason, the third column (comparing the five stimulated samples only) was included in Table 4. In the stimulated tear samples, the first stimulated sample contained higher protein levels than all subsequent samples for secretory IgA, polymeric IgA 1, polymeric IgA 2, and albumin. In the case of IgM and transferrin, the first stimulated sample contained higher protein levels than the fourth and fifth samples only. No other proteins showed any significant differences among stimulated samples.

**Comparison of New Transferrin ELISA With the Original ELISA**

The original transferrin ELISA applied subsequently to SE-HPLC fraction 5 yielded lower tear transferrin levels in all samples. A correction factor was applied to allow for loss of tear transferrin activity between the time of running the assays summarized in Table 3 (including the new transferrin ELISA) and the subsequent time at which the original transferrin ELISA was run in parallel with a repeat of the new transferrin ELISA. The correction factor was the ratio of tear transferrin levels found initially by the new transferrin ELISA to those found in the repeat of the new ELISA. Mean (corrected) tear transferrin levels (± the standard error) measured by the original transferrin ELISA were 2.08 ± 0.12 μg/ml (nonstimulated), 0.33 ± 0.16 μg/ml (first stimulated), 0.19 ± 0.04 μg/ml (second stimulated), 0.13 ± 0.02 μg/ml (third stimulated), 0.14 ± 0.03 μg/ml (fourth stimulated), and 0.14 ± 0.04 μg/ml (fifth stimulated). Therefore, the new transferrin ELISA using a monoclonal antibody yielded tear transferrin levels that were higher by a factor of 7.7, 12.3, 12.2, 13.9, 11.2, and 10.2, respectively, than the original transferrin ELISA. For both ELISAs, the trend in transferrin level with progressively increasing tear flow rate was an abrupt drop between nonstimulated tears and the first stimulated sample followed by relatively little change thereafter. Both ELISAs therefore demonstrated that the change in tear transferrin with progressively increasing tear flow rate was more consistent with the pattern for other serum proteins than the pattern for constitutive lacrimal gland proteins.

**Discussion**

In this study, the magnitudes of differences found between protein levels in nonstimulated tears and high flow rate-stimulated tears agreed well with a previous study for most proteins. Two exceptions were IgA2 and transferrin. In both cases, the redesigned ELISAs showed improved assay performance, and the current values were considered to be more reliable. However, the substantial difference in transferrin results raises a question about the new ELISA, particularly since the transferrin levels measured by the old ELISA were in agreement with other literature reports. The current results also provide insight into the nature of the transition between the extremes of flow rate used in the earlier study. For all proteins subject to decreasing concentration with stimulus, the greatest decrease almost invariably occurred between the nonstimulated and first stimulated tear sample. The only exception occurred with secretory IgA levels, where a slightly greater decrease was seen between the first and second stimulated samples.

Considering the entire series of tear samples, three different types of trends in protein levels were identified in this study: (1) for proteins in SE-HPLC fraction 2 (secretory IgA and IgM) an initial, relatively
rapid decline in concentration occurred between the nonstimulated and second stimulated tear sample, followed by a further, more gradual, decrease for the remaining samples; (2) for predominantly serum-derived proteins (IgG, transferrin, and albumin), a precipitous drop in protein concentration occurred between the nonstimulated and first stimulated tear sample followed by relatively little change between the first and second stimulated tear sample apart from albumin, and there was a slight further decrease in protein concentration for the other tear samples; and (3) for the main lacrimal gland proteins and peroxidase, protein levels appeared to be more or less independent of stimulus.

These trends correlate well with the secretion mechanisms involved in production of each type of protein. IgA-SC is known to be a constitutively secreted protein. For constitutively secreted proteins, the rate of protein secretion depends on rate of synthesis and not on the rate of fluid secretion. This means that,
with increasing stimulus, IgA-SC levels lag behind fluid secretion rate. It would appear that IgM may follow a similar secretion mechanism as its tear levels followed a very similar pattern to IgA-SC. Others found interstitial and plasma cell staining for IgA and IgM in the main and accessory lacrimal glands of humans. IgG-containing plasma cells have been detected at low levels in lacrimal gland tissue. However, the trend in tear IgG levels with progressively increasing flow rate is closer to that of serum-derived proteins with no known source in lacrimal gland tissue (transferrin and albumin) than to trends seen with constitutively secreted lacrimal gland proteins, such as secretory IgA (Figs. 2A, 2C). This indicates that the lacrimal gland is probably only a minor source of tear IgG.

Levels of serum proteins (IgG, transferrin, and albumin) varied as would be expected from their origin. High concentrations in nonstimulated tears were consistent with the mixing of these proteins with very low tear volumes. However, with the onset of stimulus, lacrimal gland fluid diluted and “flushed out” serum proteins. This caused an immediate, large drop in their tear concentrations.

The main lacrimal gland proteins, lactoferrin, TSP, and lysozyme showed very little variation with increasing stimulus. Peroxidase followed the same pattern, stimulus having little effect on its concentration in tears. All four appear to be “regulated” proteins, their rate of secretion by the lacrimal gland increasing with the rate of stimulation. Slightly higher levels of lactoferrin, TSP, and lysozyme in nonstimulated tears may be due to evaporation. It would also indicate that the accessory lacrimal glands are not producing these proteins at significantly higher concentrations than the main lacrimal gland—if they are contributing at all.

Although levels of lactoferrin, TSP, and lysozyme in stimulated tears appear to be independent of flow

Table 4. Differences in [protein] between tear samples (n = 10 subjects): two-way analysis of variance and Tukey’s test (P < 0.05)

<table>
<thead>
<tr>
<th>Protein</th>
<th>All tear samples (NS, S1, S2, S3, S4, S5)</th>
<th>Stimulated only (S1, S2, S3, S4, S5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE-F2</td>
<td></td>
<td></td>
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<tr>
<td>IgM</td>
<td>NS &gt; S1-S5*</td>
<td>S1 &gt; S4, S5</td>
</tr>
<tr>
<td>IgA-SC</td>
<td>NS &gt; S1-S5</td>
<td>S1 &gt; S2-S5</td>
</tr>
<tr>
<td>IgA1</td>
<td>NS &gt; S1-S5 S1 &gt; S4, S5</td>
<td>S1 &gt; S2-S5</td>
</tr>
<tr>
<td>IgA2</td>
<td>NS &gt; S1-S5</td>
<td>S1 &gt; S2-S5</td>
</tr>
<tr>
<td>SE-F3</td>
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<td></td>
</tr>
<tr>
<td>Peroxidase</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>SE-F4</td>
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<tr>
<td>IgA</td>
<td>NS &gt; S2-S5</td>
<td>nd</td>
</tr>
<tr>
<td>IgG</td>
<td>NS &gt; S1-S5</td>
<td>nd</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>NS &gt; S2-S5</td>
<td>nd</td>
</tr>
<tr>
<td>SE-F5</td>
<td></td>
<td></td>
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<tr>
<td>Lactoferrin</td>
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<td>nd</td>
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<tr>
<td>Albumin</td>
<td>NS &gt; S1-S5</td>
<td>S1 &gt; S2-S5</td>
</tr>
<tr>
<td>Transferrin</td>
<td>NS &gt; S1-S5</td>
<td>S1 &gt; S4, S5</td>
</tr>
<tr>
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</tr>
<tr>
<td>TSP</td>
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<td>nd</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>TOTAL [PROTEIN]</td>
<td>NS &gt; S1-S5</td>
<td>S1 &gt; S5</td>
</tr>
<tr>
<td>SE-F2 [PROTEIN]</td>
<td>NS &gt; S1-S5</td>
<td>S1 &gt; S2-S5</td>
</tr>
</tbody>
</table>

* Significant differences in [protein] between tear samples according to Tukey’s test (P < 0.05).
NS = nonstimulated tear sample; S1-S5 = stimulated tear samples 1 to 5; SE-F2 = size exclusion HPLC fraction 2; nd = no significant differences.
rate in the short term, the concentrations of all three can be depleted if large tear volumes are collected on a regular basis. In an unrelated study, a subject collected large volumes of highly stimulated tears (up to 9 ml/day) over 24 days. A total of 50 ml of tear fluid was collected in 1.2-ml aliquots. No lacrimators were necessary, and the tears were not "emotionally stimulated." Based on SE-HPLC profiles, some clear trends emerged in the tear concentrations of lactoferrin, TSP, and lysozyme. Results for lactoferrin and TSP are illustrated in Figure 3. Levels of all three proteins decreased through the day. If up to 6 ml was collected on a particular day (day 9, Fig. 3), lactoferrin levels decreased by approximately 25%, TSP by 27%, and lysozyme by 20% through the course of the day. On the day that 9 ml of tear fluid was collected (day 21, Fig. 3), lactoferrin decreased by 61%, TSP by 64%, and lysozyme by 26%. These changes were not simple diurnal variations because, when small daily tear volumes (less than 500 µl total) were collected, none of the proteins showed any clear tendency to decrease in concentration through the course of the day. Another more long-term trend also was evident. When large tear volumes were collected daily, the levels of lactoferrin, TSP, and lysozyme showed progressive day-to-day declines in addition to decreases within each day (days 1–3, days 8–9, and days 21–22, Fig. 3). However, these depleted protein levels were quickly reconstituted after discontinuing tear collection for several days.

In the current study, monomeric IgA and ceruloplasmin levels followed less clearly defined patterns through the series of tear samples. For monomeric IgA, the pattern was closer to that of serum proteins than for IgA-SC—the largest concentration decrease occurring between nonstimulated tears and the first stimulated tear sample.

Many of the proteins studied showed a wide concentration range through the series of tear samples. In fact, eight of the 13 specific protein assays revealed a range of mean protein levels spanning more than an order of magnitude. In the group of stimulated samples, some proteins still exhibited a reasonably large concentration range—in some cases, approaching an order of magnitude. It is therefore not surprising that tear protein levels reported in the literature vary so widely.

The considerable decrease in concentration of IgA-SC with progressively increasing stimulus is of particular importance. This protein has been designated by several investigators as a key ocular surface component in health and disease. In our study, IgA-SC levels in nonstimulated tears are more than twice as high as in any stimulated sample. If stimulated tears are collected at a relatively low flow rate (and no fluid is discarded), the levels of IgA-SC will remain relatively high. However, if more rapid reflex tear flow rates are established (as may occur with ammonia inhalation or direct ocular stimulation), IgA-SC levels will be significantly lower—particularly if the initial tear fluid is discarded or "flushed out." Therefore, different laboratories may disagree on the tear levels of IgA-SC in control groups and in various types of pathology as a result, not only of differences

**TIME COURSE OF [PROTEIN] CHANGES WITH COLLECTION OF LARGE TEAR VOLUMES**

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933162/)  
Fig. 3. Effect on tear lactoferrin and TSP levels of collection of large stimulated tear volumes (50 µl/min) over a 24-day period. Each data point corresponds to 1.2 ml of tear fluid. Results are for an individual subject.
in assay technique, but also of differences in tear collection procedures.

Nonstimulated tear IgA-SC levels found in the current study were significantly higher than previously reported. This was not an unexpected result because all participants were experienced in maintaining true nonstimulated tear flow rates (< 0.5 µl/min) throughout the collection procedure. In the earlier study, the average level of subject expertise was less, and the average flow rate during collection was 0.5–1.0 µl/min. Differences in nonstimulated tear IgA1 and IgA2 levels (in SE-HPLC fraction 2) between studies were consistent with secretory IgA levels.

An interstudy comparison of SE-HPLC peak integration areas for nonstimulated tears revealed similar trends to those seen for some specific proteins. Based on total HPLC integration area, nonstimulated tears in the current study contained an average of 55.6% more protein than in the earlier study. Moreover, the high molecular weight fraction (F2, in which IgM and secretory IgA were assayed) contained more than twice as much protein in the current study versus the earlier study. This is consistent with differences in secretory IgA and polymeric IgA1 and IgA2 between studies. However, it cannot account for the approximately sixfold higher IgM levels seen in the current study. It may be that considerably higher tear IgM levels are secreted at extremely low tear secretion rates, or that the accessory glands are producing tears with significantly higher IgM levels than minimally stimulated lacrimal gland fluid. Mean nonstimulated tear collection rates were almost twice as slow in the current study compared with the previous study.

These results indicate that slower collection rates are responsible for higher protein levels in the high molecular weight SE-HPLC fraction. Because different subject populations participated in each study, this cannot be stated conclusively. However, five of the ten subjects in the current study participated over a period of several years in numerous studies involving nonstimulated tear collection. A consistent pattern emerged in all cases [for each individual subject: (1) total tear protein increased with decreasing tear collection rate and (2) peak area of the high molecular weight SE-HPLC fraction was highly inversely correlated with tear collection rate].

As mentioned previously, a modified IgA2 ELISA was used in this study. This involved the use of a new IgA2 monoclonal antibody and coating of the 96-well plates with this antibody in PBS, pH 7.2, instead of pH 9.6 carbonate buffer. In addition, an affinity-purified biotinylated Fab fragment of goat antiserum to the α chain, followed by peroxidase-conjugated streptavidin were used as layers 3 and 4 of the ELISA. Decreased scatter of data at the lower end of standard and test sample dose–response curves and an improvement in assay sensitivity resulted. Therefore, the IgA2 levels in SE-HPLC fraction 2 for stimulated tears were considered to be more reliable in the current study than in the earlier one. The IgA1 ELISA was modified similarly for the detection steps (biotin-streptavidin steps), but assay performance was unchanged.

The IgA subclass distribution in the SE-HPLC fraction containing IgA-SC (fraction 2) was close to 50% for types 1 and 2 in nonstimulated tears. This agrees with the findings of others. Through the sequence of stimulated samples, a trend toward increasing proportions of IgA1 was evident (76% IgA1 in stimulated sample 3). The reason for the differences in IgA subclass distribution with increasing stimulus was unclear. One possible explanation is that the secretion mechanisms for the two subclasses, although both constitutive, may not be equivalent. In general, the sum of polymeric IgA1 plus IgA2 was consistent with IgA-SC levels, indicating that all three ELISAs were reliably done. In four tear samples (nonstimulated, first stimulated, second stimulated and fifth stimulated), the IgA1-SC level exceeded the sum of IgA1 and IgA2, and the reverse was true for the other two tear samples (third stimulated and fourth stimulated). The fact that polymeric IgA1 and IgA2 levels were less than secretory IgA levels in some tear samples and exceeded IgA-SC levels in others may indicate that polymeric IgA1 and IgA2 levels are actually more or less equal to IgA-SC levels.

IgM levels varied widely through the sequence of tear samples. Nonstimulated tears contained a significantly higher IgM concentration than previously reported. Again, the more controlled nonstimulated tear flow rates in the current study may partially explain this finding, as for IgA-SC. However, the IgM levels remained relatively high in the first stimulated sample, the 5.71 µg/ml value matching the level found by others. As with IgA-SC, the wide range of IgM levels from nonstimulated tears to the highest flow rate-stimulated sample indicates the importance of using controlled tear collection techniques.

Monomeric IgA was detected consistently in all tear samples, levels decreasing with stimulus. In the earlier study, monomeric IgA was always found in nonstimulated and stimulated tears of the 30 normal subjects that were tested. This result contrasts with the finding of others who found monomeric IgA to be present in the tears of multiple sclerosis patients but absent from normal subjects. These authors used a different approach to detect monomeric IgA, involving electrophoresis and immunoblotting versus the HPLC-ELISA approach we used. All ELISAs were validated before this study, including specificity test-
ing against a panel of negative controls. The possibility that the IgA ELISA was recognizing another antigen therefore was eliminated. In addition, all secretory and polymeric IgA was separated into a different SE-HPLC fraction before ELISA, meaning that only the monomeric form of IgA was present in the HPLC fraction being tested by the monomeric IgA ELISA. Russell et al.20 noted that both polymeric and monomeric forms of IgA were normal constituents of external secretions, including tears.

Nonstimulated tear albumin and IgG levels were similar to previously reported values.1 However, the concentration range was wider for both through the entire range of six tear samples than was evident from the two types of samples assayed in the earlier study. The overall range of IgG levels was consistent with other reports, where values from 0–32 μg/ml were found.19,21,22 Stimulated tear albumin levels in our study fell below the typically reported range of 12–54 μg/ml21–23 for samples collected at high flow rate. A new ELISA for transferrin, including an affinity-purified coating antibody and a monoclonal antibody, resulted in considerably higher tear transferrin levels than previously reported.1,22,24 The new transferrin ELISA was validated and found to be more sensitive and reliable than the previous assay1 in which sheep antisera had been used. Running the original transferrin ELISA in parallel with the new ELISA showed that the higher transferrin levels found here must have been a result primarily of the new transferrin ELISA.

The reason for the large discrepancy between transferrin ELISAs is unclear. Although the new transferrin ELISA was considered to be more reliable, the tear values did not agree with previous reports by others,22,24 nor did they agree with values reported by our group using the alternative transferrin ELISA. Both assays were also run using several different transferrin standards (including apo- and Fe-saturated transferrin) and found consistently to yield a similar magnitude of difference in tear protein levels. This new transferrin ELISA requires further investigation.

Lactoferrin and TSP levels were consistent with earlier reports.1,21,22,25,26 Lysozyme levels were lower in our study than reported previously1 but fell within the range reported in the literature.21,22,25,27 Peroxidase levels correlated with earlier findings for nonstimulated tears.1 However, the first two stimulated samples in our study were closer to the stimulated tear value found previously for peroxidase (in higher flow rate-stimulated tears). An increase in peroxidase concentration in the fourth and fifth stimulated samples was more consistent with the findings of others.28 One possible explanation for increased tear peroxidase concentrations in later stimulated samples in the tear sequence is that the peroxidase level is a function of total tear volume secreted after the onset of stimulus, rather than flow rate. However, through the range of tear samples collected in our study, peroxidase levels appeared to show no strong trend, supporting the conclusion of others28 that peroxidase is probably relatively constant at all flow rates. It therefore appears that peroxidase is behaving as a regulated lacrimal gland protein.

This study demonstrated that for most tear proteins it is not sufficient to distinguish simply between nonstimulated and stimulated tears. With progressively increasing stimulus, there is significant variation in the concentration of many proteins in stimulated tears. In addition, the variation is not consistent for all proteins. Two factors may be influencing the change in protein concentration through the sequence of five stimulated tear samples: (1) change in tear flow rate with each successive tear sample and (2) total volume of stimulated tear fluid secreted before collection of each new sample. Although the relative contributions of these two factors were not addressed specifically in our study, they can be inferred from our current knowledge of tear protein secretion mechanisms. The first factor would be the primary determinant of protein levels for lacrimal gland-derived proteins, although the second would also contribute ultimately through depletion of secretory granules. For serum-derived proteins, ocular surface epithelium and ocular surface gland-derived proteins, the second factor would predominate. In this case, lacrimal gland fluid would act essentially as a diluting and flushing agent, reducing ocular surface protein levels progressively with increasing tear secretion volume. However, flow rate would also be a factor.

Therefore, to address the effect of tear flow rate on protein profiles fully, several aspects of the tear collection procedure should be controlled and reported: (1) collection device used and steps taken to assure that the tears were obtained noninvasively; (2) whether nonstimulated or stimulated samples were being collected; (3) for nonstimulated tears, a measure of flow rate during collection or actual collection time; and (4) for stimulated tears both a measure of flow rate during collection and, when applicable, the amount of tear fluid discarded before collection the sample of interest.

Key words: tears, nonstimulated, progressive stimulus, ELISA, HPLC

References