Membrane Array Characterization of 80 Chemokines, Cytokines, and Growth Factors in Open- and Closed-Eye Tears: Angiogenin and Other Defense System Constituents

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PURPOSE. To adapt membrane-bound antibody array (MA) technology to characterize the distribution of a wide range of bioactive trace proteins in reflex (RTF) and open-eye (OTF) and closed-eye (CTF) tear samples.

METHODS. Tears were collected by capillary tube and centrifuged. A commercially available standard MA and a custom array were modified to maximize the sensitivity of detection and the signal-to-noise ratio, to assay RTF and individually pooled CTF and OTF samples for 80 chemokines, growth factors, cytokines, and angiogenic modulators. The reliability of data was assessed by Western blot and other methods.

RESULTS. Coupling an ultrasensitive chemiluminescence substrate system to an MA and optimizing conditions enhanced the sensitivity several hundredfold, allowing the detection of ~40 of the 79 probed proteins on the standard array, most of which were shown to be elevated in CTF. Identified entities include the known constituents epidermal growth factor (EGF), monocyte chemotactic protein (MCP)-1, IL-8, tissue inhibitor of metalloproteinase (TIMP)-1 and -2, and numerous previously undetected tear components, such as angiogenin (ANG), growth factors, and the CXC and CC chemokines IFN-g inducible protein (IP)-10, growth-related oncogene (GRO), epithelial neutrophil-activating protein (ENA)-78, and macrophage inflammatory protein (MIP)-3a. Identification of other proteins was hindered by high background on the negative control array. Using a less complex custom array dramatically reduced background and allowed the visualization in CTF of proteins, such as VEGF, that were not detected with the standard array.

CONCLUSIONS. MAs are powerful tools for differential screening of tears for large numbers of trace proteins. Analysis allowed the identification of previously undetected proteins that may participate in the host defense system as well as demonstrated the profound change in tear composition associated with prolonged eye closure in a manner reflective of physiological function. (Invest Ophthalmol Vis Sci. 2005;45:1228–1238) DOI:10.1167/iovs.04-0760

The precorneal tear layer plays a critical role in the innate and adaptive host defense systems and participates in many other processes that are central to the maintenance of the integrity of the ocular surface tissues. This complex mixture has been only partially characterized.1–5 Mass spectrometric (MS) analysis of open-eye tear fluid,5 for example, yields an incomplete listing of more than 100 low-abundance proteins, including dozens of cytokines, chemokines, growth factors, angiogenic modulators, and other proteins, such as enzymes and inhibitors, that are bioactive in very low concentrations. These substances are of heterogeneous origin, derived from cellular debris, lacrimal gland and accessory gland secretions, ocular surface tissue products, serum exudate, and transient or resident inflammatory and immune cells recruited into the local environment.1,2,4,6 As such, the concentrations and relative distribution of these entities in tear fluid is likely to be reflective of and contribute to a wide range of homeostatic processes. Understanding this relationship has been the subject of considerable attention, with much of the work particularly focused on the distribution of cytokines, chemokines, angiogenic modulators, and growth factors. The scope of the work is vast and is only partially referenced herein (Kitagawa K, et al. IOVS 2004;45:ARVO E-Abstract 83; Wirthlin A, et al. IOVS 2004;45:ARVO E-Abstract 3456).17–21 The parameters investigated have included changes associated with the age and sex of the donor, the rate of tear flow,1,2,5–8 open- and closed-eye phases of the diurnal cycle,9,10 wound healing subsequent to trauma or refractive surgeries,11–16 dry eye diseases (Kitagawa K, et al. IOVS 2004;45:ARVO E-Abstract 83; Wirthlin A, et al. IOVS 2004;45:ARVO E-Abstract 3456); and infectious, inflammatory, and immune (Leonardi A, et al. IOVS 2004;45:ARVO E-Abstract 625).20–28 Most of these studies have used microwell plate ELISA protocols to obtain data. Sample size, assay sensitivity limits, and time and cost constraints have usually limited analysis to the measurement of one or a few proteins in individual or pooled tear samples. Integration of the accumulated data has been hindered by many interstudy variables, including differences in the methods of tear collection and differences in the sensitivities among commercially available kits. Given the complexity of biological processes, it has long been recognized that it would be of great value if one could obtain differential data on a broad range of proteins in tear samples.

Several methods of proteomics analysis have been explored with this objective in mind. These have included use of two-dimensional (2-D) gel electrophoresis, coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS),30–31 MALDI analysis,32 trypsin digestion with differential labeling followed by tandem mass spectrometry (MS-MS), and/or quadrupole time-of-flight (MALDI QqTOF-MS) analysis.33 use of biochips coupled with surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF-MS). (Kitagawa K, et al. IOVS 2004;45:ARVO E-Abstract 83; Wirthlin A, et al. IOVS 2004;45:ARVO E-Abstract 3456) and HPLC separation using electrospray ionization-(ESI)-MS as the detector.34 MS analysis has been particularly valuable, yielding important data on the post-
translational processing of lacrimal-derived proteins.39 Despite much progress (Kitagawa K, et al. JOVS 2004;45:ARVO E-Abstract 83; Wirthlin A, et al. JOVS 2004;45:ARVO E-Abstract 3456), major problems remain, especially in adapting the power of MS technology to differential analysis. For example, although open-eye (OTF) and closed-eye (CTF) fluids differ radically in protein composition,30–36–42 the low molecular weight MALDI profiles of these two samples have been reported to be virtually indistinguishable.32 Although differential labeling techniques employing MALDI QqTOF-MS analysis can in theory allow for the differential analysis of OTF and CTF, results have so far been disappointing,33 in part because of the high levels of antiproteases and heavily glycosylated proteins unique to CTF,35,37 which makes proteolytic digestion and product solubilization problematic. As better techniques of MS analysis evolve, one would expect a wealth of meaningful data to emerge.

Antibody (protein) array technology offers an alternative approach for obtaining proteomic data. As detailed elsewhere, advances in miniaturization, rapid expansion of libraries of available matched, high-affinity antibody pairs; the development of newer methods of orientation of antibody binding to the support, to enhance the capture efficiency; and the use of ultrasensitive detection systems35 have revolutionized the potential for array analysis.44 These developments make it probable that microarrays will be available in the near future that will allow the simultaneous assay of biological fluids for hundreds, if not thousands, of biologically important human proteins. Several laboratories have successfully used multiplex analyses in the form of moving-phase immunobead arrays coupled with flow cytometry to assay individual normal and pathologic tear samples for as many as 14 cytokines (Leonardi A, et al. JOVS 2004;45:ARVO E-Abstract 625).25 Although this mode of analysis is sensitive, it requires a large initial capital investment in the form of dedicated equipment and trained personnel. Moreover, at present, only a limited number of arrays have been validated for use only with OTF. Extending the range of assay to other trace proteins and validating the methodology for use with CTF may be problematic. Tear fluid contains a highly potent clumping factor that is found in high concentrations in CTF and that aggregates IgG-bound red blood cells22 and precipitates other IgG-bound particulate matter (Sack R, unpublished observations, 2002). In addition, tear fluid contains blocking factor(s) that interfere with the binding and efficacy of ELISAs.44–47 In related work (Sack RA, et al. JOVS 2004;45:ARVO E-Abstract 3880), we presented preliminary data that documented the impact of these and other tear-specific matrix effects on the capacity to use a microwell-plate antibody array system for quantitative assay.

In this study, we report the use of a much simpler membrane-bound antibody array (MA) system for qualitative analysis of low-abundance tear proteins. This system, which is less vulnerable to matrix effects, is designed to allow the simultaneous screening of samples for the relative distribution of as many as 120 growth factors, chemokines, cytokines, angiogenic modulators, and other trace proteins, using a dot sandwich ELISA protocol.

MAs are ideally suited to serve as initial screening agents to identify and determined the relative distribution of potential biomarkers in biological fluids for further quantitative study. In this role, these arrays have enormous potential. Once established, MAs are inexpensive to custom manufacture and can be easily constructed in a laboratory. They can be run with minimal laboratory facilities by individuals without extensive laboratory training and without the need for investment in expensive instrumentation.

The particular MA system that we used in this study is sold in a kit that is designed for differential screening of biological fluids that are relatively enriched in the targeted proteins. As configured, these kits are far too insensitive to be used for tear analysis. In this study, we optimized the assay protocol and coupled the array to an ultrasensitive detection system, thereby increasing the sensitivity of detection several hundredfold or more. This allowed us to perform differential analysis of reflex (RTF) and individually pooled OTF and CTF samples for the relative distribution of 80 chemokines, growth factors, cytokines, and angiogenic modulators. Data revealed the presence of several previously undetected proteins that are likely to play important roles in the innate and specific ocular defense systems and in homeostatic processes. The findings also reinforce earlier studies documenting a profound change in the composition, origins, and probable functional roles of the tear film proteins in the open- and closed-eye environments.

Materials and Methods

Tear Collection

Tear samples were routinely recovered over a several-month period from six normal men and women, who ranged in age from 25 to 59 years, with informed consent obtained according to the guidelines set down by the Declaration of Helsinki and the institutional review board. RTF was collected with a 50-μL glass capillary tube at a rapid rate of tear flow after nasal stimulation. OTF was collected slowly over a several-minute period with a 5-μL calibrated glass micropipette. Immediately on eye opening after overnight sleep, similar sized (∼5 μL) CTF samples were collected. Samples were transported to the laboratory on ice and centrifuged (11,000 rpm, 30 minutes, 4°C), and the OTF and CTF supernatants from each donor were separately stored at −78°C until analyzed. Analysis was performed with samples that were individually pooled from each donor. To obtain sufficient volume for analysis with the standard array, we pooled samples over a 3- to 4-week period. For most donors, several sets of pooled samples were assayed over a several-month period.

Membrane Microarray Assays

Most of the work was performed either with an off-the-shelf array (RayBio Human Cytokine Array V, referred to hereafter as standard array; RayBiotech, Inc., Norcross, GA) or a variant of this array, in which the concentrations of the positive controls were reduced 10-fold. The standard array matrix consists of an 11 × 8-dot grid on a 20 × 30-mm nitrocellulose membrane (Hybond; Amersham Biosciences, Arlington Heights, IL) with 79 unique capture antibodies (Table 1), 6 identical positive control antibodies containing a biotinylated protein standard, and 3 negative controls consisting of two dots of bovine serum albumin (BSA) and one dot of the sample buffer. A second custom array was used in the study—a prototype provided as a gift by the manufacturer (RayBiotech Inc.). The overall matrix spacing and the complexity of the array were reduced to form a 4 × 4-dot grid on a 12 × 8-mm nitrocellulose membrane (Hybond; Amersham Biosciences) that consisted of 5 positive controls (at one-tenth the standard concentration), 1 negative control, and 12 capture antibodies. The capture antibodies were specific for insulin-like growth factor binding protein (IGFBP)-3, angiopeptin (Ang-2), epithelial neutrophil-activating protein (ENA)-78, oncostatin M (OSM), tissue inhibitor of metalloproteinase-1 (TIMP)-1, eotaxin-1, neurotrophin (NT)-3, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-7, tumor necrosis factor (TNF)-α, interleukin (IL)-8, and angiogenin (ANG). The array composition was selected in part to complement quantitative data obtained by microwell-plate array assays (SearchLight Array; Pierce, Rockford, IL). The latter results will be presented elsewhere, since they deal with several areas that are germane to the design of quantitative assays.

Array kits included an instruction manual, a reaction-well tray, blocking and washing buffer solutions, biotinylated secondary antibodies, streptavidin-linked peroxidase (SPO), and a luminol-amplifier-
Eotaxin-3 (MIP-4α/TSC-1)
Epidermal growth factor (EGF)
Epithelial neutrophil-activating protein (ENA)-78
Fibroblast growth factor (FGF)-4
FGF-6
FGF-7
FGF-9
Fractalkine (FKN)
Fms-like tyrosine kinase-3 ligand (Flt-3 ligand)
Glia-derived neurotrophic factor (GDNF)
Granulocyte chemotactic protein 2 (GCP-2)
Granulocyte colony-stimulating factor (GCSF)
Granulocyte–macrophage colony-stimulating factor (GM-CSF)
Growth-related oncogene (GRO)
Growth-Related Oncogene-α (GRO-α)
Hematopoietic growth factors, hepatocyte growth factor (HGF)
I-309
IFN-γ-inducible protein (IP)-10
Insulin-like growth factor (IGF)-1
Insulin-like growth factor binding protein (IGFBP)-1
IGFBP-2
IGFBP-3
IGFBP-4
IFN-γ
Interleukin (IL)-1α
IL-1β
IL-2β
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-10
IL-12
IL-13
IL-15
IL-16
Leptin
LIGHT (homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed on T cells)
Leukemia inhibitory factor (LIF)
Macrophage Inflammatory protein (MIP)-1β
MIP-1α
MIP-3α
Macrophage colony-stimulating factor (MCSF)
Macrophage-derived chemokine (MDC)
Mesoderm-inducing factor (MIF)
Monokine induced by γ-interferon (MIG)
Monocyte chemoattractant protein (MCP)-1
MCP-2
MCP-3
MCP-4
Neutrophil activating peptide (NAP)-2
Neutrophilin (NT)-5
NT-4
Oncostatin M (OSM)
Osteoprotegerin (OPG)
Placenta growth factor (PIGF)
Platelet-derived growth factor BB (PDGF-BB)
Regulated upon activation, normal T-cell expressed and presumably secreted (RANTES)
Pulmonary and activation-regulated chemokine (PARC)
Stem cell factor (SCF)
Stromal cell-derived factor (SDF)-1
Thrombopoietin (Tpo)
Thymus and activation-regulated chemokine (TARC)
Tissue inhibitor of metalloproteinase (TIMP)-1
TIMP-2
Transforming growth factor (TGF)-β1
TGF-β2
TGF-β3
Tumor necrosis factor (TNF)-α
TNF-β
Vascular endothelial growth factor (VEGF)
diluted in an equivalent amount of blocking buffer. After 2 hours of incubation with tear samples, the membranes were washed four times for 5 minutes each with 2-mL aliquots of PBS containing 0.05% Tween, followed by a second series of four 5-minute washes in PBS without detergent. The membranes were then incubated with the supplied cocktail of biotinylated secondary antibodies diluted to one half the recommended concentration in 1 mL of biotin-free casein colloidal buffer (RDI, Flanders, NJ). After incubation for 2 hours at room temperature, the washing sequence was repeated. The membranes were then incubated with 2 mL of the supplied SPO diluted 1:2000 (one half the recommended concentration) in the casein blocking solution. After 1.5 hours the membranes were washed. For maximum sensitivity, each of the membranes was incubated with 1 mL of a freshly prepared solution of the luminol-based substrate (SuperSignal West Femto; Pierce) for 1 minute. For comparative analysis, matched sets of samples and control arrays were developed and imaged in tandem. The membranes were then incubated with the supplied protein in question and that of TIMP-1 and IL-8. The validity of this relative area intensity ratios of the dot ELISA data obtained for the manufacturer’s Web site; the known concentration range established for this complex biological fluids such as tears. In evaluating our data, it is possible in instances in which the intensity of background on the array was not uniform, the intensity of the signals from the positive controls on the top and the bottom of the membrane differed significantly, in standard arrays in which the intensity of luminescence on the positive controls exceeded the linear range of either the film or camera, or with the capped precoupled membranes. In these situations, arrays were visually analyzed, and the results were blindly and independently scaled by three observers and compiled.

Although the obtained data were nonquantitative in nature, at times we used it to obtain a crude estimate of the concentration range of some of the detected proteins. This estimation was based on the relative ratio of the sensitivities of each of the dot ELISAs as posted on the manufacturer’s Web site; the known concentration range established for Tip-1 and for IL-8 in RTF, OTF, and CTF and the relative area intensity ratios of the dot ELISA data obtained for the protein in question and that of Tip-1 and IL-8. The validity of this analysis is predicated on the assumption that the signal for each ELISA (minus that on the negative control array) is within the linear range of the assay, is specific, and can be attributed solely to the probed protein, and that the sensitivities of the assays as listed on the manufacturer’s Web site are accurate.

The specificity and lack of cross-reactivity of the ELISAs found in the array have been determined by the manufacturer with recombinant standard proteins and with the array partially validated for analysis of serum and urine. This relationship does not necessarily hold for other complex biological fluids such as tears. In evaluating our data, it is therefore critical to recognize that we are reporting the presence of an antigenic species that most likely, but not necessarily, represents the probed protein. It is plausible that tear fluid contains unique cross-reacting species that may render some of the dot ELISAs inaccurate and that it contains factors that can interfere with and selectively block or

Protein Array Characterization of Bioactive Tear Proteins

**FIGURE 1.** RTF (300 μL) was probed with a standard array and accompanying kit reagents and protocol. The array is arranged in the form of an 11 × 10-lane grid with the following configuration: Lane 1: positive control (Pos), Pos, Pos, Neg, negative control (Neg), Neg, ENA-78, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, and IL-10; lane 2: IL-12p40p70, IL-13, IL-15, IFN-γ, MCP-1, MCP-2, MCP-3, M-CSF, MDC, MG, and MIP-1β; lane 3: MIP-1α, RANTES, SCF, SDF-1, TARC, TGF-β1, TNF-α, TNF-β, EGF, IGF-1, and ANG; lane 4: OSM, Tpo, VEGF, PDGF-BB, leptin, BDNF, BLC, CK β1-1, coktin, coktin-2, and coktin-5; lane 6: FGF-4, FGF-6, FGF-7, FGF-9, Fb ligand, fractalkine, GCP-2, GDNF, HGF, IGF-1B, and IGF-1B-2; lane 7: bFGBP-3, IGFBP-4, IL-16, IP-10, LIF, LIGHT, MCP-4, MIF, MIP-3α, Neg, and NT-3; lane 8: NT-4, OPG, PARC, PIGF, TGF-β2, TGF-β3, TIMP-1, TIMP-2, Neg, Pos, and Pos. The control membrane (not shown) was devoid of any nonspecific reactivity. See Table 1 for expansions of abbreviations.
enhance a given dot ELISA. It is also plausible that one or more of the probed proteins may be present in tear fluid in a complex or a modified form that is not recognizable by the antibodies used. For all these reasons, results of each of the individual dot ELISAs should be viewed as tentative, requiring independent verification. In many instances, however, we believe that sufficient confirmatory data exist in the literature or have been obtained by parallel semiquantitative micro-well-plate array assays (to be described elsewhere) or Western blot assays, to allow the definitive identification of the reactive species.

**Western Blot Analysis**

Western blot analysis was used to confirm the presence and to determine the approximate concentration range of ANG in tear fluid using a modification of a protocol that we detailed earlier. In this instance RTF, OTF, and CTF samples and a serial dilution of recombinant ANG were separated on a 16% SDS-polyacrylamide minigel under reduced conditions and blot transferred onto nitrocellulose, according to a previously published protocol. Probing was performed with a goat polyclonal antibody specific for ANG as the primary antibody (antibodies and recombinant ANG (R&D Systems, Inc., Minneapolis, MN). Detection was performed with an AP-linked anti-goat IgG (Sigma-Aldrich) and nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP; Sigma-Aldrich) as a chromogenic substrate.

**RESULTS**

Figure 1 depicts the results of a typical assay of a 300-μL RTF sample using the standard array kit as configured. Under these conditions, only three signals were detectable. These consisted of an intense signal for ANG (a protein not previously known to be present in tear fluid) and far less intense signals for two previously documented tear constituents, TIMP-1 and -2.10 Western blot analysis confirmed that ANG is present in RTF in the form of a ~14-kDa species in concentrations in the sub-nanogram-per-microliter range (Fig. 2).

Coupling the array to an ultrasensitive substrate system and optimizing the assay protocol greatly enhanced the sensitivity of detection, allowing the visualization of strongly positive signals for at least 11 antigenic species in 50- to 100-μL tear samples obtained from all six donors (Table 2), with the intensity of all these signals markedly increasing in a gradient fashion in CTF compared with OTF (not shown) and RTF (Fig. 3) samples.

Unfortunately, a high level of nonspecific luminescence accompanied the increased sensitivity of the assay on nearly all the capture antibodies on the negative control array (Figs. 3, 4), making definitive identification of many other less reactive species problematic. Increasing the sample size to several hundred microliters of RTF increased the signal-to-noise ratio, thereby allowing the unequivocal identification of numerous additional species in all three tear fluids with most present in much higher levels in CTF than in RTF and OTF (Table 3).

A further increase in the signal-to-noise ratio was obtained by the partial removal of nonspecific interacting species from both the array and the cocktail of biotinylated probe antibodies before the assay, allowing the clearer visualization of ≤39 antigenic species in 50- to 60-μL CTF samples from all individuals (Fig. 4A, top; Table 3). This includes numerous chemokines and leukochemokines (Table 4). Stripping of the membrane followed by re-probing with SAP further improved the signal-to-noise ratio and allowed the visualization of several previously cryptic species (Fig. 4A, bottom).

To explore further the sensitivity limits of this technology, a custom array was constructed that was smaller and less complex, probing for only 12 proteins. Decreasing the complexity of the cocktail of biotinylated antibodies resulted in a profound decrease in the level of nonspecific reactivity on the negative control array (Fig. 5). This allowed us to detect up to 10 of the 12 probed proteins in 20-μL CTF samples (Table 2). Detected species included trace levels of several entities, such as VEGF, that could not be detected above the noise with a fourfold larger CTF sample with the standard array. Calibrating the array assay with a known mixture of recombinant protein standards suggests a threshold of sensitivity for some of the assays extending well down into the sub-picogram-per-milli-liter range.

**DISCUSSION**

The MA system used in this study has been extensively used by others to perform differential analyses of trace proteins in tissue extracts, tissue culture filtrates, serum, and urine samples, and they also have noted the relative lack of sensitivity of the system. In this study, we partially circumvented the problem of lack of sensitivity by altering the assay conditions and using large pooled tear samples. This allowed us to document the presence of 39 of 79 low-abundance proteins in tear fluid using a single array. Given the sensitivities of these assays, these findings suggest that many of these entities are present in

**TABLE 2. Major Antigenic Species Detected in All RTF Samples**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sensitivity* (pg/mL)</th>
<th>Corroborating Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG</td>
<td>10</td>
<td>WB</td>
</tr>
<tr>
<td>EGF</td>
<td>1</td>
<td>(4, 5, 6)</td>
</tr>
<tr>
<td>ENA-78†</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>GRO</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>IL-8</td>
<td>1</td>
<td>WPMA, WB, (9, 10)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>3</td>
<td>(9)</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>10</td>
<td>(72)</td>
</tr>
<tr>
<td>IP-10</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>100</td>
<td>WPMA, WB, (44)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>1</td>
<td>WB, (44)</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>10</td>
<td>—</td>
</tr>
</tbody>
</table>

WB, western blot; WPMA, well plate membrane array assay; () reference.

* As determined by the manufacturer.
† Detectable in 75% of all samples.
tear fluid in physiologically significant levels. Moreover, we unequivocally demonstrated a profound increase in the concentration of many of these entities in tear samples collected after overnight eye closure with patterns of distribution suggestive of physiological functions.

Strongly positive signals were consistently obtained from 11 proteins in all types of tear samples from all donors (Table 2). Not surprisingly, nearly all of the signals came from dot ELISAs that exhibited a low threshold of sensitivity (the exception being TIMP-1). Five of the signals came from proteins IL-8, epidermal growth factor (EGF), TIMP-1, TIMP-2, and monocyte chemoattractant protein (MCP)-1, which have been documented in tear fluid in concentration ranges consistent with the obtained data (in the case of MCP-1, detection has been seen in ELISA solely in CTF). These data parallel results obtained by microwell-plate array analysis, which will be detailed elsewhere. Because different sets of capture and secondary antibody probes were used for these assays, this provides confirmatory data regarding the specificity of the detected proteins.

The intensity of the signals for most of these and other detected proteins were significantly higher in the CTF than in the RTF and OTF samples. In some instances, the magnitude of the difference was exponential. This finding is strikingly different from the pattern of distribution of the three major inducible lacrimal secretory proteins (lysozyme, lactoferrin, and tear lipocalin) that have been shown to remain relatively constant in tear fluid, irrespective of the mode of sample collection. This relationship holds true for EGF as well as for trace levels of hepatocyte growth factor (HGF). These two cytokines have been attributed in the open-eye condition to a lacrimal gland origin. Because inducible lacrimal secretion nearly ceases after eye closure, we conclude that the inducible lacrimal gland secretion cannot be the major source of these proteins, at least in the closed-eye environment.

The other six prominent signals detected on the standard array represent proteins that have not been reported in tear fluid. These include IFN-γ inducible protein (IP)-10, growth-related oncogen (GRO generic), insulin-like growth factor binding protein (IGFBP)-2, macrophage inflammatory protein (MIP)-1β, ANG, and ENA-78. Perhaps the most surprising of these findings was the highly intense signal for ANG in virtually all tear samples. Given the critical need for maintaining corneal avascularity and the highly angiogenic nature of this protein, at first blush, this finding seemed to be artifactual. However, semiquantitative Western blot data confirmed the result, suggesting a minimal concentration in RTF in the sub-nanogram-per-microliter range. This finding is consistent with reports of high levels of ANG in other mucosal secretions and is compatible with the recent discovery of the antimicrobial properties of this family of proteins. In this respect, it is of interest to note that polymorphonuclear neutrophil (PMN) cell elastase has been shown to clip ANG, so that it no longer can undergo cellular internalization, a critical first step in the induction of angiogenesis. We have documented in an earlier study that CTF contains high levels of PMN cell elastase (primarily in the form of anti-protease complexes) and that the open-eye mucosal tear layer contains a resident pool of PMN cells. Whether the ANG that is present in tear fluid has been clipped and whether it exhibits angiogenic or antimicrobial activity remains to be determined.

The array analysis data (Tables 2, 3, 4) and data obtained from earlier studies reveal the accumulation in CTF of numerous other proteins that are potent modulators of angiogenesis. These include pigment epithelial cell–derived growth factor (PDEGF), angiotatin, plasminogen activator inhibitor (PAI)-2, α-2-macroglobulin, 12R-HETE, IL-8, and a host of cytokines, chemokines, and growth factors such as IP-10 and MCP-1 (Table 4). These findings raise several questions, such as what are the origins, the nature, and the modes of
control and relative distribution of angiogenic activators and inhibitors in OTF and CTF? How is net activity controlled during the diurnal cycle? To what degree does the net activity in the tear fluid affect the underlying cornea? Collaborative studies are under way to address some of these questions (Sack RA, et al. IOVS 2003;44:ARVO E-Abstract 1385).

Array analysis reveals the accumulation in CTF of several CXC chemokines, the most prominent being IL-8, ENA-78, IP-10, and GRO, as well as two CC macrophage-specific chemokines, MCP-1 and MIP-1β (Table 3). All these proteins were also detected in RTF and OTF, albeit in much lower concentrations. Many of these entities are known to be upregulated and secreted by epithelial cells and corneal epithelial cells specifically, as well as by kerocytes and PMN cells in response to exposure to proinflammatory mediators or secondary to viral invasion.55–59 This implies that the ocular epithelium and most probably the recruited PMN cells are major sources of these entities in CTF.

The much lower levels of these chemokines in the open-eye environment probably serve to drive a low level of recruitment of PMN cells into the tear layer continually, resulting in a small pool of resident sentinels.38 The size of this pool is presumably upregulated by increased levels of chemokine secretions in inflammation and other pathologic conditions.1,19,41

On eye closure, the situation changes radically. The rate of inducible lacrimal secretion dramatically decreases, if not ceases,39 with ongoing tear flow continuing in the form of a constitutive secretion composed primarily of surface (SIgA resulting in “closed dry eye.” Prolonged eye closure is also associated with another phenomenon: the induction of a sub-
clinical state of inflammation, as evidenced by the build-up in CTF of serum exudative proteins, the build-up and conversion of complement, the recruitment and partial activation of a massive number of PMN cells in the aqueous tear layer, and the accumulation of several proinflammatory lipids and cytokines.

In terms of proinflammatory cytokines, our work complements and expands earlier ELISA-based studies that revealed that overnight eye closure is associated with a marked increase in the level of IL-8 and the emergence of detectable levels of macrophage-colony stimulating factor (MCSF), MCP-1, and IL-6 in CTF. IL-1α and -1β were not detected. In the present study, by using individually pooled tear samples, we increased the sensitivity and detected all these proinflammatory cytokines in all types of tear fluids with elevated levels common to all CTF samples. In addition to these particular cytokines, analysis revealed elevated levels of several other leukochemokines, including ENA-78, GRO, and occasional trace levels of oncostatin M. This finding suggests that PMN cell recruitment is a multifactorial process, likely to be driven by a cascading series of events that ultimately result in the induction of an inflammatory reaction. Determining the relative importance of chemotactic factors in this process is fraught with difficulty, especially since CTF contains proteases that are known to clip specific chemokines and thus alter their chemotactic index. A better understanding of the dynamics of this process may be of clinical significance, especially given that excess

### Table 3. Proteins Detected in Individually Pooled CTF, by MA

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sensitivity* (pg/ml)</th>
<th>High Background</th>
<th>Intense Signal</th>
<th>Moderate Signal</th>
<th>Faint Signal</th>
<th>75%–100% Samples</th>
<th>50%–75% Samples</th>
</tr>
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* As determined by the manufacturer.

### Table 4. Chemotactic, Angiogenic, and Antibacterial Characteristics of CXC Chemokines in Tears

<table>
<thead>
<tr>
<th>Protein</th>
<th>Chemotactic Properties</th>
<th>Angiogenic Properties</th>
<th>Antibacterial Properties</th>
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</tr>
<tr>
<td>GRO</td>
<td>Np, Ba, EC</td>
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<td>-</td>
</tr>
<tr>
<td>NAP-2</td>
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<td>-</td>
</tr>
<tr>
<td>ENA-78</td>
<td>Np, EC</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GROα</td>
<td>Np, T, B, Ba, EC</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IP-10</td>
<td>Tac, M, NK, EO Angiostatic</td>
<td></td>
<td>+</td>
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<tr>
<td>MIG</td>
<td>Tac, EO Angiostatic</td>
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</table>

B: B-lymphocytes; T: T-lymphocytes; Tac, activated T-lymphocytes; EC, endothelial cells; Np, neutrophil granulocytes; Ba, basophil granulocytes; Eo, eosinophil granulocytes; M, monocytes; NK, natural killer cells.
PMN recruitment and activation have been implicated in the pathophysiology of a wide range of corneal diseases. This model would imply the presence of several potential targets for therapeutic intervention to restrict the extent of PMN cell recruitment in a manner consistent with differences in the pathophysiological pathways.

Once recruited, PMN cells are likely to interact with numerous chemokines, cytokines, and growth factors in a manner that enhances the capacity to process SIgA and surfactant D-SSiA complexes to opsonize bacteria (Ni M, et al., IOVS 2004;45:ARVO E-Abstract 4635) and further dictating inflammatory and immune processing, stabilizing the PMN cells, and thereby preventing inappropriate activation and apoptosis. Several studies have provided convincing evidence that granulocyte–macrophage colony-stimulating factor (GM-CSF) upregulates the expression of the high-affinity IgA receptors on PMN cells in CTF. Other interacting species, including IL-8 and ANG, are known to suppress apoptosis and retard the release of MMPs by neutrophils. In acute respiratory distress syndrome, ANG is thought to function in this manner by protecting the epithelial surfaces from autolytic damage. These findings suggest the addition of several new proteins to an expanding list of multifunctional antimicrobial entities that accumulate in CTF, including specific leukocyte protease inhibitor (SLPI), elafin, neutrophil gelatinase associated lipocalin (NGAL), and now, several CXC chemokines.

The high levels of chemokines in normal CTF raise questions concerning control of the innate and adaptive immune processes. For example, MCP-1 and IP-10 are two potent macrophage-specific chemokines that accumulate in high concentrations in CTF. Yet significant numbers of macrophages have not been observed to accompany the massive build-up of PMN cells, even after 6 to 8 hours of eye closure. This suggests that macrophage recruitment is somehow tightly regulated, perhaps by a parallel accumulation of migratory inhibitory factors such as mesoderm-inducing factor (MIF), TNF-α, IFN-γ, IL-1, IL-2, and GM-CSF (Table 3). One might also speculate that under pathologic conditions such as allergic conjunctivitis, in which macrophage recruitment has been documented, the balance between chemokines and inhibitors is altered. In this respect, it is of interest to note the presence of several other chemokines including eotaxin and eotaxin-2 in tear samples from individuals without overt symptoms of allergic ocular diseases. Studies are ongoing to determine whether changes in the distribution of these and other proinflammatory modulators in tear fluid can be monitored with this technology in normal and pathologic situations.

In addition to chemokines, intense signals were seen in CTF for two growth factors: EGF and IGFBP-2. EGF is a well-known tear constituent, whereas IGFBP-2 has been documented in tear fluid only anecdotally. The high levels of both entities in CTF suggest that they modulate cell proliferation and migration, two processes central to the maintenance of corneal and conjunctival integrity. Also detected were weak signals for HGF and TGF-β2 (assay very insensitive) as well as the previously unreported neurotrophic growth factors NT-3 and -4. These have been reported in the cornea and the lacrimal gland. The functional importance of these and other growth factors in tear fluid and their origins remains to be determined.

Although nearly 40 species were detected in CTF, the reliability of some of these data was made particularly tenuous by the high nonspecific signal on the negative control array. It is therefore of significance that, by using a custom array with a less complicated cocktail of biotinylated secondary antibodies, we were nearly able to eliminate this problem and greatly improve the signal-to-noise ratio. This suggests that it may be possible to identify and eliminate those antibodies in the arrays that are problematic and/or increase the sensitivity of the assay by sequentially assaying with a series of small arrays. Further improvements in the sensitivity can be obtained by replacing antibody pairs with antibodies with greater affinity and specificity as these become available. This seems very feasible, given the recent technical advances in the capacity to generate, select, and clone antibodies for greater specificity for targeted epitopes. Thus, we are optimistic that arrays can be developed with sufficient sensitivity and specificity to be of great clinical value for differential analysis that could be used for diagnostic and therapeutic intervention.

References


