Proinflammatory Cytokine Profiling of Tears from Dry Eye Patients by Means of Antibody Microarrays

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PURPOSE. In the pathogenesis of keratoconjunctivitis sicca, immune processes are thought to play an important role. However, the exact details of the pathomechanisms are still unknown. In this study, the expression patterns of proinflammatory cytokines in the tears of patients with different subtypes of dry eye were analyzed.

METHODS. One hundred forty-three subjects subdivided into healthy controls (CTRL, n = 38), patients with aqueous-deficient dry eye (DRYaq, n = 35), patients with changes of the lipid layer (DRYlip, n = 36), and patients with a combination of both (DRYaplip, n = 34) were examined. Expression patterns of proteins (e.g., IL-1β, IL-6, TNF-α, and IFN-γ) were examined using an advanced antibody microarray approach.

RESULTS. Several highly significant differences in the cytokine levels of dry eye patients compared with healthy controls were detected. Patients with DRYaq or those with DRYaplip showed elevated levels for most of the tested proteins. For example, IL-1β was found to be elevated 2.4-fold in DRYaq patients and 2.75-fold in DRYaplip patients (both P < 8.00E-6). The detected amounts of protein in DRYlip patients and in healthy controls showed only minimal differences (fold increase/decrease for all proteins >1.2; P > 5.00E-1).

CONCLUSIONS. The similarity between the profiles of healthy controls and DRYlip patients justifies the assumption that the pathomechanism of this dry eye subtype is based on mechanisms other than inflammation, whereas it seems to be the case for DRYaq patients. (Invest Ophthalmol Vis Sci. 2011;52: 7725–7730) DOI:10.1167/iovs.11-7266

Keratoconjunctivitis sicca is one of the prevalent ocular diseases in the industrial world.1,2 The disease is polymorphous and is caused by several factors. In most cases, it appears to be independent of other diseases, but it can also be associated with autoimmune disorders such as Sjögren syndrome. Furthermore, there is a significant association with aging, resulting in an increasing prevalence of dry eye in consideration of demographic changes.3

In theory, two basic mechanisms cause dry eye syndrome: a deficiency of the liquid phase because of reduced tear production or an increase of evaporation because of alterations of the lipid layer.4 In the past few years, there has been increasing evidence for the involvement of the immune system in the pathogenesis of dry eye. This hypothesis is supported by the fact that different studies were able to demonstrate an infiltration of the conjunctival epithelia with inflammatory cells, especially lymphocytes.5–7 Furthermore, changes in the expression of immune system-stimulating markers, such as the intracellular adhesion molecule I (ICAM) antigen and HLA-DR, which cause T-cell homing and antigen presentation, were noticed in the context of dry eye.8 HLAI expression can be typically found on T cells in connection with autoimmune diseases, such as systemic lupus erythematosus.9

Additionally, several studies report alterations of the protein expression profiles of cytokines in the tears of patients with dysfunctional tear syndrome, such as increased concentrations of IL-1α, IL-8, and IL-12. This suggests that keratoconjunctivitis sicca is in part the result of inflammatory processes induced by cytokines, resulting in an autoimmune response.10 Furthermore, several noncytokine proteins such as lipocalin-1 and cystatin SI00—proteins that are essential for the maintenance of tear film integrity and the regulation of its function as a natural defense barrier against microbial infections—were found to be altered in dry eye patients.11–15 For a long time, dry eye therapy was solely symptomatic, mainly by application of artificial tears. The latest findings offer a chance for a more curative, immunosuppressant treatment with cyclosporin A, even for severe forms of dry eye. Several studies show an improvement of subjective symptoms, and clinical test results show an effect of the anti-inflammatory therapy, but it is still uncertain whether all patients benefit from this treatment.16,17

In this regard, deeper understanding of the mechanisms underlying the pathogenesis of dry eye syndrome is essential for treatment strategies and for a more differentiated diagnosis of other ocular surface diseases.

The aim of this study was to gain more information on the characteristics of specific tear proteins, especially proinflammatory cytokines, with respect to the different subtypes of dry eye.

MATERIALS AND METHODS

Subjects

One hundred forty-three subjects were included in this study. A healthy control group (CTRL) contained 38 participants. Subjects with dry eye were subdivided into three groups, depending on their clinical presentation: aqueous deficient (DRYaq, n = 35), alterations of the lipid layer (DRYlip, n = 36), and a combination of both (DRYaplip, n = 34). The groups were matched in age and sex (except for the DRYaplip group; Fig. 1).

Classification of study subjects was based on the results of an ophthalmic examination and a questionnaire, with scores including objective and subjective criteria. The survey contained interrogations...
about the frequency and intensity of ocular symptoms (e.g., dryness, burning sensation, clotted cilia).

Ophthalmologic examination included a detailed slit lamp inspection of all components of the tear function unit (LIPCOF). Furthermore, we used the basis secretory test (BST; Schirmer test with anesthesia), a measurement of the TBUT (tear film break-up time), fluorescein staining, and lissamine-green staining as clinical tests (Fig. 2). These examinations and the sampling were performed in the same order in each study subject. The sampled Schirmer strips were stored at −80°C until use.

Subjects were classified as healthy controls based on a BST result >10 mm and a TBUT result >10 seconds. If the BST or TBUT, or both, were considered pathologic, the subject was classified as a dry eye patient. Detailed criteria for dry eye subtypes are shown in Table 1. Criteria for subclassification of dry eye patients were selected as follows.

**DRYaq.** Patients with a BST ≤10 and no pathologic TBUT (>10 seconds) were subclassified as having aqueous-deficient dry eye because reduced production/secretion of tear fluid would predominantly result in a lower BST value. To gain representative insight into the tear proteome of patients with different stages of aqueous-deficient dry eye, we chose a BST threshold of ≤10 mm/5 minutes, in accordance with previous studies of our group and others.21–23

**DRYlip.** Patients with a TBUT ≤10 seconds and a normal Schirmer value (>10 mm/5 minutes) were classified as having lipid-deficient dry eye. A dysfunctional, thinned lipid layer is consistent with a pathologic/decreased TBUT,24 which is caused by the lower tear film stability. However, the TBUT is not necessarily correlated with a decreased Schirmer value.26 Thus, in the present study, patients with a Schirmer value >10 mm/5 minutes and a TBUT ≤10 seconds were specifically classified as having lipid-deficient dry eye (DRYlip) because they had a normal aqueous state but a pathologic tear film stability. Further, the occurrence of a lipid layer deficiency was assured by inspection of the meibomian glands.27 Patients with a score ≥18 were classified as having a pathologic condition (lipid deficiency). Foulks and Bron27 suggested a threshold of >10. Because of the higher specificity, we set a threshold of ≥18.

**DRYaqlip.** Patients with a Schirmer value ≤10 mm and a TBUT ≤10 were classified as having a combined pathogenesis, which meant they had aqueous deficiency and lipid deficiency. It is generally accepted that symptoms of advanced stages of aqueous deficiency and lipid deficiency overlap and influence each other. As suggested by Versura et al.,25 a thin lipid layer results in an unstable tear film and an aqueous tear-deficient state; conversely, an aqueous tear-deficient state results in a thin lipid layer. Thus, we classified patients with pathologic BST, TBUT, and optionally a score ≥18 as having aqueous- and lipid-deficient dry eye.

Patients with any other ocular surface or eye disease, Sjögren syndrome, or diabetes mellitus and those who underwent ophthalmic surgery in the past 6 months were excluded. The use of any systemic drugs thought to have an influence on tear production or systemic inflammatory processes and the use of topically applied drugs in the appearance of eye or nasal drops led to exclusion from the study. Informed consent was obtained from all participants. The protocols were approved by the institutional ethics committee and conformed to the provisions of the Declaration of Helsinki.

### Sample Preparation

Schirmer strips were eluted using 500 μL 0.1% (wt/vol) dodecymaltoside and 0.1% (vol/vol) trifluoroacetic acid to dissolve proteins from the filter paper. Proteins were precipitated with acetone at −20°C.

### Table 1. Criteria for the Manifestation of Dry Eye Subtypes

<table>
<thead>
<tr>
<th>Group</th>
<th>BST (mm)</th>
<th>TBUT (s)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&lt;18</td>
</tr>
<tr>
<td>DRYaq†</td>
<td>≤10</td>
<td>&gt;10</td>
<td>&lt;18</td>
</tr>
<tr>
<td>DRYlip†</td>
<td>&gt;10</td>
<td>≤10</td>
<td>≥18</td>
</tr>
<tr>
<td>DRYaqlip†</td>
<td>≤10</td>
<td>≤10</td>
<td>≥18</td>
</tr>
</tbody>
</table>

* All three criteria had to be fulfilled.
† The first and the second criteria had to be fulfilled.
overnight and centrifuged. Resultant pellets were dissolved in 100 μL HPLC-grade water. Subsequently, a measurement of the total protein concentration was performed using a BCA protein assay kit (Pierce, Rockford, IL).

To visualize captured proteins on the microarrays, tear proteins were labeled with an amine-reactive fluorescent dye (DyLight 649 NHS Ester; Pierce) before the incubation. For labeling, 5 μg protein and 0.3 μL fluorescent dye were added and incubated in the dark for 1 hour at room temperature. Afterward, excessive fluorescent dye was inactivated by the addition of 100 mM glycine to the sample solution.

**Antibody Microarrays**

Antibody microarrays were prepared by spotting purified antibodies in triplicate on nitrocellulose-coated slides (Onyxce, nitrocellulose 16 multipad slides; Grace Bio-Labs, Bend, OR) using a noncontact array spotter (sciFLEXARRAYER 3; Scienion, Berlin, Germany). Anti-IgG/IgM/IgA (1 μg/μL) served as a positive control. For the analysis of proinflammatory cytokines, antibodies against IL-1β, IL-6, IL-8, TNF-α, and IFN-γ (0.5 μg/μL; Biologend, San Diego, CA) were spotted on slides. In addition, antibodies against lipocalin 1 (LCN1), cystatin SN (1 μg/μL), and α-antitrypsin (2.43 μg/μL) were used. As a negative control, we used spotting buffer. For incubation with tear samples, slides were covered with 16-pad frame hybridization chambers (FAST; Whatman, Maidstone, UK) followed by the blocking of unspecific binding sites using 120 μL buffer (PBS/3%, NFDM/3%, BSA/1%) per subarray for 1 hour at 10°C. The arrays were washed three times (120 μL PBS) for 5 minutes each time. Labeled sample proteins were incubated on subarrays overnight at 4°C. Afterward, slides were washed four times for 15 minutes each time using PBS-T (PBS containing 5% Tween-20). Finally, the slides were dried (SpeedVac; Thermo Scientific, Waltham, MA) and subsequently scanned using a high-resolution confocal scanner (array scanner 428 TM, AVISO; Affymetrix, High Wycombe, UK).

The generated slide images were analyzed using image-processing software (Spotfinder 3.1.1, TM4; Dana-Faber Cancer Institute, Boston, MA). Background subtraction was performed according to the formula: spot intensity = mean intensity_{sp} = \left[\frac{\text{sum}_{bkg} - \text{sum}_{top5bkg}}{\text{num}_{pixel}_{sp}}\right] / \left(\text{num}_{pixel}_{sp} \times \text{num}_{pixel}_{top5bkg}\right)

Regarding the experimental data, we first performed a comparison of healthy controls (CTRL) and people with dry eye (DRY) regardless of the pathogenesis. The mean plot of detected protein/cytokine intensities for this general comparison is shown in Figure 3. All the tested analytes were found to be distinctly elevated in the dry eye group (Tukey’s HSD, P ≤ 1.00E-3). Protein amounts measured for the dry eye group were 2 to 2.5 times higher than for healthy subjects. In dry eye patients, there was pronounced variation in the protein quantities, whereas the data of healthy control subjects seemed to be more homogeneous, except for α1-antitrypsin (Fig. 3). Cystatin SN and IL-6 revealed the most distinct differences of all

**RESULTS**

In this study we used a highly sensitive antibody microarray approach for the quantification of specific proteins, especially cytokines, in the tears of patients with dry eye considering the different clinical phenotypes. In all subjects and for all tested proteins measurable signals were obtained.

Regarding clinical parameters, we found significant correlations between the BST and the TBUT in all subgroups, except for DRYaqlip patients (Supplementary Table S1f, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7266/-/DCSupplemental). None of the sicca-specific subgroups showed relevant correlations between BST and age, BST, and vital dyes or BST and clinical scores (Supplementary Table S1c–f, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7266/-/DCSupplemental). In contrast, the DRYaq and the DRYaqlip groups showed highly significant correlations for the BST and relative amounts of tested analytes (Supplementary Table S1d–f, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7266/-/DCSupplemental). No correlations were found between TBUT and cytokines in the DRYaq and DRYlip groups, whereas all the cytokines negatively correlated with TBUT in the DRYaqlip group. Additionally, we found a strong positive correlation between protein concentrations of the different cytokines in each dry eye group (Supplementary Table S1c–f, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7266/-/DCSupplemental).

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![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933248/ on 04/28/2017)
tested proteins when comparing controls with dry eye sub-
jects. Supplementary Table S2 (http://www.iovs.org/lookup/ suppl/doi:10.1167/iovs.11-7266/-/DCSupplemental) shows the relative deviations between groups for all analyzed proteins.

As described, dry eye patients were subdivided into three subgroups: patients with dry eye caused solely by an aqueous insufficiency (DRYaq), patients with an exclusive lipid layer deficiency (DRYlip), and patients with a combined variation (DRYaqlip). Data obtained from the detailed group comparisons are shown in Figure 4. We observed a pronounced difference between the control group (CTRL) and people with dry eye, but only if associated with an aqueous deficiency (DRYaq) or a combined pathogenesis (DRYaqlip). In DRYaq patients, the values for cystatin SN are elevated up to 3-fold compared with values in controls \( (P < 9.0E-05) \). The other proteins were found to be increased up to 2-fold or 3-fold, and all of them revealed a statistically significant alteration \( (P < 8.0E-05) \).

TNF-\( \alpha \) was elevated nearly 2.5-fold compared with the control group \( (P < 8.0E-06) \). Patients with DRYaqlip exhibited protein amounts nearly to those in DRYaq patients. Considering that DRYaqlip subjects were not sex matched and to ascertain the potential influence of the distribution of the sexes in this group, we randomly excluded 18 female subjects from the DRYaqlip group, thus achieving a study population of eight men and eight women. No difference was observed in comparing the data from the “sex-matched” and the “whole” DRYaqlip group (data not shown).

Only minimal differences could be observed between protein signatures obtained from patients with a lipid layer deficiency and healthy controls. Protein patterns appeared to be similar in both groups. For the analytes, no significant differences could be detected between values of the DRYlip group and the control group \( (P > 5.00E-2) \). Concordant with all groups, IL-6 is the cytokine that was most increased in DRYlip, but it was only 1.06 times as high as in the control group. Data from the relative alterations between subgroups and those from statistical analyses are listed in Supplementary Table S2 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7266/-/DCSupplemental).

**DISCUSSION**

The focus of the present study was on comparison of the expression patterns of specific proteins in the tears of dry eye patients. With regard to tested noncytokine proteins, we detected significant alterations in dry eye patients, thus confirming previous findings from our group and others. For example, the use of mass spectrometry devices, \( \alpha_1 \)-antitrypsin and cystatin SN were found to be increased in the tears of dry eye patients and in those of contact lens wearers.\(^{30,31}\) However, the subclassification of dry eye patients in the present study revealed that only patients with aqueous deficiency-associated dry eye were affected by these alterations and that patients with exclusive lipid layer deficiency exhibited protein levels nearly similar to those of healthy controls, reflecting the different underlying pathomechanisms of dry eye. In light of the biological functions of these proteins, processes such as the transportation of small hydrophobic molecules (lipocalin 112) and the inhibition of serine and cysteine proteases (cystatin SN, \( \alpha_1 \)-antitrypsin) seem to be particularly influenced by events related to aqueous-deficient dry eye. In the second instance, elevated levels of, for example, \( \alpha_1 \)-antitrypsin may lead to an activation of neutrophils, as suggested by Janciauskiene et al.\(^{32}\)

The specific analysis of tear cytokine profiles has been the basis of several studies dealing with immunologic aspects in the context of ocular surface diseases. Alterations of these immunomodulating proteins were found for different diseases, such as allergies,\(^{33}\) ocular rosacea,\(^{34}\) and ocular pemphigoid,\(^{35}\) improving our understanding of their pathogenesis.

Recent studies\(^{36–41}\) have shown that alterations of the tear cytokine profiles occur also in dry eye patients. For example, Li et al.\(^{37}\) and Massingale et al.\(^{38}\) tested several proinflammatory cytokines (e.g., IL-6, IFN-\( \gamma \), and TNF-\( \alpha \)) and observed similar deviations between the tears of Sjögren’s syndrome/dry eye patients and controls, as demonstrated in the present study. Furthermore, the specific increases of the different cytokines coincided very well.\(^{38}\)

In general, most studies observed elevated cytokine levels in the tears of dry eye patients, with the exception of Narayanan et al.\(^{36}\) who found normal expression profiles of several cytokines in patients with moderate dry eye disease. However, a direct comparison of results from these studies is challenging because of the varying study designs and especially the heterogeneous inclusion and exclusion criteria used for patient classification and subclassification. Several studies only differentiated between the presence or absence of Sjögren syndrome\(^{36–40}\) or considered additional criteria such as the occurrence of a meibomian gland disease.\(^{41,42}\) In most studies, dry eye patients were classified on the basis of etiologic factors. In the case of Sjögren syndrome this is obvious, but in the case of patients with non-Sjögren syndrome keratoconjunctivitis, a subclassification of patients seems to be reasonable to consider inhomogeneous and multifaceted clinical presentations such as

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933248/ on 04/28/2017)
a deficiency in the aqueous or in the lipid layer of the tear film or both.

Comparing other studies that consider different subtypes of dry eye with our results, several concordances exist. For example, Lam et al. performed a study subdividing patients with dysfunctional tear syndrome based on the absence or the existence of a meibomian gland disease (MGD). Compared with healthy subjects, they detected higher protein levels for cytokines such as IL-1β, IL-6, or IFN-γ in patients with dry eye syndrome without MGD, corresponding to patients with a deficiency in the aqueous phase. In the present study, patients classified as DRYaq exhibited nearly similar alterations, thus confirming the findings of Lam et al. within a greater patient population. The high SD observed for measured protein levels in the DRYaq group was also noticed by Lam et al.

However, we found no deviation in protein amounts of the tested cytokines within the tears of DRYlip patients compared with healthy control subjects. The DRYlip group specifically included patients with lipid deficiency and no deficits in the aqueous phase: the lipid deficiency might have been caused by different triggers such as MGD or bacteria. In contrast to our data, Lam et al. found cytokines such as IFN-γ, IL-1β, and IL-8 to be strongly increased in subjects with MGD-induced dry eye. This discrepancy suggests that the use of etiologic aspects results in a different subclassification of dry eye subjects than a classification by clinical aspects, as performed in the present work. An explanation for this contradistinction might be that some of the MGD patients analyzed by Lam et al. also had aqueous deficiency. This assumption is supported by our observation that patients with a combination of aqueous and lipid deficiency (DRYaqlip) had alterations in cytokine levels, as detected by Lam et al. for the MGD group. Furthermore, we found negative correlations between the BST and cytokines (e.g., IL-6, IL-8, and IL-1β) in the DRYaq and the DRYaqlip subgroups but not for patients with an exclusive deficiency in the lipid phase.

The biological relevance of these increased levels of pro-inflammatory cytokines is given by their suspected promotion of the differentiation of naive CD4+ cells to Th17 T cells, which have been identified as key effector cells in autoimmune processes. Zheng et al. could demonstrate that ocular surface tissues subjected to desiccation stress are capable of inducing dendritic cells to express increased levels of cytokines such as IL-1β, IL-6, IL-23, and TGF-β1. As further shown by Zheng et al., this cytokine cocktail is of great importance for the differentiation of Th17 cells, which emerge from naive CD4+ T cells and, in turn, are known to occur in increased numbers in ocular surface epithelia in an animal model of dry eye disease. Thus, the elevated amounts of cytokines such as IL1-β and IL-6 detected in the present study have to be considered as a Th17-promoting response of dendritic cells to desiccative stress, but only in the case of aqueous-deficient dry eye patients and not those with deficiency of the lipid layer. Increased protein levels of TNF-α and IFN-γ were shown to alter the expression levels of the membrane-associated mucins MUC1 and MUC6, thus probably affecting the tear film integrity and acting as a counterbalance for desiccative stress. However, the exact mechanism for this is still unknown.

In principle, our results support the hypothesis of an immunologic component within the pathogenesis of dry eye syndrome, but this applies exclusively to patients with aqueous-deficient dry eye or to those with an additional lipid layer alteration. Cytokine profiles of patients solely with lipid layer insufficiency did not significantly differ from those of the control group, leading to the assumption that mechanisms other than inflammation are causing the dry eye syndrome in these patients. This finding is highly important with respect to the therapeutic treatment of patients with lipid-suppressing substances such as cyclosporin A. The question that arises is to what extent patients with lipid layer insufficiency benefit from the therapy.

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References
