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Protein Analysis of Human Lacrimal Fluid in Varying Age Groups

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Abstract

Purpose: The objective of this research project was to identify proteins secreted from human lacrimal fluids onto the extra-ocular surface of the eye that could be later used to predict eye health, disease, and age-related changes. The identification of specific lacrimal proteins in relative quantities and patterns in younger versus older patients may reflect both ocular and extra-ocular disease states.

Methods: This observational study collected samples of lacrimal fluid from 20 subjects between the ages of 18 and 25 years and 20 subjects over the age of 50 years with the use of Schirmer strips. The protein composition of these lacrimal fluid samples was then analyzed to determine specific proteins that evidenced unique patterns among the subject populations.

Results: The protein concentrations between the two age groups ($n = 40$) was significantly higher in the younger patient group (1408.3 ug/mL versus 1152.5 ug/mL, $p = 0.03$). No consistent qualitative differences in the protein bands were observed between the two different patient age groups. However, excising and analyzing the outlying protein bands revealed unique proteins within the older patient group (aldehyde dehydrogenase and serotransferrin precursor). Preliminary attempts were made to determine the presence of proteins in lacrimal fluid that may originate from cells lining the ducts and blood vessels associated with the ocular environment.

Conclusion: These preliminary results in age related differences in eye lacrimal fluid will contribute to future research endeavors in order to determine which specific proteins were increased or decreased quantitatively in the younger population, if any, and what role they might have in eye health, disease, and age-related changes.

Keywords

Protein, lacrimal, eye, protein analysis

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Cover Page Footnote

We would like to acknowledge Dr. Lindsay Florkey, a local optometrist, for her assistance and expertise on methods of lacrimal fluid collection. We would also like to thank Brad Pauley and Rebecca Kyper for assistance in photographs and gel picture documentation.

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Protein Analysis of Human Lacrimal Fluid in Varying Age Groups

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Abstract

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Introduction

The eye is an ocular organ capable of distinguishing between various shapes and colors in order to produce an elaborate vision field for humans. It is considered to be one of the most complex organs within the human body. It is often cited as an example of a supposed irreducible complexity due to its intricate anatomical parts, all of which are dependent upon each other for appropriate functionality.¹

An often-overlooked feature of the eye that truly contributes to its complex functionality is its surrounding lacrimal fluid.² This extracellular fluid is secreted by the acinar and ductal cells of the lacrimal gland, located in the upper temporal part of the orbit within the lacrimal fossa.^{2,3} The secreted lacrimal fluid consists of water, electrolytes (sodium, potassium, calcium, magnesium, chloride, phosphate, and bicarbonate, etc.), protein (enzymes, growth factors, cytokines), and other small molecule metabolites (amino acids, urea, glucose, lactate, etc.), which serve to lubricate the ocular surface, provide oxygen to the avascular cornea and inhibit bacterial growth.^{2,4,5} Additionally, without this fluid, light would not be appropriately refracted onto the retina.^{2,4}

Lacrimal fluid comprises the aqueous component of the eye's tear film, which is classified into three main layers: the outer lipid layer, middle aqueous layer, and inner mucin layer.^{2,6} The lipid layer's primary source is the Meibomian glands, which produce both non-polar and polar lipids dispersed with various proteins serving to stabilize the tear film.^{2,6} The mucin layer's primary source is the goblet cell, and serves as a border between the tear film and the ocular surface.^{2,6} It has been demonstrated that the total protein concentration of normal lacrimal fluid within healthy individuals ranges anywhere from 6 to 11 mg/ml and contains anywhere from 500 to 1500 different proteins, of which 10 to 15 percent are considered to be extracellularly derived.⁶

These proteins present within lacrimal fluid not only function to protect and nourish the eye, but may also serve as biomarkers to indicate the presence or absence of an underlying pathology.⁶ The variations in tear compositions have been shown to correlate with both ocular and extra-ocular conditions.⁶ Therefore, they are an ideal source for the identification of biomarkers that may indicate the presence or absence of disease, and have the potential to offer greater insight into the

underlying pathology of different disease states.⁶ By identifying specific lacrimal proteins in relative quantities, their close relationship to both ocular and extra-ocular disease states may be better understood.

A total of 1543 lacrimal fluid proteins were recently discovered and developed into a reference list.⁷ Lysozyme, lactoferrin, sIgA, and GIIA phospholipase (PLA₂) are only several of the important tear proteins that offer defense against pathogens through broad-spectrum antimicrobial activity.⁶ Calgranulin A (S100 A8), an inflammatory-related protein, has been revealed to be present in higher quantities in individuals with keratoconjunctivitis sicca.⁶ Patients with allergic conjunctivitis were observed to have significant increases in tear IgE levels, osteopontin (an adhesive matrix protein), and substance P (a neurotransmitter involved with neurogenic inflammation).⁶ Collagenase 2 (MMP-8) and gelatinase B (MMP-9) were both increased in people with ocular rosacea, a chronic inflammatory disease affecting the eye.⁶ Additionally, treatment with oral doxycycline in these individuals was shown to decrease levels of MMP-9 from a mechanism independent of its antimicrobial activity.⁶

Among the extra-ocular disease states that have been linked to an increase in lacrimal proteins are diabetes mellitus and various cancers.⁶ Tear specific pre-albumin (TSPA) concentrations were decreased and lysozyme increased in the tear fluid of diabetics with pre-proliferative diabetic retinopathy.⁶ Concentrations of nerve growth factor (NGF) were increased in the tear fluid of diabetics with proliferative diabetic retinopathy.⁶ These findings indicate the potential to identify early-onset diabetic ocular dysfunction.⁶ Lacryglobin was detected in higher concentrations in patients with breast, lung, colon, and prostate cancers when compared to non-cancer patients.^{6,8} In a study with 50 subjects, roughly 80 to 90 percent of patients with breast and lung cancer and 100 percent of patients with colon and prostate cancer had detectable levels of lacryglobin in their lacrimal fluids, relative to the 60 percent of control patients.⁸ Overall, the variations in tear fluid composition suggest that proteins and other tear components could become useful biomarkers of disease presence and severity.

Because the collection technique can drastically affect the analysis of the tear fluid, a brief discussion of the advantages and disadvantages between the various lacrimal fluid collection techniques is warranted.⁶ The Schirmer's strip test and glass capillary tubes are two of the most popular collection techniques.^{6,9} The

Schirmer's strip test is commonly used in ophthalmology clinics to aid in the diagnosis of dry eye.^{6,9} It consists of a strip that is placed inside the conjunctival sac (eyelid) for roughly five minutes or until the strip is filled with tear fluid.^{6,9} Although commonly used, this method can often cause discomfort and has the tendency to collect unwanted epithelial proteins from the surface of the cornea or conjunctiva.^{6,9} The glass capillary tube method involves placing the tip of the capillary tube in the lower tear meniscus to allow for the fluid to be naturally drawn into the tube.⁶ Although this technique theoretically allows for minimal contact, it assumes the person is experienced with the procedure.⁶ There is a risk of the capillary tube touching the ocular surface, especially with inexperienced individuals.⁶ Additionally, this process is generally very slow and is usually not possible in patients with a low tear fluid volume (dry eye, elderly, etc.).⁶

A biomarker is typically defined as any biologic substance that can be objectively measured to indicate the presence, absence, or severity of a normal or pathological process, or a pharmacological response to a therapeutic intervention.¹⁰ Common biomarkers include, but are not limited to: B-type natriuretic protein (BNP) for heart failure, troponin for myocardial infarctions, low-density lipoprotein (LDL) for atherosclerotic cardiovascular disease, hemoglobin A1c for diabetes, and prostate specific antigen (PSA) for prostate cancer. The typical process for a biomarker development includes its initial discovery, where a potential biomarker is identified.¹⁰ Once identified, the biomarker can be linked to a normal process, pathological process, or response to a pharmacological intervention within the confirmation stage of development.¹⁰ If the biomarker is successfully linked to a specific process, it typically enters the validation and refinement stage where its relevance to the population can then be established.¹⁰ Lastly, the biomarker is adopted once its clinical utility has been identified.¹⁰

The initial discovery stage of biomarker development, as its main purpose is to identify potential biomarkers that can be linked to various processes at a later time. Our goal was to identify proteins located in human lacrimal fluids that could be later used to predict eye health, disease, and age-related changes. The purpose of the research was to determine the age-related differences in tear fluid composition between different age groups. We analyzed human lacrimal fluid from individuals of varying ages, and subsequently determined a relationship between the findings and the

disease state, eye health, and age-related changes of the corresponding individuals. By looking at the variation in proteins within lacrimal fluids of patients in different age groups we hope to identify any variations that could potentially link a patient with a particular age group and possibly, at a later time, a specific disease.

Materials and Methods

Subject Enrollment

Subjects were enrolled in the study using a quota sampling strategy. A quota of 20 to 25 individuals was set for the age groups of 18 to 24 years old and over 50 years old. Individuals were conveniently selected from the Cedarville University School of Pharmacy student population and a local practicing optometrist's patient database. Individuals who have worn contacts within the past year were excluded from the study. All of the sample collection and analysis was completed by professional students of the Cedarville University School of Pharmacy during the time period of August 2013 to January 2015, under the instruction and validation of a faculty advisor and a practicing optometrist. IRB approval was obtained from the Institutional Review Board for Human Subjects Research of Cedarville University.

Sample Collection

Each subject was provided with information about the study upon their arrival at the Health Sciences Center of Cedarville University. The subject then entered the simulation clinic with two researchers and was asked to sign a consent form. If the subject indicated his or her willingness to participate in the study, he or she was assigned a subject number and the data collection process began. The first component of the process consisted of a short interview to record any aspects of the subject's personal history that could affect the protein components of his or her lacrimal fluid, such as age, occurrence of laser eye surgery, use of eye drops, use of eyeglasses, any current or chronic health problems they may have, and birth control status. Once the interview concluded, a researcher collected a sample of the subject's lacrimal fluid, one from each eye. While wearing gloves, the researcher used a Schirmer strip to collect a 30-50 microliter sample of lacrimal fluid from the lacrimal lake of the lower eyelid of each eye. The Schirmer strip was inserted inside the lower eyelid of each eye and remained there for approximately 2-5 minutes, depending on the time needed for the correct

amount of sample to be collected. Strips were stored in a secure minus 80° C freezer until the time of analysis.

Data Analysis

The mass of sample collected with each Schirmer strip was recorded by taring a scale with a microfuge tube containing an unused Schirmer strip and then obtaining the mass of each microfuge tube containing a patient sample Schirmer strip.

The lacrimal fluid sample was extracted from each Schirmer strip using 100 microliters/strip of tear extraction buffer (500mM NaCl, 0.5% tween 20, phosphate buffered saline, pH 7.4, 1% bovine serum albumin, protease inhibitors, plus 1 mM NaVO₃, 1mM PMSF, and 1mM benzamidine). Extraction was performed for 1 hour at 4 °C with end-over-end rotation and collection of fluid by centrifugation for 5 minutes at 13,500 rpms. A standard BCA protein assay was utilized to quantitate the protein content of each sample.¹¹ Polyacrylamide gel electrophoresis was utilized to separate collected proteins based on charge and molecular weight.¹²

Proteins were localized in each gel using the Pierce® Silver Stain for Mass Spectrometry method.¹³ This method is compatible with the process of protein digestion by enzymes to create peptide fragments for mass spectrometry analysis.¹³ Gel images were collected and photographed utilizing an iPad camera application. Unique protein bands from the gels were excised using a sterile razor, and each excised band was placed into individual microfuge tubes. Images of the gels after the excision of the unique protein bands were obtained to confirm identity. The images of the gels were also recorded on a CCD camera with Alpha imager software.

Excised protein bands were processed for analysis by mass spectrometry by de-staining the bands with freshly prepared buffers.¹³ Following the completion of this protocol, selected excised gel pieces were sent to the University of Cincinnati Proteomics Laboratory where the preliminary analyses were completed utilizing mass spectrometry. Additional analyses of the tear samples were conducted to determine the presence of proteins possibly shed from endothelial cells during normal cell turnover. To accomplish this secondary objective, we performed a standard dot blot method to search for the presence of immobilized proteins on nitrocellulose membranes.¹⁴

Five micrograms of lacrimal tear fluid were spotted onto the nitrocellulose membrane using a narrow-mouth pipette tip and dot blot apparatus. Once the membrane dried sufficiently, the non-specific sites were blocked by soaking the membrane in 5% BSA in TBS-T (20 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.5% Tween 20) for one hour at room temperature. Each membrane was incubated with the primary antibody diluted in BSA/TBS-T (0.1% BSA in TBS-T) for 30 minutes at room temperature. Membranes were washed three times with TBS-T for 3-5 minutes per wash. Secondary antibodies conjugated with HRP were added to the membranes for 30 minutes at room temperature. Final washes were performed with TBS-T (first wash, 15 minutes; second and third washes, 5 minutes each). After a final wash with TBS for 5 minutes, membranes were processed for enhanced chemiluminescence (ECL, GE Healthcare) for 1 minute before exposing it to the alpha-imager.

Results

Patient demographics

Patients enrolled in this study were selected conveniently from the surrounding Cedarville/Xenia, Ohio area. Students within the Cedarville School of Pharmacy were selected for the young group and patients from the Cedarville and Xenia practice sites of Dr. Florkey were selected for the older patient group. A total of 40 patients were enrolled in the study (20 patients per age group), and prior to providing samples patients were asked about their past medical history regarding: eye health, health concerns, chronic disease states, menopausal status, other factors that alter hormone levels, and age. All results were strictly patient reported. Chronic disease states were more prevalent in the old group (6 patients from the old group compared to 1 from the young group). The young group had 10 males and 10 females, whereas the old group had 8 males and 12 females. The average age for the young group was 22 years old, with an average age of 60 years old for the old group (one patient in the old group did not have an age recorded). Male average age in the study was 21.7 years old and 59.8 years old for the young and old groups respectively. Female average age in the study was 22.2 years old and 60.2 years old for the young and old groups respectively.

Lacrimal Fluid

The young group produced an average larger volume of lacrimal fluid (LF) than the older group; 0.0314 ± 0.008 g of LF collected from the young group compared to 0.0263 ± 0.005 g of LF from the old group. Eyes within the groups produced on average different volumes of LF. In the young group the right eye produced a greater volume of LF, 0.0323 ± 0.008 g compared to 0.0304 ± 0.008 g of LF from the left eye. Conversely, in the old group the left eye produced, on average, a greater volume of LF, 0.0272 ± 0.008 g compared to 0.0253 ± 0.003 g of LF collected from the right eye.

BCA Protein Assay

To examine the difference in protein concentration between groups, as well as between the left and right eyes, samples were analyzed by a BCA assay. Which revealed that the young group had a greater average concentration of protein in LF than the old group, 1408.33 ± 17.0 $\mu\text{g/mL}$ compared to 1152.46 ± 16.3 $\mu\text{g/mL}$. A paired two-sample t-test revealed a statistically significant difference between protein concentrations for each group. In each group, the eye that produced more volume also had the greatest protein concentration. The young group averaged a protein concentration of 1578.91 ± 21.5 $\mu\text{g/mL}$ for the right eye and 1224.94 ± 21.4 $\mu\text{g/mL}$ for the left eye. The old group's protein concentration did not vary as greatly as the young group. The old group's left eye averaged a greater protein concentration (1159.93 ± 31.0 $\mu\text{g/mL}$) than the right eye (1144.99 ± 27.2 $\mu\text{g/mL}$).

Pierce® Silver Stain

In order to find and extract unique proteins from the samples, all samples were analyzed using the silver stain method. This analysis allowed the research team to observe all proteins found in LF that was collected, and select unique bands for further identification. Figures 1A and 1B are representative gels showing the migration of proteins and level of clarity. Initial observations were to identify bands that were found to be unique and presented differently from other proteins within the same gel. Any protein that was unique was excised, de-stained, and sent for mass spectrometry analysis. For this study proteins from the left eye of an older patient were analyzed in more detail.

Figure 1A

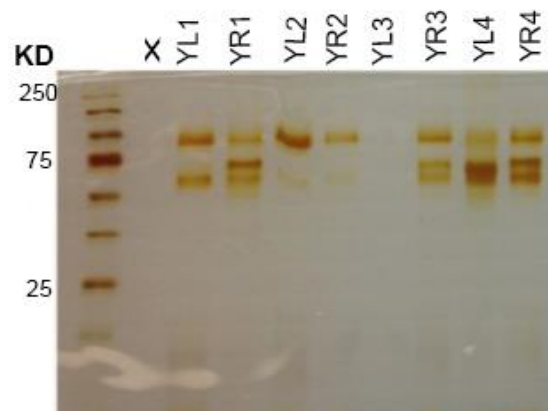


Figure 1B

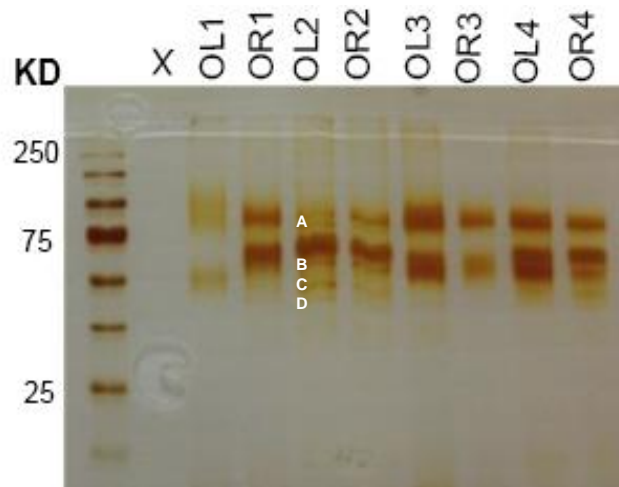


Figure 1A and 1B. Representative protein gels of separated lacrimal proteins obtained from human lacrimal fluid obtained from the left (L) and right (R) eyes absorbed onto Schirmer Strips. Proteins were separated on 8% percent PAGE gels at 100 nanograms per well. Molecular weight markers are indicated in kilodaltons (BioRad protein plus). Pierce silver stain protocol was used to stain proteins prior to mass spectrometry analysis. Figure 1A represents samples from young patients. Figure 1B represents Older patients, patient OL2 and OR2 were of interest in the initial mass spectrometry search. In figure 1B, labels A, B, C and D represent bands isolated for more detailed analysis.

MALDI-TOF/TOF and MASCOT

After bands were excised, they were de-stained and sent for mass spectrometry in order to be identified. These 4 protein bands (Labeled A-D in figure 1B) were reduced, alkylated, digested with trypsin and identified by MALDI-TOF/TOF and a MASCOT search of the mammalian proteome. The proteins were cleaved into peptides for peptide identification and potential proteomic identification. Band A (OL2) was determined to be made of human serum albumin in a complex with myristic acid and tri-iodobenzoic acid, and Ig alpha-1 chain (C region). Band B (OL2) had the exact same identification as Band A. Band C (OL2) contained aldehyde dehydrogenase, another tentative identification of the contents (meaning further confirmation would be required to accurately identify the protein) was IgG1 (Heavy chain H Lambda). Finally, Band D (OL2) contained serotransferrin precursor and albumin (isoform CRA_t).

Fibronectin Type III Repeat in Lacrimal Fluid

All lacrimal fluids from Schirmer strips from young and older patients were bound to nitrocellulose in a dot blot format and probed for proteins known to be associated with endothelial cells, CD31 and fibronectin type III repeats. Two patient samples had detectable levels of fibronectin type III repeat protein, in Row A spot A2 and A10 (numbering pictured below each dot blot). The dot blot was stripped and re-probed with anti-tubulin antibody as a negative control, results are shown in figure 3, no detectable reactivity.

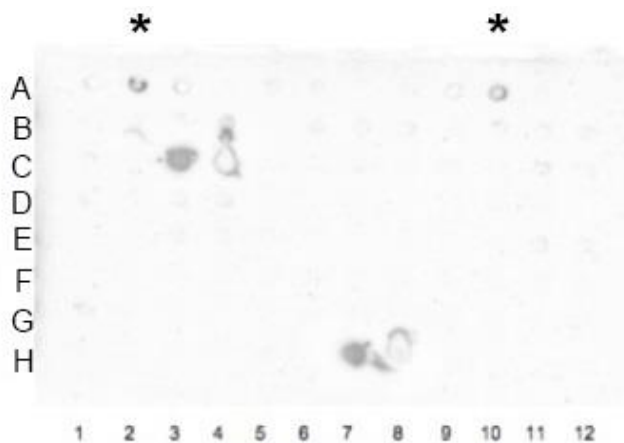


Figure 2. Dot blot demonstrating fibronectin type III repeat like protein present in extracted tear fluid. Spots

A2 and A10 (asterisk) are samples of young patients that did not wear contacts or have any diagnosis related to an eye disorder and are positive for reactivity to fibronectin type III repeat. Spots C3 and H7 are positive controls, purified protein from HEK293 cells. D8 is fluid from tear blink an artificial wetting agent for eyes.



Figure 3. The dot blot used for fibronectin type III reactivity was stripped and re-probed with a mouse anti-tubulin antibody to demonstrate non-reactivity with an unrelated antibody.

Discussion

The purpose of this study was to identify any differences in the protein makeup of lacrimal fluid between young and old patients. The resulting differences could then be utilized as a baseline for future studies to identify which specific proteins might correlate with disease and eye health. Preliminary gel electrophoresis runs revealed some minor but distinct variations between young and old patient groups. There were three unique protein bands located in one particular patient (Left eye patient 5), and one was from the young group (Left eye patient 4). After excising the three outlying protein bands and sending them to be analyzed, the proteins were identified to be albumin complexed with myristic acid, albumin isoform CRA, partial immunoglobulin, aldehyde dehydrogenase, and serotransferrin precursor. Both albumin with myristic acid and partial immunoglobulin were found in the young and old groups. However, aldehyde dehydrogenase and serotransferrin precursor were unique to the outlying protein band within the old group.

The presence of aldehyde dehydrogenase and serotransferrin precursor protein may indicate the occurrence of age-related changes to the eye. However,

these proteins were only linked to the left eye of one specific subject. The subject, in which these proteins were identified, was a female, 66 years of age, with a past medical history of osteoporosis. Additionally, her eyes were dilated 20 minutes prior to a lacrimal sample being taken. Thus, the presence of aldehyde dehydrogenase and serotransferrin in the lacrimal sample could have been due to inflammation from her chronic osteoporosis or due to the fact that her eyes were dilated beforehand. Because the dilatory drops get metabolized very quickly on the ocular surface and should not be present after roughly 10 minutes, it is not believed that the dilatory drops themselves had an effect on the lacrimal fluid composition. However, the dilatory effects were still present, and could have contributed to an increase in certain proteins within the subject's lacrimal fluid. Because only one elderly subject's lacrimal fluid was observed to contain these unique proteins, future studies would need to be conducted in order to hypothetically link these proteins to actual ocular age-related changes or inflammatory disease states and if dilation in some way affects protein expression independently of disease status. No other patients in the study had pupil dilation prior to sample collection.

During the course of the study, the concentrations of the proteins in the lacrimal fluid were able to be calculated. When comparing the means, it was found that the young subjects had a significantly higher protein concentration within their lacrimal fluid ($p=0.03$). Future studies could be aimed at determining what specific proteins, if any, were increased in younger

patients, and what role they might have in eye health, disease, or various age-related changes.

A secondary objective was to determine if proteins known to be present on normal endothelial cells such as CD31, fibronectin type III repeats and Von Willebrand's factor could be detected in lacrimal tear fluid from young and older patients. Out of a total of 40 patients, two young patients had detectable levels of fibronectin type III protein. Further analyses are being conducted to determine contribution to the tear fluid in these patients. Various eye inflammatory or injury settings could present with shed endothelial cell components because of leaky vessels or exuded cell pieces due to apoptosis and cell fragmentation.¹⁵

Conclusion

Protein content within the lacrimal fluids was significantly higher in the younger population relative to the older group. Although there were no significant differences in the type of proteins found between the two groups, there were obvious but small variations from the protein bands from samples of two subjects. These unique bands revealed two different proteins. Work is ongoing to determine the reason for the proteins in the lacrimal fluid. The data that was gathered can be used in future studies in order to determine which proteins were increased in the younger population, and what role they might have in eye health, disease, and age-related changes.

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