Characterization of Ocular Mucus Extracts by Crossed Immunoelectrophoretic Techniques

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Crossed immunoelectrophoresis (CIE) and crossed immunoelectrofocusing (CIEF) were used to characterize proteins and mucosubstance in the saline extract of human ocular mucus pooled from the normal eyes of donors. CIE resolved more components with greater specificity than previous techniques. Up to 25 components were identified. Lactoferrin, protein G, tear prealbumin, and ocular mucosubstance were found to be ocular-specific. CIE also allowed for the study of protein associations of: 1) albumin-α1-antitrypsin; 2) albumin-tear prealbumin; and 3) IgA-secretory component and lactoferrin-mucosubstance. CIEF revealed that most of the proteins were in the pI range of 4.6–7.4. Up to 19 components were identified. Protein associations revealed by CIE were not evident by CIEF. These results provide a basis for future comparative analyses of tear and mucus from normal and diseased eyes, essential for a better understanding of tear and mucus function. Invest Ophthalmol Vis Sci 31:1127–1135, 1990

A clearer understanding of the structure and functions of tear film requires characterization of its macromolecular components. We employed human ocular mucus in addition to tears to characterize tear/ocular mucus components. The mucus, which coats the epithelial surface of the eye, is generally considered to provide physical and enzymatic protection, similar in function to the mucus of other body secretions.1,2 The soluble components of the mucus are believed to contribute to tear film stability.1 Since the mucus forms an aggregate at the outer canthus of the eye, it can be easily collected and pooled for analysis. The collection area is also bathed by tears. In addition to mucus components, the ocular mucus contains tear-specific proteins secreted by the lacrimal gland.3

Our previous one-dimensional immunoelectrophoretic analyses of the saline soluble human ocular mucus from normal eyes have shown component similarities to tears; seventeen serum- and tear/ocular mucus-specific proteins plus ocular mucosubstance were identified.4 The major proteins in the soluble mucus extract included albumin, IgA, IgG, lactoferrin, lysozyme, and ocular mucosubstance; minor proteins included α1-antichymotrypsin, α1-antitrypsin, ceruloplasmin, α1-acid glycoprotein, Zn α2-glycoprotein, haptoglobin, IgE, IgM, α2-macroglobulin, tear prealbumin, transferrin, and serum prealbumin. Due to the overlap and complexity of the lines in the one-dimensional immunoelectrophoretic gel pattern, it was of interest to acquire more detail in the pattern of the components in the saline extract of the ocular mucus by two-dimensional (crossed) immunoelectrophoresis (CIE) and crossed immunoelectrofocusing (CIEF). CIE and CIEF techniques have proven useful in characterizing proteins in many biologic specimens.5,6 The significance of this study lies in 1) employing antisera to saline-extractable components of human ocular mucus for CIE and CIEF to characterize in more detail both tear/ocular mucus components; 2) establishing a basic CIE gel pattern for future comparison of the gel patterns of tear/mucus specimens from donors of various ages and eye diseases; and 3) examining occurrences of microheterogeneity, polymorphism, or complex formation among proteins of normal ocular mucus specimens for a better understanding of the component interactions relevant to tear film and mucus gel formation.

Materials and Methods

Mucus Collection and Preparation of Saline Extract

Collection of human ocular mucus and the procedure for saline extraction of the mucus were essentially as reported previously.4 Briefly, the mucus was collected from the outer canthus of normal eyes of adults 20–35 yr old, and stored at −20°C. No attempt
was made at this point to subdivide the group of donors according to age, sex, or other parameters. A concentrated solution of human ocular mucus, resulting from exhaustive extraction with saline, generally gave a protein concentration (assayed by the method of Bradford) between 0.8–1.4%.4

For the removal of a specific protein from the saline-extractable components of the mucus, the mucus extract was passed through an immunoadsorbent column of glutaraldehyde reacted beforehand with antibody to the specific protein similar to the method of Avrameas and Ternynck.8

Isolation of Ocular Mucosubstance, Mucus Glycoprotein, and Tear Prealbumin

Ocular mucosubstance was a high molecular weight (>105 D) fraction isolated from the saline-extractable human ocular mucus by Sepharose CL-4B (Sigma, St. Louis, MO) chromatography as described previously.9 The mucosubstance was characterized as a crude glycoconjugate containing mucins in complex with lipids, some glycosaminoglycans (GAGs), and proteoglycans (PGs).4,10

Ocular mucus glycoprotein (mucin) was isolated from the brief saline extract of human ocular mucus in this laboratory.11

We also isolated tear prealbumin from the saline soluble mucus.12 In the immunoelectrophoresis of tear and the saline extract of human ocular mucus, a second minor component of tear prealbumin with a β-mobility consistently appeared along with the tear prealbumin with anodic mobility.4,13 We previously designated the tear prealbumin with the anodic mobility as TPA1 and that with β-mobility as TPA2.12

Chemicals

Agarose for electrophoresis was purchased from Bio-Rad (Richmond, CA). Isof gel agarose for isoelectric focusing and Sea Plaque agarose for crossed immunoelectrophoresis were from FMC (Rockland, ME). Sea Plaque agarose was specifically chosen for its low gelling temperature, and relatively higher resolution, sensitivity, and reproducibility among other gels tested. Ampholytes and pI markers were purchased from Bio-Rad and FMC.

Human albumin, IgA, IgG (Fab and Fc), IgM, lactoferrin, and transferrin were purchased from Accurate Chemical (Westbury, NY). α1-acid glycoprotein, α1-antitrypsin, ceruloplasmin, complement C3, 3d, and 4, haptoglobin, IgA, IgE, IgM, lysozyme, α2-macroglobulin, β2-microglobulin, serum prealbumin, and human serum standards were purchased from Calbiochem-Behring (San Diego, CA). All other chemicals were either analytical or reagent grades.

Antisera

Antisera to human albumin, α1-acid glycoprotein, α1-antichymotrypsin, α1-antitrypsin, ceruloplasmin, complement C3, 3d, 4, 5, C-reactive protein, group-specific component (Gc)-globulins, haptoglobin, hemopexin (β1 B globulin), α2-HS-glycoprotein, IgA α-chain, IgD κ-chain, IgE ε-chain, IgG γ-chain, IgM μ-chain, lactoferrin, α- and β-lipoprotein, lysozyme, α2-macroglobulin, β2-microglobulin, secretory component, serum prealbumin, serum retinol-binding protein, transferrin, whole serum protein, and Zn α2-glycoprotein were purchased from Accurate Chemical or Calbiochem-Behring. The validity of each of the antisera was checked using human serum standard or pure antigens against the antiserum in an immunoelectrophoresis run.5

Antisera to saline-extractable components of human ocular mucus, tear (ocular surface) specific proteins, human ocular mucosubstance, and crude TPA1 (TPA1 associated with minor albumin) were prepared using procedures described previously.4

This study was performed in accordance with the ARVO Resolution on the Use of Animals in Research.

Crossed Immunoelectrophoresis (CIE) and Crossed Immunoelectrofocusing (CIEF)

The procedure of CIE was similar to that of Clarke and Freeman, with minor modification.6,14 Twenty microliters of saline-soluble mucus, containing approximately 180 μg protein, was applied to a well 1.5 cm from the cathode edge of a gel strip 12 cm long and 1.5 mm thick composed of 1% (w/v) agarose in barbitol buffer, pH 8.6. The first-dimensional electrophoresis was carried out at 10 V/cm and 15°C. Preliminary CIE studies indicated that lysozyme, the only major basic protein in the mucus extract, produced a downward peak migrating opposite to the direction of the other protein peaks. Therefore, the current CIE studies focused on the greater resolution of acidic and neutral components by placing the sample closer to the cathode (ie, 0.5–1.5 cm). After this separation of proteins, a 2-cm-wide gel strip was cut and placed on a new plate. A 14.4-ml quantity of antibody-containing solution of 1.1% (w/v) Sea Plaque agarose gel in a pH 8.6 barbital buffer was cast onto the same plate adjacent to the gel strip and covering an 8 × 12 cm² area. The second electrophoresis was run perpendicular to the first at 2 V/cm and 12°C for 30 hr.

CIEF initially involved the separation of protein components in the first dimension based on their characteristic isoelectric points in the range of pH 3.8–8.3, similar to the procedure of Vesterberg and
Vesterberg and Holmberg. The ampholine gradient used was: pH 3–10 (40% w/v), 0.8 ml; pH 8–10 (20%), 0.13 ml; pH 4–7 (40%), 0.07 ml; and pH 3–5 (20%), 0.13 ml. One milliliter ampholine solution was added to 17.0 ml gel solution (0.8% Isogel agarose and 10% sorbitol, in deionized water) so as to obtain a final ampholine concentration of 2% (w/v). This solution was cast onto a piece of Gel Bond (FMC), giving a final gel volume of 9 × 12 × 0.15 cm³. Four samples (25 μl containing up to 160 μg protein each) and one pH marker (2 μl containing 200 μg protein) were applied to strips of filter paper (0.5 × 1.0 cm²) placed 1.0 cm apart on the gel. Isoelectric focusing was performed at 12°C for 1.5 hr at a constant power of 10 W. After completion, the gel strip containing the focused pH marker was stained with Coomassie Blue, while the gel strips containing the focused proteins were subjected to electrophoresis in the second dimension against agarose gel containing specific antibody, as described for CIE. In addition, the pH gradient was determined by excising a blank gel strip into 24 0.5 × 1.0-cm² areas across the length of the gel and placing each in 2 ml deaerated H₂O. After shaking for 10 min, the pH of each solution was measured. In both CIE and CIEF studies, the proportion of antisera to the saline-extractable components of human ocular mucus used in the second dimensional electrophoresis was 1.2 ml to 13.2 ml of Sea Plaque gel solution. To develop or enhance a component peak using a monospecific antiserum, the amount of antiserum was calculated based on the relative concentration of that antigen in the total amount of protein in the mucus extract, multiplied by 1.2 ml (volume of mixed antiserum added).

Three methods were used to identify a protein or mucosubstance in the saline extract of the ocular mucus. Method 1 involved first-dimensional electrophoresis of the saline extract of the mucus, and development in the second-dimensional electrophoresis with a monospecific antiserum and anti-albumin. Method 2 involved first-dimensional electrophoresis of a specific antigen and reference antigen albumin, and development in the second dimension with antiserum to the saline-extractable components of human ocular mucus. These two methods generally would produce a gel pattern showing precipitin peaks corresponding to the specific antigen tested and reference albumin. Method 3 involved first-dimensional electrophoresis of the saline extract of the mucus plus a 5–10 μg specific antigen to be tested, and development in the second dimension with the antiserum to the saline-extractable components of the mucus. Subsequent steps were 1) examination of the peak produced by the specific antigen tested, and calculation of the distance between the midwell and the midpeak of the specific antigen relative to that of albumin; and 2) detection of the increase in peak height of the specific antigen tested in the overall gel pattern resulting from the addition of the specific antigen. In CIEF, identification also was achieved through one-dimensional immunoprecipitation with monospecific antiserum after isoelectrofocusing of the specific antigen.

Results

CIE Gel Pattern of the Proteins and Mucosubstance in the Saline Extract of Human Ocular Mucus

Using antisera to saline-extractable components of human ocular mucus (hereafter referred to as anti-HOM), the CIE of the saline-extractable components of the mucus revealed multiple peak and line patterns after staining with Coomassie Blue. Except for two or three component peaks (described below), the relative peak shape (symmetric/asymmetric), size (height/width), and shade did not vary significantly among specimens. An overall gel pattern is shown in Figure 1. Peaks and lines of up to 25 components were identified. Albumin was a predominant peak. Other major peaks (those greater than 1 cm in height) from cathode to anode included TPA2, combined peak of lactoferrin/mucosubstance, combined peak of IgA/secretory component/lactoferrin/mucosubstance, transferrin, hemopexin, haptoglobin, protein G, Gc-globulins, α1-antichymotrypsin, α1-antitrypsin, α1-acid glycoprotein, TPA1, and occasionally, serum albumin. Minor peaks/lines included IgG, IgM, IgE, complement C3, C3d, C4, Zn-α2-glycoprotein, α2-macroglobulin, and ceruloplasmin. Other peak characteristics also were observed. Albumin and TPA1 each appeared as a peak with a left fused shoulder. α1-antitrypsin gave a peak with one or two shoulders on its left. The shape of IgG appeared low and extensive. IgA, secretory component, lactoferrin, and mucosubstance appeared as an unsymmetrical combined peak. Lactoferrin and mucosubstance also appeared as another unsymmetrical combined peak. The peak shape or size of TPA2, lactoferrin–mucosubstance, and Gc-globulins often varied notably with specimens, and their peak height ranged from 1–6 cm.

All of the component peaks were identified by the three general methods of identification (see the section concerning CIE, under Materials and Methods), except for protein G the identification of which is described in the following section. Components tested but not identified included complement C5, C-reactive protein, α2-HS-glycoprotein, IgD, and β-lipoprotein, β₂-microglobulin, and serum retinol binding protein. Components newly identified by CIE included complement C1q, C3d, C4, Gc-globu-
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Fig. 1. CIE gel pattern of the saline-extractable components of human ocular mucus. Major component peaks (>1 cm): 1) TPA; 2) combined peak of lactoferrin and mucosubstance; 3) combined peak of IgA, secretory component, lactoferrin, and mucosubstance; 4) transferrin; 5) hemopexin; 6) haptoglobin; 7) protein G; 8) Gc-globulins; 9) α1-antichymotrypsin; 10) α1-antitrypsin; 11) α1-acid glycoprotein; 12) albumin; 13) TPA; and 14) serum prealbumin. Minor peaks/lines: 15) IgG; 16) IgM; 17) IgE; 18) C3d; 19) C3a; 20) C4; 21) Zn α2-glycoprotein; and 22) α2-macroglobulin; and 23) ceruloplasmin.

Fig. 1. CIE gel pattern of the saline-extractable components of human ocular mucus. Major component peaks (>1 cm): 1) TPA; 2) combined peak of lactoferrin and mucosubstance; 3) combined peak of IgA, secretory component, lactoferrin, and mucosubstance; 4) transferrin; 5) hemopexin; 6) haptoglobin; 7) protein G; 8) Gc-globulins; 9) α1-antichymotrypsin; 10) α1-antitrypsin; 11) α1-acid glycoprotein; 12) albumin; 13) TPA; and 14) serum prealbumin. Minor peaks/lines: 15) IgG; 16) IgM; 17) IgE; 18) C3d; 19) C3a; 20) C4; 21) Zn α2-glycoprotein; and 22) α2-macroglobulin; and 23) ceruloplasmin.

Gc-globulins and hemopexin are two known serum proteins,\(^{17}\) whereas protein G is a tear-specific protein found and designated by Gachon et al from the gel pattern of human tears using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).\(^ {18}\) Since the protein G was never chemically isolated, a special procedure for the peak identification was used, as follows. A saline extract of human ocular mucus containing about 180 ng proteins was subjected to SDS-12.5% PAGE with and without reducing agent, similar to the procedure of Gachon et al.\(^ {18}\) The 31-kD band was seen in the PAGE gel of both reduced and nonreduced extract, but was more pronounced in the reduced mucus extract. A gel piece of reduced sample corresponding to a molecular weight of 31-kD of protein G was cut out, placed in a 1% barbital buffered agarose gel, and subjected to CIE against anti-HOM. A parallel study was made with human tears. The protein G in the PAGE gel of tears and that in the mucus was proven identical by double immunodiffusion against anti-HOM. A parallel study was made with human tears. The protein G in the PAGE gel of tears and that in the mucus was proven identical by double immunodiffusion against anti-HOM. These gel patterns are shown in Figure 2. The CIE gel pattern of the 31-kD PAGE gel strip revealed a major protein G peak with three minor peaks, one on its left and two on its right, all exhibiting partial reactivity against anti-TPA. In both tear and soluble mucus samples, protein G gave a distinctly wide peak located near the center of the overall CIE gel pattern.

CIE Studies of Microheterogeneity, Polymorphism, and Associations of Components in the Saline Extract of Human Ocular Mucus

Components which displayed combined/shouldered peaks in the CIE gel patterns of the saline extract of human ocular mucus were examined further, for the occurrence of microheterogenity, polymorphism, and component association. The shouldered peaks of α1-antitrypsin are believed to be due to the microheterogeneity or polymorphism of the proteins, since similarly shouldered peaks were found in the CIE gel patterns of pure α1-antitrypsin produced with either anti-α1-antitrypsin or anti-HOM. The combined/shouldered peaks as observed for the other components—albumin, IgA, secretory component, lactoferrin, mucosubstance, and TPA—were found to arise from association with other protein(s) based on the following observations.

Albumin-α1-antitrypsin: The CIE pattern of the saline-extractable components of human ocular mucus, produced with anti-albumin, displayed a major albumin peak having a small fused shoulder on its left side (Fig. 3A). However, the shoulder of the albumin peak in the overall gel pattern (Fig. 1) was not clearly demonstrated. This shoulder peak was found to result from some association of albumin...
with $\alpha_1$-antitrypsin, since this shoulder disappeared upon removal of $\alpha_1$-antitrypsin (but not any other antigens) from the saline soluble mucus by immunoadsorbance (see Materials and Methods).

**TPA-albumin:** The CIE pattern of the saline-extractable ocular mucus, produced with antiserum to albumin associated TPA$_1$, displayed a major peak having a shoulder (and occasionally, another hump) on its left side (Figs. 1, 3B). The TPA$_1$ peak in the overall gel pattern of the soluble mucus also appeared with a hump and a shoulder (Fig. 1). Upon removal of albumin (but not other antigens) from the saline soluble mucus by immunoadsorbance, both the hump and shoulder of the TPA$_1$ peak became less evident.

It should be pointed out that the albumin-$\alpha_1$-antitrypsin association was revealed only in the albumin peak but not in the albumin peak (Fig. 1). Here, the albumin peak shape was not changed upon the removal of the TPA$_1$ from the soluble mucus.
IgA-secretory component: The CIE peaks of IgA and secretory component (SC), of the saline extract of the mucus produced by either anti-HOM, anti-IgA, or anti-SC, appeared in almost all CIE analyses as a single unsymmetrical peak identical in position to the combined peak 1 of lactoferrin/mucosubstance (Figs. 1, 4A). The IgA-SC association appeared strong since neither protein could be separated from the other by immunoadsorbance.

Lactoferrin-mucosubstance and IgA-SC-lactoferrin-mucosubstance: Two types of combined peaks of lactoferrin and mucosubstance were found in the overall CIE gel pattern of the saline extract of the ocular mucus, produced by anti-HOM, anti-lactoferrin, or anti-ocular mucosubstance (Figs. 1, 4A, 4B). Similar immunoadsorbance techniques revealed that the combined peak 1 appeared to be a peak of lactoferrin associated with IgA/SC and mucosubstance, and peak 2 appeared to be a peak of mucosubstance associated with lactoferrin. In normal mucus specimens, the size and shape of peak 2 varied. However, preliminary CIE studies on a few specimens from donors of >55 yr of age and from donors with seasonal allergy also consistently revealed a more predominant combined peak 2 in the CIE gel pattern (Fig. 4C).

It was noted also that all of the above peak associations in CIE were most evident at protein levels of >0.7%, but could be visualized at as low as 0.3% concentration of the saline extract of the ocular mucus.

CIE Pattern of Ocular-Specific Protein Components in the Saline Extract of Human Ocular Mucus

Using antisera to ocular-specific proteins, the CIE pattern of the saline-extractable components of human ocular mucus revealed four to five peaks (Fig. 5). These were identified as lactoferrin/ocular mucosubstance (one or two peaks), TPA₂, protein G, and TPA₁. The peak shape of lactoferrin/mucosubstance differed from that in the gel pattern produced with anti-HOM (Fig. 1), and varied with the specimen analyzed. A very faint peak of IgA but not of SC was found in the peak of lactoferrin/mucosubstance. The size and shape of the peak of TPA₂ also varied with specimens analyzed. The peak shape of TPA₁ was more symmetrical, with little or no appearance of a shoulder due to its albumin association. Although lysozyme is also an ocular specific protein, it does not lie in the CIE pattern under the current investigation.

CIEF Pattern of the Proteins and Mucosubstance in the Saline Extract of Human Ocular Mucus

Application of CIEF in the analysis of the saline-extractable components of human ocular mucus resulted in the following gel pattern (Fig. 6). Figure 6A represents the separation of the components after isoelectrofocusing in the first dimension. Figure 6B shows the gel strip in Figure 6A after electrophoresis in the second dimension against anti-HOM. The bands and peaks were concentrated in the pI range of 4.6–7.4, indicating that the majority of the components were in the acidic to neutral range. The pI values of the components, except those with major peaks (ie, albumin, TPA₁, and α₁-antitrypsin), were best determined by immunoprecipitation against monospecific antibodies after one-dimensional isoelectric focusing. Many component pI values were found to be located within a wide range. Ten peaks were identified: 1) albumin (pI, 4.85); 2) TPA₁ (4.80); 3) α₁-antitrypsin (4.5–4.7); 4) transferrin (6.1); 5) haptoglobin (5.1–5.8); 6) ocular mucin (5.0–6.4); 7)
Fig. 5. Crossed immunoelectrophoretic gel patterns of ocular specific mucus proteins produced with antisera to ocular specific mucus proteins. Peaks: 1) TPA\(_1\); 2) lactoferrin/mucosubstance; 3) protein G; and 4) TPA\(_4\).

\(\alpha_1\)-antichymotrypsin (5.4); 8) protein G (4.8–5.2); 9) lactoferrin (4.8–7.4); and 10) Gc-globulins (4.5). The following two peaks received tentative identification: 11) lysozyme residue (11.2) and 12) fraction of ocular mucosubstance (5.9–6.2). Other line and small overlapped peak components which could not be clearly identified on the two-dimensional gel pattern were: ceruloplasmin (pI, 4.4–4.6), IgA (4.9–5.8), IgE (4.7–6.7), IgG (5.5–7.3), IgM (4.7–6.1), \(\alpha_2\)-macroglobulin (5.3), and serum prealbumin (4.7). The TPA\(_1\) peak appeared clearly nonshouldered. Other combined/shouldered peaks shown in the CIE gel pattern were neither evident nor identified. Proteins not found in the range under study were \(\alpha_1\)-acid glycoprotein (2.7), hemopexin (3.1), and Zn \(\alpha_2\)-glycoprotein (3.8).

Discussion

Although CIE and CIEF were proven useful for the analysis of other body secretions,\(^5,6\) this is the first time these techniques applied in characterizing proteins and mucosubstance in the saline extract of human ocular mucus. In CIE, the size and shape of a precipitin peak produced in the second dimension depends on antigen and antibody concentration, mobility of each antigen-antibody complex, and the polymorphism, heterogeneity, and complex formation of an antigen.\(^5\) CIE allowed for component resolution with greater specificity than the one-dimen-
sional immunoelectrophoresis reported previously.\(^4\) Twenty-five components, 6 more than were previously found, were identified. Of the newly identified proteins, complement, hemopexin, and Gc-globulins are serum proteins,\(^19\) and protein G is tear-specific.\(^18\)

Fig. 6. Gel patterns of isoelectrofocusing (A) and CIEF (B) of the saline-extractable components of human ocular mucus. Ten peaks were identified (B): 1) albumin; 2) tear prealbumin; 3) \(\alpha_1\)-antitrypsin; 4) transferrin; 5) haptoglobin; 6) hemopexin; 7) \(\alpha_1\)-anti-chymotrypsin; 8) protein G; 9) lactoferrin; and 10) Gc-globulins. Two peaks were given tentative identification: 11) lysozyme residue, and 12) fraction of ocular mucosubstance.
Trace amounts of complement also were found in tears, but it is not clear whether its role in tears is similar to that in humoral immunity. In serum, hemopexin functions in heme binding. Gc-globulins are synthesized in the liver, subject to genetic modulation, and their function in serum is not known. In ocular secretion, these proteins are present possibly due to vascular leakage; their specific functions require further investigation. The current CIE study revealed a characteristically wide peak of a tear/ocular mucus-specific protein identical to the 31-kD range protein G shown by SDS-PAGE of nonreduced tear samples. In CIEF, the protein G peak was notably decreased in size, indicating dissociation of some protein G subunits by isoelectricfocusing. Such dissociation also was found in the protein G of reduced tears by SDS-PAGE. Isolation and characterization of protein G will be necessary to define its function.

Polymorphism or microheterogeneity were characteristic of several proteins in the ocular mucus extract. These were found in α1-antitrypsin by CIE, and in ceruloplasmin, protein G, IgA, IgE, IgM, lactoferrin, and ocular mucosubstance by isoelectricfocusing. The results correlated well with those reported previously. CIE also revealed protein associations of: 1) albumin-α1-antitrypsin; 2) albumin-TPA1; 3) lactoferrin-ocular mucosubstance; 4) IgA-secretory component; and 5) IgA/secretory component-lactoferrin/mucosubstance. The association of minor amounts of α1-antitrypsin to albumin was never found in other mucus secretions. Albumin migrates slightly faster than α1-antitrypsin electrophoretically, and these proteins also have similar pls (4.80 and 4.5–4.7, respectively). Co-migration could be one factor contributing to the shouldered peak of α1-antitrypsin.

Albumin–TPA1 association was revealed previously in our isolation study of TPA1 from human ocular mucus, where 1–3% of albumin was tightly associated with TPA1. IgA/secretory component was reported to be associated with mucin glycoproteins (mucins) in mucus secretions of the ocular surface and other body organs. Lactoferrin was shown capable of forming complexes with mucins and IgA. IgA, lactoferrin, and albumin also may participate in interglycroprotein linkages of the mucus. In the saline extract of the mucus, lactoferrin and mucosubstance appeared to bind in a manner in which either a minor portion of mucosubstance becomes associated to lactoferrin or a minor portion of lactoferrin associated to mucosubstance. IgA and secretory component seemed to be structurally linked to each other and also to lactoferrin/mucosubstance. Only in rare cases, a small IgA/secretory component peak in addition to the combined peak was revealed in the CIE gel pattern. The result was contrary to that in human tears, in which a IgA/secretory component peak was always distinctively resolved. This finding could be due to the higher level of mucosubstance present in the saline soluble human ocular mucus (12%) than in tears (1–4%). Other studies have shown an increase in viscosity of a mucin solution by albumin, indicating some structural interaction between albumin and mucin. All the component associations/interactions can be summarized as follows:

\[
\text{prealbumin—albumin—α1-antitrypsin}
\]

\[
\text{IgA/SC—ocular mucosubstance—lactoferrin}
\]

(lipids/ocular mucin/GAGs)

Albumin, IgA, lactoferrin, and mucosubstance are the major constituents in mucus. IgA plays a role in blocking the attachment of microorganisms to mucus cells. Lactoferrin suppresses the growth of iron-dependent bacteria, and also has been characterized as a biosurfactant. The moieties of ocular mucosubstance, lipids, mucin, GAGs, and PGs have been recognized to have protecting and lubricating functions. TPA1 is a newly isolated ocular surface-specific protein, and is predominant in tears. Aside from being a bactericide and a retinol carrier, its functions have not been well defined. It is possible that the albumin-α1-antitrypsin and albumin–TPA1 associations may also have functional implications in ocular mucus secretion. All of the component associations are noncovalent, arising instead from hydrophilic/hydrophobic interactions. These associations may contribute to the gel formation and protective properties of the ocular mucus.

The CIEF pattern reveals that the major components are concentrated in the pI range 4.6–7.4. This may explain the near neutral pH of the mucus extract. As a consequence, individual component peaks (except albumin, TPA1, and α1-antitrypsin) were not well resolved. The shoulder peaks of albumin and TPA1 were not evident, indicating that the protein associations found in CIE patterns are noncovalent. Thus, isoelectric focusing may be used to purify these associated proteins. The isoelectric point is a physicochemical characteristic of a protein. The pI values of protein components in the mucus were similar to those reported previously. CIE is a combination of the techniques of one-dimensional electrophoresis and rocket immunoelectrophoresis which can be used both qualitatively and quantitatively. Further analysis of mucus specimens from normal and diseased eyes will yield spe-
specific information of the component composition and associations as they relate to the functions of the ocular mucus.

Previous one-dimensional immunoelectrophoretic studies demonstrated component similarities between human tears and the saline extract of ocular mucus. Studies of tears have been limited due to unstandardized methods of collection and tear sample quality evaluation. Ocular mucus, with its relative ease of collection, generally comprises all of the same components as tears, including minor components. Therefore, antisera to the saline extract of human ocular mucus is a more practical alternative for CIE analysis of tear specimens. The current work sets a basis for the future CIE study of human tear composition and component association, which in turn may lead to a better understanding of the structure and formation of tear film.

Key words: crossed immunoelectrophoresis/immunoelectrofocusing, human ocular mucus tear, proteins, mucosubstance, protein association

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References