Immunological Study of Proteins and Mucosubstance in Saline Soluble Human Ocular Mucus

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Proteins and mucosubstance of the saline extract of human ocular mucus were studied by immunological analysis. A minor study was made with human tears for comparison. Immunoelectrophoresis of proteins from these two sources consistently revealed similar characteristic gel patterns. Proteins were found as the major constituents of both samples. However, more mucosubstance was present in the saline extract of human ocular mucus than in tears. Seventeen proteins were identified in the mucus extract. Albumin, IgA, and lactoferrin appeared to be the three major proteins, while lysozyme, lactoferrin, tear prealbumin, and ocular mucosubstate were tear and ocular mucus specific. Although saline soluble mucosubstate is complex in structure, it seemed to resist electrical dissociation, producing only one major precipitation line along with a line of IgA during immunoelectrophoresis. The ocular mucosubstate accounted for about 12% of the saline extractable proteins of human ocular mucus. Invest Ophthalmol Vis Sci 28:546-554, 1987

An essential step toward obtaining a clearer understanding into the structure and function of the tear film and ocular mucus requires analyses of their macromolecular components. The mucus gel, which coats the epithelial surface of the eye, is generally considered to provide protection against physical and enzymatic injury, similar in function to the mucus of other body organs. The major constituents of mucus include glycoproteins of mucus, plasma and non-plasma type proteins, lipids, and perhaps glycosaminoglycans. In particular, the mucus glycoproteins (mucins) greatly contribute to the physicochemical properties of the mucus. Up to 15 proteins in the mucus secretions of other body organs have been detected, of which albumin, secretory IgA, IgG, lactoferrin, and lysozyme were shown to predominate, also playing essential roles in the protective function of mucus.

In our preliminary studies on the fractionation and characterization of human ocular mucus, we chemically isolated four major macromolecular fractions. The highest molecular weight fraction of greater than 10^5 daltons was likely to consist of mucosubstate containing mucins in complex with lipids, some glycosaminoglycans, and proteoglycans. The remaining fractions, which constituted about 67% of the total macromolecular substances, were comprised mostly of proteins. It was expected that ocular mucus collected from the outer canthus would contain tear components, since this area is also bathed by tears. Twelve of these identified proteins were found to be common to tears.

The major proteins of tears are believed to originate from the secretory activity of the lacrimal gland and from leakage of serum proteins from the circulation. The lacrimal gland has been reported to secrete more than 20 protein components, of which have been identified by various immunological techniques. Tear-specific proteins, which are products of local synthesis, are reported to include lactoferrin, lysozyme, and tear prealbumin. The tears also contain mucinous secretions which may represent a dissolved form of mucus originating from the conjunctival goblet cells. The soluble mucins, together with lipids and perhaps glycosaminoglycans, contribute to the formation of viscous tear fluid and serve as lubricants and surfactants, thereby stabilizing the tear film.

This previous knowledge concerning the ocular mucus and its relationship to tears has led to this study, conducted with the purpose of providing additional insight into the following areas. First, very few studies have centered on the analysis of human ocular mucus. Comparisons of the macromolecular composition of ocular mucus to the mucus of other body organs may lead to a greater understanding of the true functions of ocular mucus. Second, the saline extract of the ocular

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546

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mucus may resemble a concentrate of tears, and therefore could provide an alternate source of tear components for analysis. This article reports studies of the proteins and mucosubstance in the unfractionated saline extract of human ocular mucus by immunological analysis. It also attempts to correlate components in tears with those found in ocular mucus. Many studies have employed immunological techniques in the analysis of human serum and mucus secretions.15,16

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.6 After informed consent had been obtained, the mucus was collected from the outer canthus of normal eyes of adult lab personnel 20–45 years 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 pooled sample of mucus, about 100 mg in wet weight, was stirred with about 5 volumes of 0.154 M NaCl containing 0.02% NaN3 for 2 hr at 4°C, centrifuged at 12,000 × g, and the supernatant collected. The insoluble mucus was further extracted repeatedly with saline up to 8 times until no serum proteins in the solution could be detected by immunodiffusion. All extracts were then combined, dialyzed, and lyophilized. The lyophilized material was redissolved into the first extract to produce a solution that contained all of the saline extractable components of human ocular mucus. This solution, which generally gave a protein concentration (assayed by the method of Bradford)17 between 0.8–1.4%, was considered a concentrated saline extract of human ocular mucus.

Tear Collection

Tears were also collected from the normal eyes of the same mucus donors by a microcapillary pipette from the medial canthus, similar to the technique used by Fullar and DeLucas.18 At a collection rate of about 1 µl per min, about 20–50 µl of tear specimens were collected each time from both eyes. The tear samples were pooled and centrifuged at 12,000 g, and the supernatant stored at −20°C. The protein concentrations of the tears were in the range of 0.7–1.0%.

Ocular Mucoisolate

The ocular mucoisolate was a high molecular weight (> 105 daltons) fraction isolated from the saline extractable human ocular mucus by Sepharose CL 4B (Sigma, St. Louis, MO) chromatography, equivalent to the fraction previously designated as S1.6

Tear Prealbumin

The prealbumin used in this study was isolated from the saline extract of the human ocular mucus according to the procedure of prealbumin isolation from tears by Selsted and Martinez.19

Chemicals

Agarose HSA used for immunodiffusion was purchased from Accurate Chemical Co. (Westbury, NY). Agarose M for immunoelectrophoresis was obtained from LKB (Gaithersburg, MD). Human albumin, α1-antitrypsin, ceruloplasmin, α1-acid glycoprotein, haptoglobin, IgA, IgE, IgG, IgM μ chain, lactoferrin, lysozyme, α2-macroglobulin, serum prealbumin, transferrin, and human serum standard were from Calbiochem-Behring Corp. (San Diego, CA). Human albumin, α-globulins, γ-globulins, IgA, IgG, transferrin, and human serum were also purchased from Miles Labs (Naperville, IL), human α1-acid glycoprotein was from Accurate Chemical Co., and lactoferrin and lysozyme were from Cappel Labs (West Chester, PA). All other chemicals were either of reagent or analytical grades.

Antisera

Antisera to human proteins: Antisera to human albumin, α1-antichymotrypsin, α1-antitrypsin, ceruloplasmin, haptoglobin, IgA α chain, IgE ε chain, IgG γ chain, IgM μ chain, lactoferrin, lysozyme, α2-macroglobulin, orosomucoid (α1-acid glycoprotein), serum prealbumin, secretory component of IgA, whole serum protein, and transferrin were purchased separately from two different sources, Accurate Chemical & Scientific Co. and/or Calbiochem-Behring. Anti-Zn α2-glycoprotein was a gift from Dr. J. V. Sieber, Behringwerke (West Germany) and also was purchased from Calbiochem. The validity of each of the antisera was checked using human serum standards and/or pure antigens against the antiserum in an immunoelectrophoresis.

Antisera to human tears or to saline extractable components of human ocular mucus: The antisera were prepared from New Zealand rabbits by intramuscular injections initially of 0.5 ml of human pooled tears or a concentrated saline extract of human ocular mucus emulsified with 1.5 ml of complete Freund’s adjuvant and 1.0 ml of phosphate buffered saline (PBS), pH 7.41. After 30 and 45 days, additional boosters of 0.5 ml of tears or the saline extract of the mucus, mixed with incomplete Freund’s adjuvant, were subcutaneously injected. The antisera were isolated at 60 days, and weekly thereafter. During the immunization period, the antibody titers were monitored by double immunodiffusion.

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anti-human ocular mucoisolate: The antiserum was prepared from rabbits by an initial immunization with 1 mg of the mucoisolate in 1.5 ml PBS emulsified with 1.5 ml of Freund’s adjuvant. Additional boosters, each containing 1 mg of the isolate, were given after 30 and 45 days with incomplete Freund’s adjuvant. The antiserum was isolated at 52 days and weekly thereafter.

Anti-human tear prealbumin: The immunization procedure was the same as that outlined above for anti-human ocular mucoisolate, with the exception that 500 µg of tear prealbumin was administered in each injection.

Anti-human tear specific proteins: The antisera were prepared by passing antisera to saline extractable components of human ocular mucus through a column of immunoadsorbent which previously had been bound with human serum proteins according to the procedure of Avrameas and Ternynek.20 The completeness of adsorption of the anti-serum proteins from the antiserum to the saline extractable components of human ocular mucus was checked using both immunoelectrophoresis and immunodiffusion. Neither technique showed positive results as to the presence of any anti-serum proteins, thus indicating an antiserum specific to human tear proteins.

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

Immunodiffusion and Single Radial Immunodiffusion (SRID)

Gel double immunodiffusion was performed on diffusion plates (95 X 45 mm) covered with 2 mm 1% agarose HSA in PBS, pH 7.41. Proteins and mucoisolates in tears and in the saline extract of human ocular mucus were identified by filling three basins (1, 2, and 3) with the respective samples in either of two arrangements: (1) saline extract of human ocular mucus, monospecific antiserum, and human serum or pure antigen; or (2) antigen, monospecific antiserum, and antiserum to saline extractable components of human ocular mucus. Suitable concentrations of antigens and the samples to be tested were estimated by applying a serial dilution of a specific antigen with a constant amount of antiserum.

SRID for the quantitation of proteins in the saline extract of human ocular mucus was essentially the method of Mancini.21 Quantitation was based on the radius of diffusion of the sample in reference to standards of known concentration of antigen. Pure antigens and/or human serum standard were used as standards and were run at least five times per plate in serial dilution. A standard curve was plotted for each plate. A magnifying viewer (Kallestad, Austin, TX) was used to measure precipitin ring diameters in 0.1 mm increments. The quantitation limit was 1 µg/ml. Quantification of individual components was calculated as a percentage of the protein vs total protein content, and data was expressed as mean ± SD. Due to the unavailability of the pure antigens, α1-antichymotrypsin and Zn α2-glycoprotein were quantified using human serum standard, assuming mean normal adult serum concentrations of 48.7 mg per dl and 4.8 mg per dl, respectively.22

Agarose Gel Electrophoresis and Immunoelectrophoresis

Electrophoresis was performed on a 12 X 14 cm glass plate covered with 1.2 mm of 1% barbital buffered (pH 8.6) agarose M at 10 v/cm and 15°C, using a standard technique as described by Ouchterlony and Nilsson.22 A drop of bromophenol (0.01%) was used as an indicator for the completion of an electrophoresis. Albumin was used as a reference protein. Generally 120 µg of sample protein was applied. To locate and immunologically identify a protein or mucoisolate in the saline extract of human ocular mucus in an immunoelectrophoresis, the general scheme of sample application was followed as shown in Figure 1. The relative position of a line formed by a monospecific antiserum was used for its recognition in the multi-line pattern.

Results

Immunoelectrophoretic Analyses of Proteins and Ocular Mucoisolate in the Saline Extract of Human Ocular Mucus as Compared to Those in Human Tears

All immunoelectrophoreses of the saline-extractable components of human ocular mucus and tears, when developed with either anti-human tears or antisera to saline-extractable components of human ocular mucus (hereafter referred to as anti-HOM), revealed multiple line patterns after having been stained with Coomassie...
Blue (Bio-Rad, Richmond, CA) for protein identification. The patterns from different donor samples were more or less alike. Typical patterns are represented in Figs. 2A and 2B. There was an overlapping and/or partial overlapping of the precipitation lines. When developed against either antisera and compared, the saline-extractable mucus and tears produced line patterns which were found to be similar. Variations were observed in the staining intensities of several corresponding lines on the patterns of tears and the saline extract of the mucus, and may possibly have been due to inconsistencies in the concentration of some protein components of these two sample solutions.

Immunoelectrophoretic gels of the two samples were also stained with PAS for carbohydrate detection. Each revealed a single, long precipitation line extending from the point of sample application to the anode side (Fig. 2C), indicating the direction of mobility of mucosubstance in an electric field at alkaline pH. However, the PAS-positive line produced from tears was less intense and shorter than that occurring from the saline-extractable mucus.

The mucosubstance in tears and in the saline-extractable mucus were further investigated using ocular mucoisolate and antiserum to the mucoisolate. Although the available mucoisolate is not a purified product, examining its behavior in immunoelectrophoresis could provide insight for future studies. The immunoelectrophoretic patterns of the mucoisolate, developed with either anti-mucoisolate or anti-HOM, when stained with Coomassie Blue, consistently produced two long precipitation lines, one positioned on the anode side, and the other beginning on the cathode side (Fig. 3Aa). These same two lines also appeared in similar positions in the immunoelectrophoretic pattern of the saline-extractable mucus when developed against anti-mucoisolate (Fig. 3Ad). Tears also produced two lines when developed against anti-mucisolate, although its anodal line was not positionally identical to that occurring from the mucus (Fig. 3 Ae). In each case, only the line positioned on the anode side was proven to be derived from mucoisolate, since staining with PAS produced a positive line on the anode but none on the cathode (Fig. 3B). It should be noted that in the figures of gels stained by PAS, staining intensities of the soluble mucus developed with anti-tears, anti-HOM, or anti-mucoisolate were two to three times stronger than those of tears. Because the samples consisted of similar amounts of protein, these results may indicate that the mucoisolate is present in greater amounts in mucus than in tears.

It was proposed that the protein line shown on the cathode side may be a precipitation line of IgA, as mucosubstances or mucins have been reported to be bound with IgA.24 To study this possibility, human serum, after an electrophoresis, was allowed to react with anti-human IgA on one side and anti-mucoisolate on the other (Fig. 3Ag and 3Ah). In addition, samples of mucosubstance, saline extract of the mucus, and tears were electrophoretically separated and then individually reacted against anti-IgA (Fig. 3Ab, 3Ac, and 3Af). Results indicated that it was indeed IgA which contributed to the precipitation line on the cathode, confirming the binding of IgA with ocular mucoisolate.

We further tested the immunological identity between mucoisolate in tears and that in saline extractable mucus using double immunodiffusion (Fig. 4). The mucus extract, tears, mucoisolate, and IgA were each allowed to react against anti-mucoisolate. Protein staining showed that the mucus extract, mucoisolate, and tears all produced one distinct precipitate line (outer), and another fainter line (inner), and IgA only one distinct line. One inner precipitation line produced by both mucus and tears fused together with that of mucoisolate, which seemed to indicate identical mucoisolate in tears and in the mucus, though differing from the immunoelectrophoretic pattern of mucoisolate in the mucus and tears as described above. The second precipitation line (outer) shown for the samples of the mucus, mucoisolate, and tears fused together in ring fashion with that of IgA, thus identifying this second component as IgA. The fact that the tear protein concentration required to produce a clear Ouchterlony immunodiffusion was 2.5 times greater than that of the mucus extract also indicates that the mucoisolate component of tears is present in a lesser amount in tears than in the mucus.
Immunological Identification of Tear-Specific Prealbumin and Haptoglobin in the Saline Extract of Human Ocular Mucus

The identification of tear-specific prealbumin was achieved through immunoelectrophoretic analysis of tear prealbumin, isolated from the saline extract of human ocular mucus according to the method of Selsted and Martinez. The isolated prealbumin from the mucus was tested against antiserum to the mucus prealbumin (Fig. 5A). Two precipitation lines were observed: one extending to the far end of the anode side (showing a mobility similar to serum prealbumin) and the other beginning on the cathodal side (showing β-mobility). When human tears were also tested against the antiserum to the human prealbumin, the same set

positionally identical to tear prealbumin at the far anode. However, because the precipitation line obtained was always faint, its identification at this time is inconclusive. With the exception of haptoglobin, all proteins found in the saline-extractable human ocular mucus have also been variously reported in tears.

Immunological Identification of Proteins in the Saline Extract of Human Ocular Mucus

By immunoelectrophoresis and/or Ouchterlony’s immunodiffusion, 17 proteins were identified in the saline extract of human ocular mucus. Albumin, α1-antichymotrypsin, α1-antitrypsin, ceruloplasmin, α1-acid glycoprotein, Zn α2-glycoprotein, haptoglobin, IgA α chain, IgE ε chain, IgG γ chain, IgM μ chain, lactoferrin, lysozyme, secretory component, and transferrin were identified by both techniques. α2-Macroglobulin was identified by immunoelectrophoresis, but not by immunodiffusion. The identification of tear-specific prealbumin is described in the following section. It should also be mentioned that a trace amount of serum prealbumin was occasionally found in the saline extract of the mucus by immunoelectrophoresis.
of two precipitation lines, positionally identical, were produced. These two lines as observed were also positionally similar to the two precipitation lines referred to by Gachon as PMFAs (proteins migrating faster than albumin) in their immunoelectrophoretic analysis of tears.13

The immunoelectrophoretic identification of the precipitation line of haptoglobin is shown in Figure 5B. Samples of the saline extract of the mucus, when tested against anti-haptoglobin and anti-HOM, revealed in each instance a precipitation line positionally and serologically identical to that produced when human serum or haptoglobin was developed with anti-haptoglobin (Fig. 5Bb-g). However, no precipitation line of haptoglobin was observed when tears were tested against anti-haptoglobin (Fig. 5Ba), indicating that haptoglobin was not identified in human tears.

Quantitation of the Saline Extractable Components of Human Ocular Mucus by SRID

The identified pure protein components as well as mucosiolate were further quantified by SRID. The major components, greater than 5% of the total amount of protein in the saline extractable mucus based on analyses of 15–25 mucus samples, are as follows: albumin was present in the greatest amount, at 30.7 ± 4.1% (coefficient of variation, CV, 13.4%); IgA, 11.2 ± 3.0% (CV, 26.8%); IgG, 6.1 ± 2.5% (CV, 41.0%); lactoferrin, 10.1 ± 2.2% (CV, 21.8%); lysozyme, 6.2 ± 2.7% (CV, 43.0%); and ocular mucosiolate, 12.3 ± 2.1% (CV, 17.0%). Proteins present in the range of about 2–5% (CV, 22–61%) were after sample analyses were α1-antitrypsin, α2-macroglobulin, and transferrin. Other trace proteins present in the range of 0.01–0.05% (CV, 20±75%) calculated from 6–15 analyses were α1-antichymotrypsin, ceruloplasmin, α1-acid glycoprotein, haptoglobin, IgE, IgM, and Zn-α2-glycoprotein, collectively accounting for about 1–3% of the total saline soluble proteins of the soluble mucus.

Correlation of Patterns to Electrophoretic Methods For the Analyses of the Saline Extract of Human Ocular Mucus

The resolution and identification of the gel patterns of the saline extractable components of human ocular mucus by agarose gel and immunoelectrophoreses are illustrated schematically in Figure 6, which is a common method of presentation in the studies of proteins of serum and other secretions.15,16 Electrophoretic mobilities of the mucus components, stained with Coomassie Blue for proteins, are shown in Figure 6B. Quantitative estimations of protein bands were based on their staining intensities measured at 565 nm (in the electrophoretic diagram A), and showed that the protein distributions, using conventional mobility classifications of the plasma proteins, were approximately: albumin, 24%; albumin-α1 globulin, 14%; α1-α2 globulin, 4%; α2β globulin, 12%; β-γ globulin, 28%; and lysozyme, 7%.

The immunoelectrophoretic analysis of the saline extractable mucus, after development with anti-HOM, consistently produced 19 lines. The overall gel pattern did not illustrate well all of the component lines, especially for the trace proteins. For a clearer presentation of the identified components, details of lines were

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Fig. 5. Immunoelectrophoretic identification of (A) tear prealbumin and (B) haptoglobin. (A) Well 1 was filled with prealbumin isolated from mucus, well 2 with human tears, and the trough with anti-prealbumin. (B) Well 1 was filled with human tears, well 2 with human serum, well 3 with saline extract of the mucus, and well 4 with haptoglobin. T1 and T3 were filled with anti-haptoglobin, and T2 and T4 with antiserum to the saline extractable mucus.

Fig. 6. Schematic representation of electrophoretic pattern of the saline extract of human ocular mucus in pH 8.6 buffer. (A) Quantitative estimation of protein band in simple agarose gel electrophoresis. (B) Gel strip stained with Coomassie Blue. (C) Scheme of the immunoelectrophoretic analysis.
Resolution and Identification of Ocular Mucus/Tear-Specific Proteins

When the anti-serum proteins were removed from anti-HOM using an immunoadsorbent gel (see Methods), the immunoelectrophoretic pattern showed only six lines, representative of the ocular mucus specific proteins (Fig. 7). The lines were identified as (1) tear-specific prealbumin (appearing as two lines), (2) human ocular mucosololate (appearing as a long line of mucosololate along with a line of IgA), (3) lactoferrin, and (4) lysozyme. Although the line of IgA also appears in the gel pattern, it was considered to be partially bound to the mucosololate complex.
α2-glycoprotein, have been studied and their functions discussed. In serum, haptoglobin is known as one of the triumvirate of plasma proteins involved in the transport of hemoglobin complex, and under certain conditions, also exhibits peroxidase activity. Due to the trace levels of haptoglobin in the ocular mucus, and the improbability that it performs these identical functions in the mucus, its role cannot be defined at this time. We would like to point out that albumin, IgA, IgG, lysozyme, and lactoferrin, which are major components of ocular mucus, are also predominant components in the mucus of other body organs. Albumin is reported to raise the viscosity of mucin in solution. It is generally believed that the two immunoglobulins, particularly IgA, play a role in blocking the attachment of microorganisms to mucus cells. Lysozyme acts as an inhibitor to the growth of some bacterial cells, and lactoferrin suppresses the growth of iron-dependent bacteria. In addition, IgA, lactoferrin, and albumin may participate in interglycoprotein linkages of the mucus. Thus, these proteins in ocular mucus may play a special role in mucus formation, in its lubrication, or in defense functions.

Another aspect of this study deals with our discovery of the ocular mucosoluate component in tears. The mucosoluate was immunologically identical in the saline extract of the mucus and in tears, though it appeared in less amounts in tears. Because of its physical and chemical properties, the soluble mucosoluate may play a part in the stabilization of the precorneal tear film, and the insoluble portion may participate in the formation of mucus gel. The ocular mucosoluate was previously characterized as a complex glycoconjugate containing lipids, mucins, some glycosaminoglycans, proteoglycans, and possibly other substances. Although it was not in a state of desired purity, the complex binding in this isolate was apparently strong enough to resist dissociation in the electric field in immunoelectrophoresis. It also appeared that a certain amount of IgA is covalently (or tightly) bound to the mucosoluate, similar to IgA-type binding in the mucus of some other body organs. The bound IgA apparently is either located in the domain of the mucosoluate, or may be present in small amounts so that it remained associated with the mucosoluate even after passage through the immunosorbsorbent column of anti-serum proteins. Of the mucosoluate glycoconjugates, two major moieties, mucins and lipids, have been studied and discussed often in past works. Other moieties, such as glycosaminoglycans, proteoglycans, and glycolipids, remain to be further purified, analyzed, and characterized. Such characterization should help clarify the contribution of each component in shaping the physiological function of ocular mucus.

Key words: immunological analysis, human ocular mucus, tears, proteins, ocular mucosoluate

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