Origin and Biosynthesis of Human Tear Fluid Proteins

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In tear fluid, a large number of proteins can be detected with electrophoretic or immunologic techniques. The composition of serum proteins in tears resembles that of whole serum. By comparison of the protein patterns resulting from different sampling methods, it was shown that serum albumin is not present in the secretion of the lacrimal gland, but is mixed with the tear fluid in the conjunctival sac. The in vitro synthesis and excretion of more than 20 protein components by the lacrimal gland could be demonstrated. Among these were lactoferrin, tear-specific prealbumin, lysozyme, and secretory IgA. The complexity of the electrophoretic protein pattern of tear fluid can be explained from the combination of the secretory activity of the lacrimal gland and the leakage of serum proteins from the circulation into the tear fluid. Invest Ophthalmol Vis Sci 24:623-630, 1983

In electrophoretic analysis of tear fluid, at least 60 protein components were detected by Gachon et al.1 With some simplification, the tear fluid proteins can be divided in three groups: proteins present in serum as well as in tears, proteins present in tears but not in serum, and proteins present in epithelial cells as well as in tears.

This last group of proteins can be detected in tear samples, collected with filter paper or by other methods that cause slight epithelial damage.2 In general, however, their concentration will be below the detection limit of electrophoretic techniques, currently used for protein analysis.

Lactoferrin, lysozyme, and tear-specific prealbumin (TSPA) are the most important examples of proteins present in tears, but not in serum to any extent.1,3 The results of immunofluorescence studies of the lacrimal gland4,5 and the simultaneous disappearance of these proteins in the tear fluid of keratoconjunctivitis sicca patients3,6 indicate a common origin of these proteins. Lysozyme was shown to originate from the lacrimal gland.7

There is no agreement in the literature on the way the serum proteins enter the tear fluid. Chao et al8 could not exclude synthesis or modification of some of the serum proteins within the lacrimal gland. Grabner et al9 suggested that serum proteins are present in basic tear flow from the lacrimal gland. From the strong coincidence of high serum albumin levels in tear fluid and hyperemia of the conjunctiva, as well as their decrease after administration of calcium dobsilate, a drug that reduces vasopermeability, we concluded that serum proteins are mixed with the tear fluid by leakage from the conjunctival capillaries.10

The present study determines which components were the product of local synthesis in the lacrimal gland and which components originated from the serum within the complex protein pattern observed after immuno-electrophoresis and SDS polyacrylamide gel electrophoresis.

Materials and Methods

Reagents

Unless stated otherwise all reagents were analytical grade, obtained from British Drug Houses Ltd., United Kingdom.

Tear Samples

Tear samples were collected from the conjunctival sac on Whatmann 3 MM filter paper discs (6 mm in diameter) or in glass capillaries. Sometimes tear fluid was also collected in a glass capillary near the lacrimal gland slightly above the lateral palpbral ligament or from the meniscus above the lower lid. Volumes of tear fluid collected were determined by weighing.

A part of the tear samples was analyzed separately, and another part of the samples was pooled, dialysed by ultrafiltration on a Diaflo YM 5 membrane (Amicon Corp., USA), and lyophilized.

Antisera

Pooled normal serum was obtained from a clinical laboratory, and normal tears were obtained from a group of volunteers. Human serum albumin was ob-
tained from Sigma Chemical Corp., USA, and purified further by gel filtration on Sephadex G-100 (Pharmacia, Sweden) in a buffer containing 0.05 mol/l Tris-HCl pH 7.5 and 0.15 mol/l NaCl. Lysozyme was isolated from leukemic urine by the method of Alderton et al. 11 As a final purification step, the lysozyme was chromatographed on Ultrogel AcA 54 (LKB, Sweden) in a buffer containing 0.05 mol/liter Tris-HCl pH 7.0 and 0.3 mol/l NaCl.

Antisera against human serum proteins, serum albumin, tear fluid proteins, and lysozyme were raised in New Zealand White rabbits as described previously. 3

Antisera against human lactoferrin, secretory component, and the α-chain of IgA were purchased from Behringwerke, German Federal Republic.

**Immunodiffusion**

Immunodiffusion was performed on 75 × 25 mm glass slides covered with 4 ml of 1.5% purified Bacto Agar (Difco Laboratories, USA) in phosphate buffered saline pH 7.3. Sample wells were made with a Gelman gelpuncher. Diffusion lasted for 48 hrs, after which the slides were washed for 24 hrs in several portions of saline. The slides were dried under filter paper at 40 C, stained with a solution of 5 g/l Coomassie Blue (Serva, GFR) in 9:9:2 water-ethanol-acetic acid, and destained in the same solvent mixture. Serum albumin concentrations in tear samples were determined by radial immunodiffusion.10

**Electrophoresis**

Immunoelectrophoresis and SDS-polyacrylamide gelelectrophoresis was performed as described previously.3

**Immunoadsorbents**

Pooled normal serum was dialysed against water and lyophilized. The γ-globulin fraction of an antiserum against human serum proteins was isolated by precipitation with 34% saturated ammonium sulphate of pH 6.5. After three precipitation cycles the precipitate was extracted once more with 1 ml H2O, and pooled supernatants were lyophilized (tear gland extract [chase]). The medium was dialysed by ultrafiltration on Diaflo YM 5 and lyophilized (pulse medium). Six pieces of tissue were transferred to a culture dish containing a medium of 0.6 ml (1.1 MBq) [U-14C] protein hydrolysate, specific activity 2.18 GBq/milliatom C (Radiochemical Center, UK), and 2.4 ml Hanks' balanced salt solution with vitamins for minimal essential medium (Flow) and 100 IU/ml penicillin and streptomycin (Difco Laboratories, USA). The tissue was incubated at 37 C for 24 hrs. After this, the medium was dialysed by ultrafiltration on Diaflo YM 5 and lyophilized (pulse medium).

The remaining six pieces of tear gland tissue were transferred to a culture dish with 1 ml medium 199 (Difco), containing 100 IU/ml penicillin and streptomycin and solidified with three drops of 5% agar. After incubation for 24 hrs at 37 C, the tissue was extracted as described above (tear gland extract [chase]). The medium was homogenized with a Teflon potter and centrifuged. The precipitate was extracted once more with 1 ml H2O, and pooled supernatants were dialysed by ultrafiltration on YM 5 and lyophilized (chase medium).

**Autoradiography**

Radioactive protein samples were analysed by immunodiffusion and electrophoresis. Immunodiffusion and immunoelectrophoresis slides were dried routinely in air; SDS-polyacrylamide gels were dried under vacuum on Whatmann 3 MM filterpaper. Au-
toradiography was performed on dried slides or gels, using X-Omat x-ray film (Kodak, USA). The film was processed with DX-80 developer and FX-40 fixer (both from Kodak) according to the manufacturer’s instructions.

Results:

Serum Proteins in Tears

With an antiserum against human serum proteins, the immunoelectrophoretic separation patterns of tear fluid and serum are very similar (Fig. 1A). Only a few lines are lacking or are less prominent in the tear sample, indicating that it is not a highly specific process by which some proteins from the serum are passed to the tear fluid.

The presence of many serum proteins in tear fluid can also be demonstrated by the presence of antibodies against these proteins in an antitear fluid antiserum. The immunoelectrophoretic separation pattern of human serum with antitear fluid antiserum is very similar to that developed with antihuman serum antiserum as shown in Figure 1B.

The serum proteins do not enter the tear fluid together with the lacrimal secretion. Serum albumin is not present in tear fluid collected with a glass pipette near the excretory duct of the lacrimal gland, but it is readily detected by radial immunodiffusion in tear fluid collected on filter paper discs from the conjunctival sac. Albumin concentrations in tear samples collected near the lacrimal gland and from the conjunctival sac of both eyes of 11 volunteers are compared in Table 1. The remarkable difference is not explained by the slight epithelial damage known to result from an insertion of a filter paper disc, as tear fluid collected with a glass pipette from the conjunctival sac also contains serum albumin, as shown in Figures 2A and B. The protein pattern of tear fluid collected by glass pipette from the tear meniscus above the lower lid, however, resembles much more that of lacrimal gland fluid than that of conjunctival sac fluid, especially after stimulation of the tearflow (Fig. 2C).

Proteins Originating from the Lacrimal Gland

Immunoadsorbents: The protein pattern of tear fluid collected near the lacrimal gland is dominated by three proteins: lactoferrin, tearspecific prealbumins, and lysozyme (Fig. 2). When tear fluid is passed through an immunoadsorbent of immobilized immunoglobulin from an antiserum against human

<table>
<thead>
<tr>
<th>Serum albumin concentration (mg/ml) in:</th>
<th>Patient no.</th>
<th>Lacrimal gland fluid</th>
<th>Conjunctival sac fluid</th>
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<tr>
<td></td>
<td>1 OD*</td>
<td>&lt;0.1</td>
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<tr>
<td></td>
<td>1 OS†</td>
<td>&lt;0.1</td>
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<td></td>
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<td></td>
<td>2 OS</td>
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<td></td>
<td>3 OD</td>
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<td></td>
<td>3 OS</td>
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<tr>
<td></td>
<td>4 OD</td>
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<td></td>
<td>5 OD</td>
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<td>5 OS</td>
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<tr>
<td></td>
<td>6 OD</td>
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<tr>
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<td></td>
<td>7 OD</td>
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<td>&lt;0.1</td>
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<td></td>
<td>8 OS</td>
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<tr>
<td></td>
<td>9 OD</td>
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<td></td>
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* OD = right eye.
† OS = left eye.
Fig. 2. Influence of sample collection method on gelelectrophoretic separation pattern of tear proteins. Samples were obtained from three healthy volunteers (A, B, and C) by means of I: a capillary pipette placed slightly above the lateral palpebral ligament; II: a capillary pipette from the conjunctival sac; III: a capillary pipette from the tear meniscus above the lower eye lid; and IV: a filter paper disc from the conjunctival sac. The arrows indicate the position of 1: lactoferrin, 2: serum albumin, 3: tearspecific prealbumin (TSPA), and 4: lysozyme.

Fig. 3. Immunoadsorbent fractionation of tear proteins. I: total tear proteins; II: tear proteins passing an immunoadsorbent of immobilized γ-globulins of an antiserum against serum proteins; III: proteins bound to the immunoadsorbent, eluting at low pH.

Fig. 4. Result of immunoelectrophoresis with absorbed antiserum. Antibodies reacting with serum components were removed from an antiserum against tear proteins by passing this antiserum through an immunoadsorbent of serum proteins coupled to CNBr-activated Sepharose. Immunoelectrophoresis of tear fluid with this absorbed antiserum resulted in the formation of four precipitation lines. Apart from these four lines, no more anodal lines were identified. Several other lines, identified as lysozyme and PMFA (proteins moving faster than albumin in nondenaturing acrylamide gelelectrophoresis), could not be identified.

Tissue culture—SDS gelelectrophoretic analysis: Tear gland tissue was incubated for 24 hrs in Hanks' balanced salt solution, containing antibiotics, vitamins, and 14C-labeled amino acids (pulse), followed...
by a 24-hr culture in medium 199 (chase). Tear fluid collected before surgery, as well as media and tissue extracts obtained after 24- or 48-hr cultures, were analyzed by SDS polyacrylamide gel electrophoresis, followed by autoradiography (Fig. 5).

Incorporation of radioactivity is observed for nearly all the protein bands of the tear gland extract, that are visible after staining (Figs. 5-III, 5-V). When chase medium (Fig. 5-VI) is added to the preoperative tear sample a blackening of the x-ray film is observed from lactoferrin, TSPA, lysozyme, and a group of protein bands below albumin (Fig. 5-II).

In pulse medium (Fig. 5-IV), apart from these proteins, considerable amounts of serum albumin and of two protein bands migrating faster than lysozyme (molecular masses: 15,500 and 14,800) are found. These proteins are also present in tear gland extracts (Fig. 5-III, 5-V), but are not synthesized there as can be concluded from the corresponding autoradiograms. Their low molecular mass and the equal staining intensity of the two bands suggested that these bands were the α- and β-chains of hemoglobin from erythrocytes, apparently not completely removed during the washing procedure of the lacrimal gland.

Fig. 5. Tissue culture of lacrimal gland. Lacrimal gland tissue was incubated for 24-hr in a medium containing 14C-labeled amino acids (pulse) followed by a 24-hr culture on a cold medium (chase). I: preoperative tear sample; II: mixture of preoperative tear sample and chase medium (VI); III: lacrimal gland extract (pulse); IV: pulse culture medium; V: lacrimal gland extract (chase); VI: chase culture medium. S indicates the separation pattern after staining of the gel with Coomassie Blue; A indicates the autoradiogram obtained after exposing an x-ray film to the dried gel.
tissue. This conclusion was supported by SDS gel electrophoresis of human erythrocyte extract and tear fluid, in which the positions of the α- and β-globin chains were found to be the same as those in Figure 5-IV. The incomplete removal of blood from the tissue can also explain the presence of serum albumin in pulse medium.

From the autoradiograms of pulse and chase media (Figs. 5-IV, 5-VI), it can be concluded that lysozyme and TSPA are rapidly synthesized and excreted in vitro by lacrimal gland tissue. The incorporation of radioactive label in lactoferrin, TSPA, and lysozyme found in chase medium is similar in magnitude, while in the pulse medium only a small amount of radioactive lactoferrin can be detected in comparison to the other two proteins. This is not caused by an accumulation of lactoferrin within the tissue because the amount of lactoferrin there (Fig. 5-III) is also low with respect to that of lysozyme and TSPA. Therefore, it can be concluded, that the rate of in vitro synthesis and excretion of lactoferrin is less than that of lysozyme and TSPA.

The separation pattern of chase medium after staining as well as autoradiography (Fig. 5-VI) resembles closely that of lacrimal gland fluid (Fig. 2: A-I, B-I and C-I) and that of tear fluid after removal of serum components (Fig. 3B). Apart from the three major components (lactoferrin, TSPA, and lysozyme), another 18 components can be distinguished in the autoradiogram of chase medium. Their presence cannot be caused by necrosis of tissue as certain intensely stained and labeled tissue components are not detected in the medium. Therefore, a selective excretion of these components by the lacrimal gland tissue into the culture medium must be assumed.

**Tissue culture. Immunologic analysis:** Equal amounts of pulse and chase media were mixed with pooled normal tear fluid and subjected to immunoelectrophoresis with antisera against tear fluid proteins, human serum proteins, and an antiserum against tear fluid proteins absorbed with serum proteins (Fig. 6). Radioactive label can be detected in about eight precipitation lines of the immunoelectrophoresis pattern developed with antitear fluid proteins antiserum. They include among others TSPA, lactoferrin, IgA, IgG, and, faintly, serum albumin. The radioactivity detected near the antiserum through the γ-region probably corresponds to lysozyme.

After absorption of this antiserum with serum proteins, the four precipitation lines remaining in the separation pattern all contained radioactive label. After immunoelectrophoresis with an antiserum against serum proteins, a very faint blackening of the x-ray film was found from albumin, IgA, and a line in the β-region.

The same antigen mixture was used in an immu-
Fig. 7. Immunodiffusion analysis of tissue culture medium. Sample wells a-f contained antisera, sample well g contained the same antigen as Figure 61-a: antiserum proteins; b: anti-IgA-α-chain; c: antiserum secretory component; d: anti-lactoferrin; e: anti-lysozyme; f: antiserum albumin. S indicates the stained immunodiffusion pattern; A indicates the corresponding autoradiogram.

Immunodiffusion test with several different antisera (Fig. 7). The radioactive label incorporated in the precipitation lines formed with antisera against lysozyme and lactoferrin confirms local synthesis within the lacrimal gland. The radioactivity in the precipitation line formed with anti-IgA-α-chain and antisecretory component antisera indicates the local production of secretory IgA. The spur in the latter precipitation line, pointing in the direction of the antisecretory component antiserum well, results from the presence of IgA in the antigen mixture, originating from the circulation and, therefore, lacking secretory component and of free IgA, produced locally, as indicated by the radioactivity incorporated in the spur. Free secretory component, resulting in the formation of a spur in the precipitation line pointing towards the anti-IgA-α-chain antiserum well could not be detected. The horizontal precipitation line formed between the antisera wells containing antiserum albumin and anti-lysozyme was caused by an interaction between these two antisera. A faint radioactive labeling was found once more for serum albumin.

Discussion

Although Balik12 reported the absence of proteins in tear fluid collected from a ductus aberans, the idea that the lacrimal gland is a major source for tear fluid proteins is generally accepted. Indications for the secretory activity of the lacrimal gland were obtained by immunofluorescence4'5 and by measurement of certain proteins—notably lysozyme—in tear fluid of patients suffering from the degeneration of the tear gland.13,14

Proteins secreted by the lacrimal gland do not originate from the blood circulation, but are the product of local synthesis, as appears from the incorporation of radioactive label in these proteins. For lysozyme this confirms the study of Covey et al.7 In immunochromical analysis of the proteins secreted by the lacrimal gland in vitro, however, an incorporation of radioactive label in some serum proteins was also observed: slightly in albumin and IgG and more pronouncedly in IgA. This confirmed the results of Chao et al.8 who also reported an incorporation of labeled glucosamine in immunodiffusion lines of the serum proteins transferrin and ceruloplasmin. From this they concluded that these serum-type glycoproteins were synthesized or modified by the lacrimal gland. After SDS gel electrophoresis, however, we could no longer detect radioactive label in the serum albumin band. This indicates that the radioactivity associated with albumin after immunodiffusion or immunoelectrophoresis results from absorbed and not from incorporated radioactive amino acids, thus ruling out local synthesis. This observation is in accordance with the transport function of albumin for various compounds.

Whether the same phenomenon is responsible for the radioactivity in IgG immunoprecipitation lines cannot be excluded completely. The possibility of local synthesis by plasma cells, however, is strongly supported by the results of immunofluorescence localization of IgG and IgA in the lacrimal gland.4'5 In vitro synthesis of IgG and IgA in conjunctiva was observed by Lai A Fat et al.15

The local production of secretory IgA forms an important specific defense mechanism against microbial attack of the mucous epithelium.16 Therefore, the IgA/IgG ratio in external secretions, such as tears, differs markedly from that in serum.17 Secretory IgA is not only synthesized in the conjunctiva,12 but also in the lacrimal gland, as indicated by the strong incorporation of radioactive activity, detected with antisera against IgA-α-chain and secretory component (Figs. 6, 7). No evidence was obtained for the presence of free secretory component in the culture medium.

With electrophoretic techniques, more than 60 protein components can be detected in normal tear fluid.1 We could show that more than 20 of these components are secreted by the lacrimal gland. Apart from these, many serum proteins could be detected
in tear fluid, and, therefore, the complexity of the protein pattern might very well be explained by the combination of the secretory activity of the lacrimal gland and the leakage of serum proteins from the circulation into the tear fluid.10

Lysozyme concentration in tear fluid is determined easily by enzymatic assays, and, therefore, lysozyme is used as a parameter for tear gland function.13,14 As several other proteins are synthesized in the lacrimal gland apart from lysozyme, it could be that among them there are some that can perform this function equally well or perhaps even better than lysozyme.

Grabner et al9 and Mackie and Seal18 reported that in normal tear samples collected from the conjunctival sac on filter paper, there is no relation between the volume of tear fluid absorbed by the disc and its lysozyme concentration; ie, tear lysozyme concentration is not dependent on the rate of tear production. Such a relation, however, does exist for serum albumin levels; high concentrations of that protein are measured in “slow tear producers,” low concentrations in “fast tear producers.” Grabner et al9 suggested, that in fast tear producers “basic” tear fluid, containing serum albumin, is diluted by a plasma filtrate, containing only proteins, smaller than serum albumin. From the present results it can be concluded, that “basic” tears and reflex tears, as secreted by the lacrimal gland, are free of serum albumin. Furthermore, dilution of “basic” tears by a plasma ultrafiltrate, would lead to a decrease in the concentration of lysozyme, which actually has not been found.9,18 Therefore, we suggest that “basic” and reflex tears, as secreted by the lacrimal gland, are essentially the same in chemical composition. The low serum albumin concentrations generally found in tear samples collected from the conjunctival sac of “fast tear producers” can be explained by a strong dilution of serum proteins, leaking from the conjunctival vessels, in a large volume of tear fluid, secreted by the lacrimal gland.

Key words: tears, muramidase, lysozyme, lactoferrin, tear-specific prealbumin, biosynthesis, tissue culture, gel electrophoresis, serum proteins, immunoglobulins, lacrimal gland

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