Investigative Ophthalmology & Visual Science, Vol. 31, No. 6, June 1990

Expression of Two Molecular Forms of the Complement Decay-Accelerating Factor in the Eye and Lacrimal Gland

Jonathan H. Lass,* Elizabeth I. Walter,† Terry E. Burris,‡ Hans E. Grossniklaus,*† Melvin I. Roat,§ Debra L. Skelnik,‖ Laure Needham,† Milton Singer,‡ and M. Edward Medof†

Complement is present in ocular fluids, but the molecular mechanism(s) restricting its activation to exogenous targets and not to autologous ocular cells are currently unknown. To clarify how this control is achieved, monoclonal antibody (mAb)-based techniques were used to examine the eye, the lacrimal gland, and ocular fluids for the decay-accelerating factor (DAF), a membrane regulatory protein which protects blood cells from autologous complement activation on their surfaces. Immunohistochemical staining of tissue sections revealed DAF antigen on corneal and conjunctival epithelia, corneal endothelium, trabecular meshwork, and retina, as well as on lacrimal gland acinar cells and in adjacent lumens. By flow cytometry, cultures of conjunctival epithelium exhibited the highest DAF levels and levels on corneal epithelium > corneal endothelium > conjunctival fibroblasts. Biosynthetic labeling of corneal endothelium yielded de novo DAF protein with an apparent molecular weight (Mr) of 75 kD, approximating that of blood cell DAF protein, and digestions of conjunctival epithelium with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme which cleaves glycoinositolphospholipid membrane anchors, released ~70% of the ocular surface DAF protein similar to leukocyte surface DAF protein. Quantitations of DAF by radioimmunometric assay employing mAbs against two DAF epitopes revealed 325 ng/ml (n = 12), 4.8 ng/ml (n = 10), and 22.0 ng/ml (n = 8) of soluble DAF antigen in tears, aqueous humor, and vitreous humor, respectively. Western blot analyses of the tear DAF antigen revealed two DAF forms, one with an apparent Mr of 72 kD resembling membrane DAF forms in other sites, and a second with an apparent Mr of 100 kD, which is previously undescribed. Since DAF activity is essential physiologically in protecting blood cells from autologous complement attack, the identification of DAF on the ocular surface, intraocularly, in the lacrimal gland, and in tears suggests that DAF-mediated control of complement activation is also required in these locations. Invest Ophthalmol Vis Sci 31:1136–1148, 1990

In the vascular space and adjacent tissues, complement promotes the elimination of foreign targets by covalent attachment of opsonins which sensitize them to lysis or phagocytosis and by generation of inflammatory mediators which attract and activate cellular effectors (reviewed in Ref. 1). In ocular tissues, the role of complement in host defense is less well understood. Several lines of evidence, however, indicate that it may function analogously. Under physiological conditions, all components of both the classical and alternative activation pathways are measurable in tears,[2–4] cornea,[5–7] and aqueous humor.[8,9] In pathologic conditions associated with ocular inflammation, complement peptides are demonstrable in conjunctiva, sclera, uveal tract, and retina. For example, in ocular cicatricial pemphigoid, C3 and C4 antigens are found at the conjunctival basement membrane.[10,11] In anterior uveitis,[12,13] C3b and C4b activation fragments are found in aqueous humor as well as in tissue sites.

Although factors that can modulate complement activation have been identified in ocular fluids, the regulatory mechanisms which operate in vivo and which control the activation process so as to focus it

From the *Division of Ophthalmology and the †Institute of Pathology, Case Western Reserve University, Cleveland, Ohio; the ‡Corneal Research Laboratory, Devers Eye Institute, Portland, Oregon; the §Eye and Ear Institute of Pittsburgh, Pittsburgh, Pennsylvania; and the ‖Department of Ophthalmology, University of Minnesota, Minneapolis, Minnesota.

Supported in part by grants from the †American Cancer Society, †American Heart Association, National Institutes of Health †AI-23598, †PO-1DK38181, and §EY-06186; the *Ohio Lions Eye Research Fund; and ‡Oregon Lions Sight and Hearing Foundation.

Presented in part at the annual meeting of the Association for Research in Vision and Ophthalmology, Sarasota, Florida, May 5, 1988, and May 4, 1990.

Submitted for publication: August 2, 1989; accepted October 30, 1989.

Reprint requests: Jonathan H. Lass, MD, University Hospitals of Cleveland, 2074 Abington Road, Cleveland, Ohio 44106.

on targets and not on ocular cells are unknown. The serum regulators, C1 inhibitor (C1INH) and C3b inactivator (Factor I), are detectable in cornea and aqueous humor,[14] but levels of both proteins are insufficient to be functionally significant. An iron binding protein, lactoferrin, which can inhibit the formation of the classical C3 complexes[15,16] is present in tears, but this molecule has no known role in complement regulation in the vascular space and undirected C3 convertase inhibition by it in ocular fluids could dampen complement interaction with targets. No investigations relevant to complement regulation in the conjunctiva, uveal tract, retina, or vitreous humor have been carried out.

Decay-accelerating factor (DAF) is a 70–80-kD glycoprotein[17] that functions intrinsically in plasma membranes of blood cells to circumvent injury that could arise from autologous complement activation on their surfaces.[18,19] Elucidation of its mechanism of action has been facilitated by the observation that the purified protein, when added to cells in vitro, incorporates into their membranes and exerts its function.[19] Insertion of DAF into sheep cell intermediates and analysis of the effects has shown that it interacts with deposited C4b and C3b fragments and interferes with ability of these covalently bound polypeptides to serve as sites for the assembly of C3 and C5 convertases, the amplification enzymes of the cascade.[19] It is expressed on blood elements[20,21] as well as on vascular endothelium, all cell types in intimate contact with plasma complement proteins.[22] In the hemolytic disorder paroxysmal nocturnal hemoglobinuria (PNH), exaggerated C3b uptake by affected cells in vivo is associated with DAF deficiency,[18,23] and reconstitution of the affected cells with exogenous DAF ameliorates the abnormal uptake of autologous C3b by the cells in vitro.[24] These findings have established that DAF activity is physiologically essential in protecting blood cells from autologous complement.

An important structural feature of blood cell surface DAF protein is that it is anchored in plasma membranes by a C-terminal glycoinositolphospholipid (GIPL) structure[25,26] rather than by a hydrophobic polypeptide membrane spanning sequence, as is present in most cell surface molecules. Structural and biosynthetic analyses of DAF[25] and other proteins anchored in this way (reviewed in Refs. 27, 28) have shown the GIPL-anchoring structure is preassembled in cells and is incorporated into nascent polypeptides immediately after they emerge from ribosomes in the rough endoplasmic reticulum. The biologic relevance of the GIPL-anchoring mechanism is incompletely understood. Enzymatic studies have shown that GIPL anchors are susceptible to cleavage by phosphatidylinositol-specific phospholipase C (PI-PLC) of bacterial origin. It is hypothesized that such cleavage by endogenous lipases could modulate cell surface levels of these proteins. Photobleaching studies[29–31] have indicated that GIPL anchoring can confer more than 10-fold greater lateral mobility than conventional anchoring to proteins in the surface membrane. It is believed that this enhanced mobility could be important in the function of DAF.

In a previous study it was shown that outside of the vascular space, DAF is demonstrable on surface epithelia lining extracellular compartments in a number of sites[32] including the eye, and that a hydrophilic form of the protein can be recovered from adjacent extracellular fluids, such as tears, saliva, plasma, and urine. The urine protein was found to be a 67-kD hydrophilic DAF molecule with regulatory activity similar to DAF but mediated extrinsically[32] rather than intrinsically. The structure or function of DAF proteins in tears or other body fluids was not elucidated. The observations, however, prompted the speculation that extravascular DAF in these sites might serve a protective role analogous to that of blood cell DAF.

In the current study, ocular and lacrimal gland tissues and fluids were examined for the presence of DAF using immunochemical, flow cytometric, and radioimmunometric assays. These analyses demonstrated DAF protein on the surface of several ocular cell types, unexpectedly in some cases in concentrations greater than on blood cells. The studies additionally established 1) that the ocular cell surface DAF protein is synthesized de novo and resembles blood cell DAF protein both in size and in GIPL anchoring; 2) that hydrophilic DAF protein is present not only in tears but also in other ocular fluids, including aqueous and vitreous humors; and 3) that tear DAF protein consists of two molecular forms, one with an apparent Mr of ~72 kD, and one with an apparent Mr of 100 kD, which is likely a product of lacrimal secretions.

Materials and Methods

Proteins, Antibodies, and Radiolabeling

DAF was purified from Nonidet P-40 (NP-40) extracts of human erythrocyte (E^{hu}) stroma by affinity chromatography on IA10-coupled sepharose.[25] Murine anti-E^{hu} DAF monoclonal antibodies (mAbs) IA10, IIH6, and VIIIA7 were obtained as described.[20] RPC5 and MOPC-1 (nonrelevant mAbs) were obtained from Biometics (Charleston, SC). Peroxidase-labeled affinity-purified goat anti-murine Ig (IgG H and L chain-specific) was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Protein A-Sepharose was obtained from Pharmacia Fine

Chemicals (Piscataway, NJ). mAb IIH6 was labeled with ^{125}I using Iodogen (Pierce Chemical, Rockford, IL), according to the manufacturer's instructions, and was purified by gel filtration followed by extensive dialysis.

Immunohistochemical Analysis of DAF in Ocular Tissues

Eyes and lacrimal gland tissue obtained within 6 hr of death (Cleveland Eye Bank and Lions Eye Bank of Oregon) were snap-frozen in liquid nitrogen. Cryostat tissue sections were fixed in acetone for 10 min at 20°C. Fixed sections were overlayered with pooled anti-DAF mAbs (IA10, IIH6, and VIIIA7) 5 μg/ml each in 0.001 M Tris-buffered saline, pH 8.0, supplemented with 1% normal goat serum (TBS-NGS), or with nonrelevant mAbs of the same subclasses (RPC5, MOPC-1) at the same concentration. The mAb-treated sections were preincubated in a moist chamber at 37°C for 1 hr, and after washing in filtered TBS-NGS and drying, the slides were incubated at 20°C for 15 min with biotinylated goat anti-mouse IgG (ICN Immunologicals, Lisle, IL) at a concentration giving optimal staining in preliminary experiments.[33] After rinsing with TBS-NGS and drying, 3 drops of streptavidin-conjugated peroxidase (ICN Immunologicals) were applied and after 30 min at 20°C, the slides were washed with 0.05 M Tris, and developed in 4.5 ml of the same buffer containing 0.5 ml of 3,3'-diaminobenzidine and 5 μl 30% hydrogen peroxide. After transfer to water to stop the reaction, sections were fixed in 0.5% osmium tetroxide for 1 min. Stained and fixed sections were counterstained for 10 sec with hematoxylin, and after extensive washing with cold water and dehydration by baths in absolute ethanol and xylene, they were mounted with eukitt.

Cultured Ocular Cells

For conjunctival epithelium and fibroblasts, 1–2-mm diameter biopsies of bulbar conjunctiva were excised from normal volunteers, as described previously.[34] These explants were placed epithelial side up in a 35-mm tissue culture dish (Falcon 3001; Becton Dickinson, Lincoln Park, NJ) and left uncovered for 5 min, and then 1.5 ml of tissue culture medium was added. To keep outgrowths of conjunctival epithelial cells free of fibroblasts, explants were removed at day 8 and grown to confluency at 37°C in 5% CO_2 in modified SHEM medium[35,36] containing Ham F-12 (Gibco, Grand Island, NY) in Dulbecco's modified Eagles medium (DMEM) (1:1) (Gibco); mouse epidermal growth factor (mEGF) 10 ng/ml (Collaborative Research, Bedford, MA); insulin 5 μg/ml (Collaborative Research); cholera toxin 0.1 μg/ml (Sigma, St. Louis, MO); L-glutamine 1 μg/ml (Gibco); fetal bovine serum (FBS) 15% (Gibco); donor horse serum 5% (Hazelton, Lenexa, KS); dimethylsulfoxide 0.5% (Sigma); and gentamicin sulfate 40 μg/ml.

For conjunctival fibroblasts, explants were removed at day 8 and placed in 35-mm dishes using DMEM containing 15% FBS and 40 μg/ml gentamicin. Cells were passaged with trypsin-EDTA (Sigma) and passages 2–9 used for analyses.

For corneal epithelial cells, rims of limbal cornea, remaining after removal of the corneal button from a corneoscleral preparation for penetrating keratoplasty, were split through the mid-stroma, and the anterior portion was divided into 2 × 2-mm blocks. These explants were placed epithelial side up in 35-mm tissue culture dishes (Falcon 3001) and left uncovered for 10 min, and then 1.5 ml modified SHEM tissue culture medium was added.[35,36] The cultures were incubated at 37°C in 5% CO_2 for 7 days. Explants were removed at day 8 (before fibrocyte outgrowth had begun) and cultured until they reached confluency.

For human corneal endothelial cells, human donor corneas were prepared for standard organ culture and stored for a minimum of 5 days.[37] Endothelial cells were separated and cultured by a modification of a previous method[38] in which corneas were removed from organ culture and rinsed for 30 sec in serum free Eagle's MEM with Earle's salts, 2 mM L-glutathione, 25 mM HEPES buffer, and 90 μg/ml gentamicin sulfate. Corneal endothelial cells were directly trypsinized with 0.83% trypsin, 0.16 M EDTA, 33 mM HEPES in serum-free MEM with Earle's salts over a 15–40-min period at 20°C. When cells were rounded and intercellular processes broken, endothelial cells were flushed gently from Descemet's membrane and placed in Chondroitin Sulfate Corneal Storage Medium (CSM; Aurora Biologicals, Williamsville, NY), supplemented with 10% FBS. The cell suspension was centrifuged at 1100 cpm for 10 min at 4°C. The cell pellet was resuspended in CSM supplemented with 10% FBS. The endothelial cells were incubated at 35°C in a humidified atmosphere of 5% CO_2. After 24 hr, the cell medium was aspirated and replaced with fresh medium. The cells were grown in 6 well dishes for 7–10 days until confluency was reached. Cells were subcultured with the same trypsin-EDTA solution after a brief exposure of 1–4 min.

Preparation of Cell Extracts

Washed cells, resuspended to 1×10^8/ml in PBS, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 12 μg/ml soybean trypsin inhibitor, and 2 mM iodoac-

etamide were extracted for 20 min at 0°C with an equal volume of 1.0% NP-40 in PBS containing the same inhibitors. The NP-40 extracts were centrifuged at 12,000 *g* for 15 min and the supernatants stored at −70°C.

Immunoradioimmunometric Assay (IRMA) for DAF

DAF concentrations in extracts were quantitated by two-site IRMA using anti-DAF mAbs performed as described previously.[20,39] Briefly, samples or purified E^{hu} DAF standards were added in duplicate 30-μl aliquots to the wells of 96 well U-bottomed plastic microtiter plates (Becton Dickinson, Oxnard, CA) precoated with 50 μl 20-μg/ml anti-DAF mAb IA10 and blocked with PBS containing 1% BSA. After incubation at 20°C for 2 hr and washing, 30 μl ^{125}I-labeled anti-DAF mAb IIH6 was added to each well, and the plates incubated for 1 hr at 20°C. After extensive washing, bound DAF antigen in the samples was quantitated by comparison of bound counts in cut-out wells that received samples to those in wells that received DAF standards.

Surface Expression of DAF Antigen and PI-PLC Cleavage

Surface DAF expression was assessed using a fluorescence-activated cell sorter (FACS; Ortho Diagnostics, Westwood, MA) as described.[20,39] Of each cell type, 10^6 cells in 25 μl PBS containing 1% BSA and 0.02% azide (AZ) were incubated at 0°C for 30 min with anti-DAF mAb IA10 (5 μg/ml each in 25 μl of the same buffer) or with a nonrelevant mAb (RPC5) at the same concentration. After washing, the mAb-treated cells were resuspended to 25 μl in 1% PBS-BSA-AZ, and incubated at 0°C for 30 min with an equal volume of a 1:50 dilution of fluorescein-isothiocyanate-goat anti-mouse Ig $F(ab')_2$.[20] The stained cells were fixed with 0.5% paraformaldehyde, washed, and resuspended to 1 ml in 1% PBS-BSA-AZ.

PI-PLC digestion studies were performed by incubating 10^6 cells in 25 μl PBS with an equal volume of 1:50 *B. thuringiensis* PI-PLC (provided by M. Low, Columbia University, New York, NY) in PBS or PBS alone for 1 hr at 37°C. The cells were washed two times in PBS and stained for surface DAF expression, as described above.

Biosynthesis of DAF Protein

Ten million corneal endothelial cells cultured for 1 wk were preincubated in cysteine (C)-free RPMI for 1 hr at 37°C, 5% CO_2. Cells were labeled with 500 μCi [^{35}S]-C for 4 hr.[25] After washing, [^{35}S]-C-labeled cells were extracted by the addition of 500 μl 2% SDS, and extracts were sonicated and boiled.

Ocular and Body Fluids Collection

Informed consent was obtained from each subject for tear and aqueous humor collection after the procedures were fully explained. To obtain tears, a 50-μl pipette was placed in the inferior fornix of the unanesthetized ocular surface while the lids were manually opened and filled by capillary action. After centrifugation at 10,000 *g* for 15 min to remove cells and debris, samples were stored at −70°C.

For aqueous humor, with the exception of subject 18, all subjects received tobramycin sulfate 4 mg/ml four times per day for 3 days prior to surgery and tropicamide 1% and phenylephrine hydrochloride 2.5% 1 drop each at 1.5, 1, and 0.5 hr preoperatively. No patients had been on topical or systemic corticosteroids or nonsteroidal agents within 1 yr of surgery. After lid and retrobulbar anesthesia (a 1:1 mixture of 2% Xylocaine with 1:100,000 epinephrine and 0.75% bupivicaine hydrochloride), a 30-gauge needle attached to a tuberculin syringe was introduced into the anterior chamber at the limbus through a grooved incision and 0.15 ml aqueous humor obtained. The sample was immediately placed on ice and after centrifugation frozen at −70°C.

For collections of vitreous humor from eye bank eyes, after the cornea with scleral rim had been removed, the zonules cut, and the lens manually expressed out of the eye, the vitreous was aspirated with an 18-gauge needle and 2 ml obtained. After centrifugation, the sample was frozen at −70°C.

Western Blotting

After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels under nonreducing conditions,[20] separated proteins were transferred to nitrocellulose using a Transblot Apparatus (Bio-Rad Laboratories, Richmond, CA). After blocking for 1 hr at 37°C with PBS containing 5% BSA and 0.05% AZ, the nitrocellulose membrane was incubated at room temperature for 1 hr with ^{125}I-labeled anti-DAF mAb IIH6, washed three times with PBS-BSA-AZ, dried, and loaded into film cassettes.

Immunoprecipitation

Samples of 2% SDS cell extracts[25] were diluted 5-fold with 50 mM Tris-HCl, pH 7.4, and 190 mM NaCl containing 100 units/ml trasylol, 6 mM EDTA (Tris-NaCl-EDTA), and 2.5% Triton X-100 (TX-100), and preabsorbed for 2 hr at room tempera-

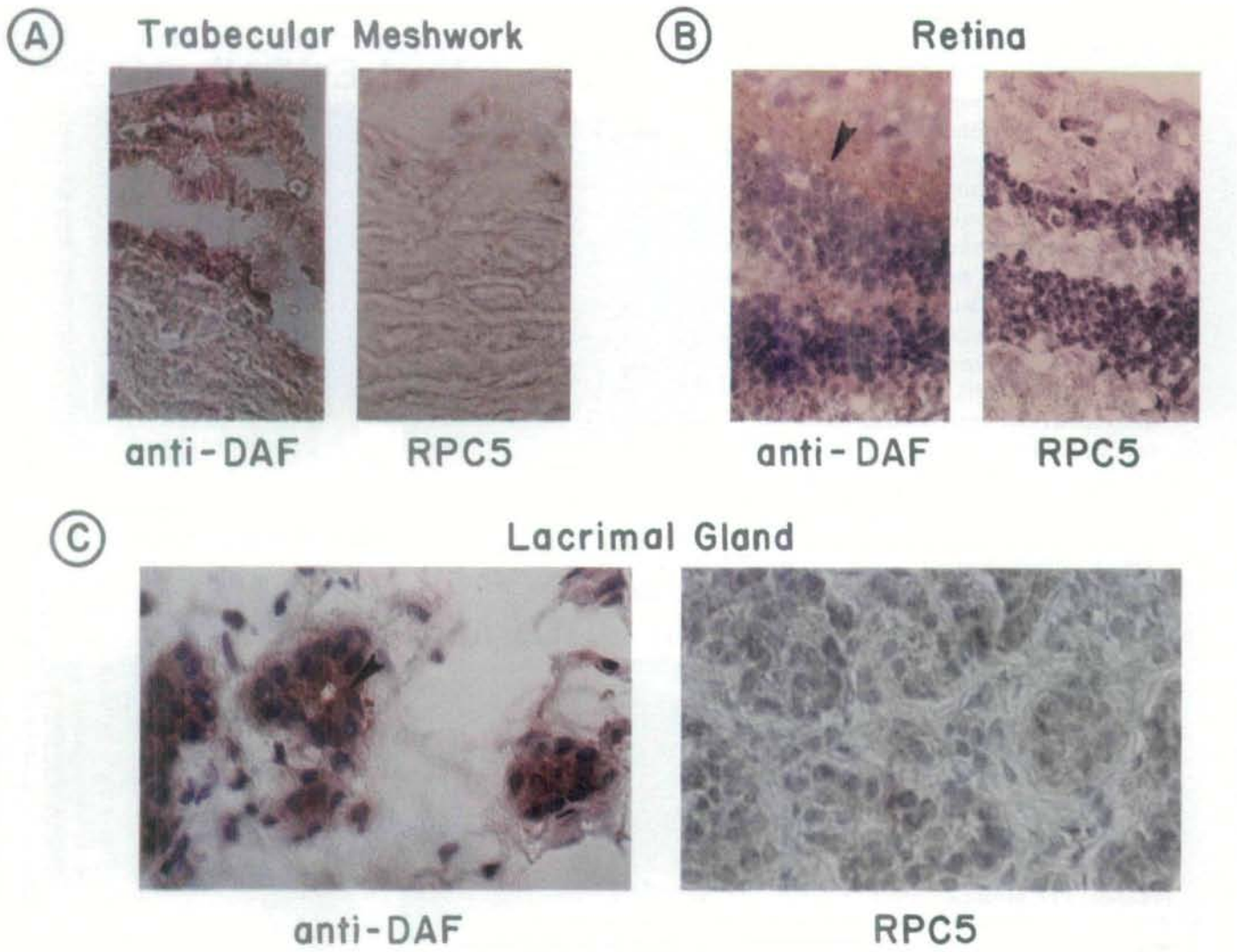

Fig. 2. Immunohistochemical analyses of ocular tissues. Sections (×128) stained with anti-DAF (**A–C**, *left*) and control nonrelevant (RPC5)-mAbs (**A–C**, *right*). (**A**) Trabecular meshwork (×40) showing weakly positive anti-DAF staining of the trabecular beams. (**B**) Retina showing anti-DAF staining of inner retinal layers (arrowhead). (**C**) Lacrimal gland showing intense anti-DAF staining of the apices of acinar epithelium and lumens of acinae (arrowhead).

from inositolphospholipids, releasing GIPL-anchored proteins from cells as hydrophilic derivatives. As seen in Figure 5, PI-PLC digestion of these cells removed 70% of the anti-DAF stainable surface antigen, a proportion comparable to that observed with leukocytes and HeLa cells, indicating that the DAF protein in these cells is GIPL-anchored.

Detection of DAF Antigen in Ocular Fluids

Because previous studies have identified soluble DAF protein in tears,[32] samples of tears and other ocular fluids from an expanded number of individuals were collected for quantitation of DAF antigen by IRMA employing two anti-DAF mAbs.[20,39] Tears were collected from healthy volunteers (n = 12; average 37 ± 12 yr of age, ±1 SD) with no previous ocular inflammation, surgery, or contact lens wear. In selected cases (n = 5), saliva, urine, and blood samples were collected simultaneously for comparative analyses. Aqueous humor specimens were collected from subjects (n = 10, 74 ± 9 yr of age) at the time of cataract surgery or cyclocryotherapy. No subject had had previous ocular surgery, trauma, or inflammation, except one (subject 18) who had chronic iritis and neovascular glaucoma at the time of surgery. Vitreous humor specimens were obtained from eye bank eyes (8 donors, 12 eyes; 44 ± 30 yr of age) enucleated and processed within 6 hr of death from individuals who died of myocardial infarction, colon and prostate cancer, cerebrovascular accident, and gunshot to the head.

The results, summarized in Table 1, revealed the presence of DAF protein uniformly in all of the ocular fluids. The mean DAF concentration measured in tears was 325 ± 289 ng/ml, whereas that measured in aqueous humor was 4.8 ± 3.7 ng/ml. It is noteworthy that one specimen (that from subject 18, with active iritis) showed a DAF concentration 5-fold higher than the other nine uninflamed specimens. Exclusion of this specimen from the data decreased the mean aqueous humor level to 3.7 ± 1.2 ng/ml, a value

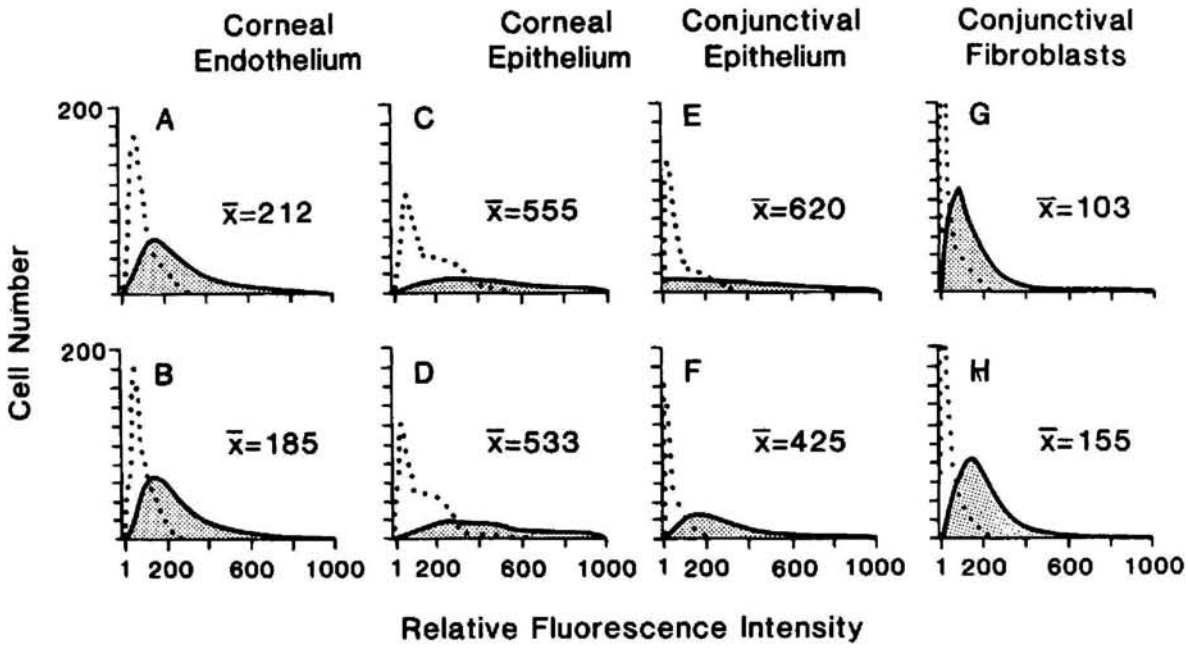

Fig. 3. Flow cytometric analyses of surface DAF levels on cultured human corneal endothelium (**A, B**), corneal epithelium (**C, D**), conjunctival epithelium (**E, F**), and conjunctival fibroblasts (**G, H**). For each cell type, analyses of two independent cultures are shown. Cells were stained with pooled anti-DAF or nonrelevant (RPC5) mAbs as control followed by fluorescein-isothiocyanate-labeled goat anti-mouse Ig. Solid lines with underlying shading show the results with anti-DAF, and dashed lines the results with nonrelevant mAbs. Conjunctival and corneal epithelium exhibit high surface DAF levels. Corneal endothelium and conjunctival fibroblasts exhibit low, but significant, surface DAF levels.

75-fold less than that in tears. The mean DAF concentration for the vitreous humor was 22.0 ± 8.2 ng/ml, a value 5-fold higher than that of aqueous humor ($P < 0.001$). Three donor pairs (donors 3, 6, and 7) showed comparable DAF concentrations between their two eyes, whereas one donor pair (donor 5) showed a variation.

The results of correlative analyses in selected individuals of ocular fluid DAF levels with DAF levels in other body fluids are given in Table 2. As can be seen among all body fluids, the mean DAF concentration in tears was consistently the highest, approximately seven times that in plasma, and approximately four times that in urine. Among different individuals, tear DAF levels paralleled saliva DAF levels. No relationship was apparent between tear DAF concentration and DAF levels in plasma or urine.

Characterization of Ocular Fluid DAF

To assess the structure of the DAF antigen detected in tears, collections from 30 normal subjects were pooled. Samples from 8 of these donors were subjected to SDS-PAGE, and after electrotransfer to nitrocellulose, the separated proteins were analyzed by Western blotting with anti-DAF mAbs. It is apparent by comparison of the bands to that of E^{hu} DAF protein, included as a control, that in addition to a DAF form with an apparent Mr of 72 kD, a larger DAF species with an apparent Mr of 100 kD was detected (Fig. 6 *left*).

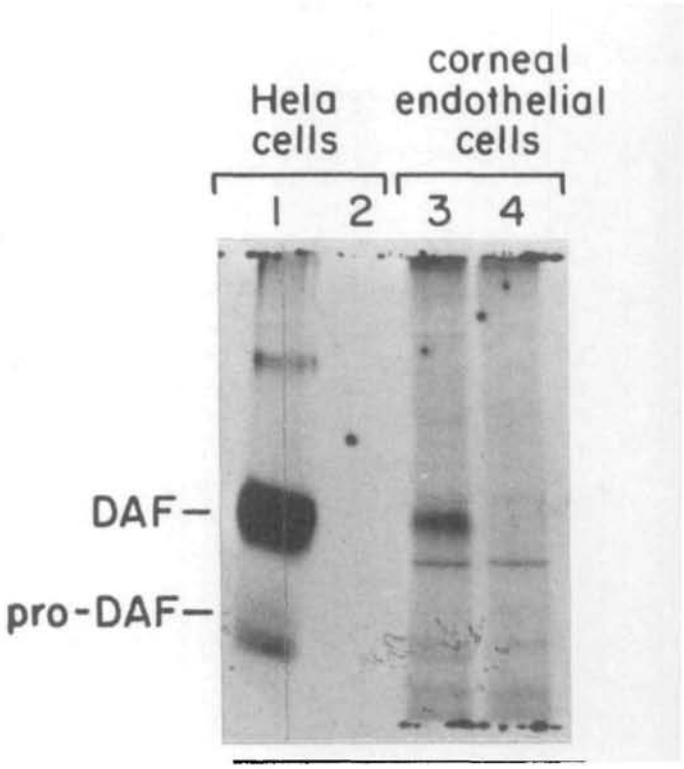

Fig. 4. Biosynthesis of DAF by corneal endothelium. Ten million corneal endothelial cells and HeLa cells were pulse-labeled with 500 μCi [^{35}S]-C. Cells were extracted in detergent, and the labeled DAF proteins immunoprecipitated with pooled anti-DAF mAb (lanes 1 and 3). Lanes 2 and 4 are the extracts, immunoprecipitated with nonrelevant (RPC5) mAb. The large shift in apparent Mr observed between 47-kD DAF precursor (pro-DAF) and mature 75-kD membrane DAF protein is due to incorporation of O-linked sugars in the Golgi.

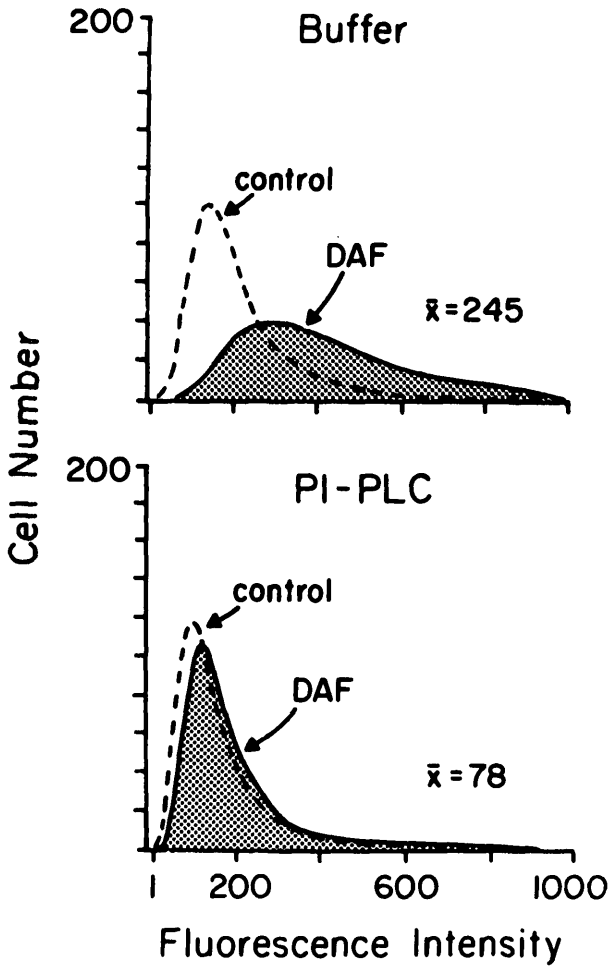

Fig. 5. PI-PLC digestion studies. One million conjunctival fibroblasts were treated with PI-PLC (1:50) or PBS for 1 hr at 37°C. After blocking in PBS, cells were stained with anti-DAF mAb, IA10 (solid lines) or nonrelevant (RPC5) (dotted lines) mAbs, followed by FITC sheep–mouse IgG (Fab'2) and analyzed by FACS. Seventy percent of anti-DAF fluorescence was removed by the enzyme.

To investigate the source of the two DAF species, tearing was induced with onion, an agent known to stimulate reflex tear secretion. After collection (n = 2) and pooling, the induced tears were analyzed as above. As is apparent by comparison of the results shown in Figure 6 (right) to those in Figure 6 (left), a markedly increased proportion of the 100 kD DAF species was observed, suggesting that this DAF form derives from the lacrimal gland.

Table 2. Comparison of tear DAF concentrations with DAF concentrations in other body fluids*

	DAF (ng/ml)			
Donor	*Tears*	*Saliva*	*Urine*	*Plasma*
1	328	71	48	20
9	249	53	28	34
3	179	49	46	31
2	172	24	33	10
12	151	28	90	49
x̄ ± SD	216 ± 73	45 ± 19	49 ± 24	29 ± 15

* Obtained simultaneously.

Effects of Ocular DAF on Complement Activation

To establish whether tear DAF proteins contain convertase decay-accelerating activity and to determine if the main C3 convertase inhibitory activity in tears resides in DAF proteins as opposed to other molecules, $E^{sh}AC142$ bearing limited C2 were incubated with serially diluted tears in the absence and presence of pooled anti-DAF mAbs and the effects on decay of $\overline{C4b2a}$ convertase activity measured. As shown in Figure 7, in the absence of anti-DAF mAbs, incubation of a 1:81 dilution of tears with the intermediates decayed the preformed $\overline{C4b2a}$ by >50%. However, in the presence of anti-DAF mAbs, incubation of as much as 1:9 dilution of tears with the intermediates had minimal or no effect. Taken together, these findings indicate that tear DAF proteins are not only functional but also comprise >90% of the convertase inhibitory activity in tears.

Table 1. Comparative DAF concentrations in different ocular fluids

Tears		*Aqueous humor*		*Vitreous humor*	
Donor/age/sex	*DAF (ng/ml)*	*Donor/age/sex*	*DAF (ng/ml)*	*Donor/eye/age/sex*	*DAF (ng/ml)*
1/25/F	328	13/72/F	5.6	1/OD/43/F	15.8
2/35/F	172	14/72/F	2.8	2/OD/54/F	35.2
3/55/F	179	15/80/F	2.4	3/OD/76/F	23.1
4/24/M	216	16/81/F	4.7	3/OS/76/F	24.2
5/27/M	138	17/86/F	3.2	4/OD/79/F	14.9
6/30/M	325	18/62/M	15.0	4/OS/79/F	19.5
7/30/M	165	19/66/M	5.0	5/OD/18/M	48.4
8/33/M	174	20/67/M	3.2	5/OS/18/M	19.7
9/35/M	249	21/69/M	2.5	6/OD/64/M	18.3
10/46/M	1100	22/88/M	3.7	7/OD/66/M	17.7
11/54/M	700			7/OS/66/M	14.5
12/54/M	151			8/OS/73/M	15.8
x̄ ± SD	325 ± 289		4.8 ± 3.7		22.0 ± 8.2*

* Calculated using average values for donors 3, 4, 5, and 7.

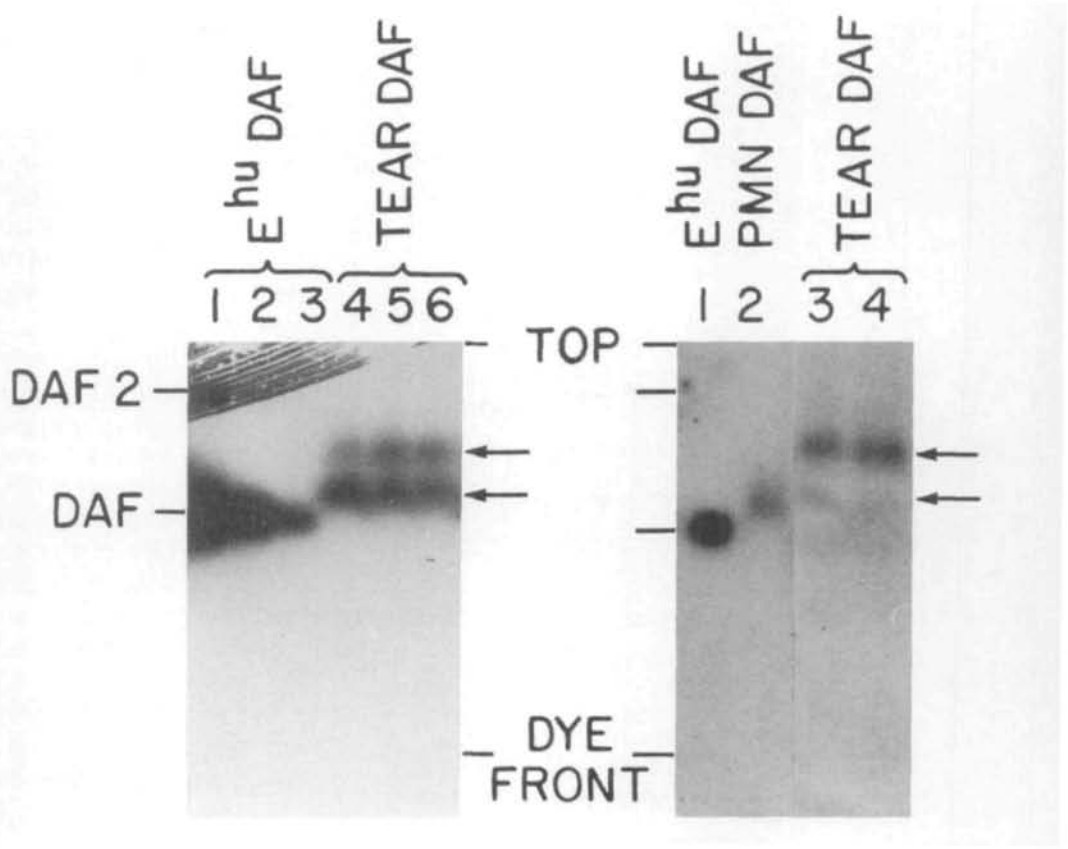

Fig. 6. Structural analyses of tear DAF antigen. *Left:* Tear samples and purified E^{hu} DAF protein were subjected to electrophoresis on 7.5% SDS-PAGE gels under nonreducing conditions. The separated proteins were transferred to nitrocellulose and the transfers were blotted with ^{125}I-labeled anti-DAF mAb IIH6. Sequential 2-fold decreasing concentrations of purified E^{hu} DAF protein are shown in lanes 1, 2, and 3. Decreasing amounts of 70-kD DAF protein are apparent. In lane 1, a minor amount of 140-kD DAF protein corresponding to DAF2 is seen. Nonstimulated tears from three healthy donors are shown in lanes 4, 5, and 6. In all cases, two DAF forms (arrows) with apparent Mrs of 72 kD and 100 kD are visualized. *Right:* Stimulated tear samples induced with onion, purified PMN DAF, and purified E^{hu} DAF were analyzed as above. E^{hu} DAF is shown in lane 1, PMN DAF in lane 2, and tears from two donors in lanes 3 and 4. Comparison with Panel A shows increased proportions of the 100-kD DAF form (top arrow).

Discussion

The principal finding of the current study is that DAF, a regulatory protein which is essential in blood cells to circumvent autologous complement activation on their surfaces,[18,19] is expressed in high levels on the ocular surface, a region not previously believed to require cell-associated regulatory mechanisms to prevent autologous complement-mediated injury. The regulatory protein is also found on conjunctival fibroblasts, corneal endothelium, trabecular meshwork, and retina, as well as on lacrimal gland acinar cells. Moreover, soluble DAF proteins are present in tears, aqeuous and vitreous humors, and in lacrimal gland acinae. The observation that levels of DAF on the corneal and conjunctival epithelial surface are high, exceeding those on blood cells,[20] argues that the protein must serve a regulatory function physiologically in this location.

The DAF protein demonstrated immunohistochemically on the ocular surface is not passively deposited there from the tear film, since our studies with in vitro cultured ocular cells showed that these same cell types synthesize DAF protein de novo. This was important to establish, since previous studies have shown that isolated DAF protein can incorporate into cell lipid bilayers.[19,24] The biosynthetic labeling performed using corneal endothelial cells, which exhibited the lowest levels of anti-mAb staining, additionally verified the DAF expression by this cell type and showed that the expressed surface protein exhibits an apparent Mr of 75 kD, approximating that of blood leukocyte and HeLa cell DAF protein.[20] PI-PLC digestion analyses demonstrated that, similar to surface DAF on these cell types, DAF proteins on conjunctival cells are membrane-anchored by a GIPL structure which is presumably, as in HeLa cells,[25,26] added posttranslationally in the rough endoplasmic reticulum.

The source of the soluble DAF detected in tears and other ocular fluids is unknown. The finding, however, by IRMA that levels of soluble DAF protein in tears are 4- to 7-fold higher than in plasma or other body fluids[32] is consistent with the notion that the protein is generated locally rather than transported in from the blood stream or from another location. Assuming that tear DAF protein arises from a local source, two possible mechanisms exist for its production. It may represent a product of preexisting membrane DAF molecules generated as a consequence of endogenous PI-PLC (or D)[40,41] digestion. Alternatively, it may represent a separate secreted DAF form. Since it is known that there is only one DAF gene,[42] the latter secreted DAF protein may arise from alter-

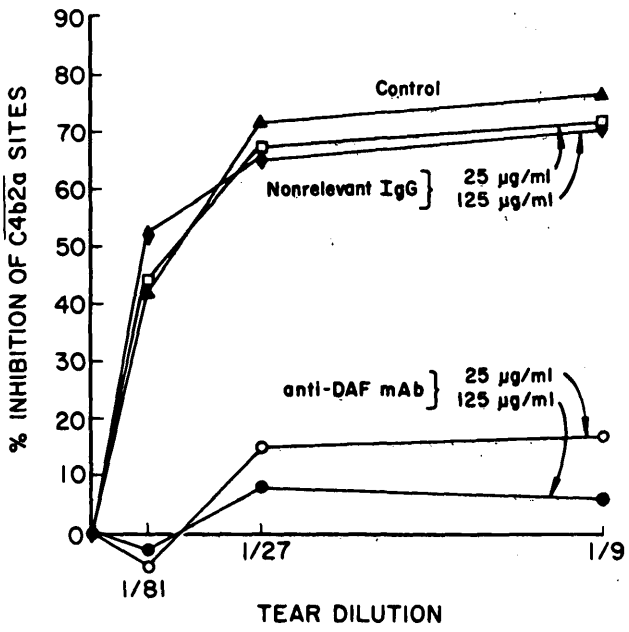

Fig. 7. Effect of tears in the absence or presence of pooled anti-DAF mAb on C4b2a hemolytic sites. E^{sh}AC142 (prepared with an amount of C2 giving ~1.0 site per cell after 15 min of decay at 30°C) were incubated alternatively with decreasing dilutions of tears, with tears preincubated for 15 min at 30°C with anti-DAF mAb IA10 and IIH6, 25 or 125 µg/ml each, or with myeloma proteins of corresponding subclasses at the same concentrations. After incubation, cells were diluted 10-fold with GVB-E and hemolytic sites developed by addition of 300 site-forming units of guinea pig C3-9. As can be seen, tears inhibited C4b2a hemolytic activity in a dose-dependent fashion and the inhibition was >90% abolished by anti-DAF mAbs, but not by nonrelevant IgG.

native mRNA splicing[43] or from differential post-translational processing pathways. Our observation by Western blotting that tear DAF consists of two molecular forms, one with an apparent Mr of 72 kD appropriate in size for a hydrophilic derivative of epithelial cell DAF,[25] and another form with an apparent Mr of 100 kD which has not been described previously for membrane DAF, raises the possibility that both mechanisms may operate. The Western blot comparison of nonstimulated and stimulated tears argues that the larger DAF form may derive from lacrimal secretions. However, further experiments with isolated lacrimal cells will be required to clarify this point.

In view of previous studies demonstrating that complement proteins are present and functionally active in tears and corneal tissue,[2-7] it seems reasonable to speculate that ocular DAF must function to protect the corneal and conjunctival surface from inadvertant autologous complement-mediated injury. There are abundant data which indicate that the ocular surface is constantly being challenged by a variety of substances, including infectious organisms, and that many of these agents activate the complement system.[44] Without ocular DAF, any C3 or C4 activation fragments which would condense with hydroxyl or amino groups on the ocular surface could serve as sites for assembly of the alternative and classical C3 convertases (C3bBb and C4b2a), resulting in amplification of C3b deposition (reviewed in Ref. 1). Unlike soluble complement inhibitors that have been described previously, such as lactoferrin,[15,16] the localization of membrane DAF within the plasma membrane of ocular cells and the anchoring of the protein by a GIPL structure would allow it to move readily in the plane of the plasma membrane to efficiently regulate assembly and expression of convertase activity wherever C4b or C3b might deposit.

Whether soluble tear DAF serves a separate or supportive complement inhibitory function is unknown. Our functional studies using sheep cell intermediates showed not only that tear DAF inhibits C3 convertases, but also that it constitutes >90% of the convertase inhibitory activity in tears, assuming no cross reactivity of anti-DAF mAb IA10. This result, obtained with whole tears, argues that although other molecules such as lactoferrin reportedly[15,16] can exhibit convertase inhibitory activity,[15,45,46] when the system is studied unseparated as it exists in vivo, convertase regulatory activity resides virtually entirely within DAF.

Whether deficiency or defective function of DAF could play a role in ocular surface disease has not yet been examined. Previous studies have established that complement-mediated damage to ocular tissues potentially can occur by two routes. In one route, injury can occur via a bystander effect. This can be observed in staphylococcal hypersensitivity marginal keratitis[47] or gram-negative bacterial keratitis,[48] wherein bacteria or bacterial antigens deposited in the peripheral cornea incite an antibody response and the resulting immune complexes activate the classical complement pathway and generate nascent C4b and C3b, which deposit on adjacent ocular tissues. In the other route, damage can occur directly. This can be observed in ocular cicatricial pemphigoid, wherein autoantibodies directed against conjunctival basement membrane antigens focus C4 (and C3) deposition at this site, inducing complement activation directly on ocular surface.[10,11,49] Similarly, this can be observed in Mooren's ulcer, in which binding of autoantibodies to corneal epithelium initiates complement activation on the corneal surface and induces corneal injury.[50] Whether ocular DAF regulatory activity in these diseases simply is overwhelmed or whether abnormalities in its expression or function can contribute to complement-mediated injury remains to be examined.

Key words: complement, decay-accelerating factor, eye, lacrimal gland, C3b

Acknowledgments

Technical assistance was provided by Dr. D. Hyder, Dr. W. Ratnoff, Dr. P. Shands, and Dr. R. Dutt. Ms. M. Abbuhl and Mr. R. Iwata assisted on immunochemical studies. Ms. G. Sossi assisted on cell cultures. Ms. B. Finke prepared the manuscript and figures.

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