

IDENTIFICATION OF THE COMPLEMENT  
DECAY-ACCELERATING FACTOR (DAF) ON EPITHELIUM  
AND GLANDULAR CELLS AND IN BODY FLUIDS

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A glycoprotein that inhibits complement activation is present on the surface of human erythrocytes ( $E^{hu}$ )<sup>1</sup> (1–3). This ~70 kD surface component (3), termed decay-accelerating factor (DAF), interacts with autologous C4b and C3b that inadvertently become associated with  $E^{hu}$  and prevents the covalently bound fragments from serving as sites for the uptake and conversion of C2 and factor B into enzymatically active C2a and Bb (4, 5). The interference by DAF with these C4b- and C3b-dependent cleavages prevents the assembly of C3 and C5 convertases of both complement pathways, i.e., all amplifying enzymes of the complement cascade (5). Unlike the serum regulatory proteins, C4 binding protein (C4bp) and factor H, which interact extrinsically with convertases on targets of complement (e.g., microorganisms or immune complexes), DAF acts only within the surface of the same cells, i.e., it is strictly an intrinsic membrane inhibitor (5). The ability of DAF to move freely in the plane of host  $E^{hu}$  membranes and efficiently restrict formation of autologous amplification convertases on their surfaces protects  $E^{hu}$  from injury that could arise from C4b or C3b deposited directly, e.g., via autoantibody, or in a bystander fashion as a result of complement activation in their vicinity.

Studies using flow cytometry and radioimmunoassays based on anti-DAF antibodies (6, 7) have shown that DAF is present not only on the surface of  $E^{hu}$  but also in the membranes of circulating neutrophils, monocytes, B and T lymphocytes, and platelets, as well as on vascular endothelium (8), all cell types that are in intimate contact with serum complement proteins. In blood cells, DAF levels are highest in neutrophils and monocytes, cell types that interact closely with complement-bearing targets during the events that precede their

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<sup>1</sup> *Abbreviations used in this paper:* A, rabbit hemolysin; AChE, acetylcholinesterase; Az, azide; C4bp, C4 binding protein; DAF, decay-accelerating factor;  $E^{hu}$ , human erythrocytes;  $E^h$ , sheep erythrocytes; GVB<sup>2+</sup>, veronal buffer with gelatin; mfVSG, membrane form variant surface glycoproteins; PNH, paroxysmal nocturnal hemoglobinuria; PPO, 2,5-diphenyloxazole; PI-PLC, phosphatidylinositol-specific phospholipase-C; SFU, site forming unit; sVSG, soluble form of VSG.

ingestion (6). Incorporation of exogenous DAF in vitro into affected E<sup>hu</sup> of patients with paroxysmal nocturnal hemoglobinuria (PNH) (9), a disorder in which DAF is deficient (4, 6, 10–12), diminishes the exaggerated uptake of C3b that characterizes the complement-sensitive PNH E<sup>hu</sup> in vivo (reviewed in reference 13). These findings that DAF deficiency is causally involved in the complement sensitivity of affected PNH cells have established that DAF activity in blood cells is essential under physiological conditions.

The present study was undertaken to investigate the distribution of DAF outside the vascular space.

### Materials and Methods

*Proteins, Antibodies, and Radiolabeling.* E<sup>hu</sup> DAF was purified from NP-40 extracts of stroma as described (5). The product appeared homogeneous upon analysis by SDS-PAGE and silver staining (see Fig. 1 of reference 5). Guinea pig C1 (14) and C3-9 (14) and human C4 (15), C2 (16), and C4bp (17) were purified as described. Murine anti-human DAF mAbs IA10, I1H6, and VIII A7 (6); anti-human C3b/C4b receptor (CR1) mAbs 57F, 44D, and 31D (18); and antimalaria mAbs 2D3, 2A10, 3D11, and 6G2 (19) were obtained as described. Each was purified from ascites fluid by ammonium sulfate precipitation, DEAE-Sephacel chromatography, and Sephadex G200 gel filtration. Peroxidase-labeled goat anti-murine Ig (affinity purified, IgG and IgM H and L chain specific) was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Protein A-Sepharose was from Pharmacia Fine Chemicals (Piscataway, NJ).

Proteins and antibodies were labeled with <sup>125</sup>I using Iodogen (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Iodinated products were purified by gel filtration followed by extensive dialysis.

*Immunohistochemical Studies.* Autopsy tissues and corneas (Oregon Lions Eye Bank, Portland, OR) preserved in RPMI medium were placed in OCT compound (Miles Laboratories Inc., Naperville, IL) and snap frozen. Cryostat tissue sections were fixed by sequential immersion for 2 min in cold acetone and 7 min in acetone/chloroform (1:1). After drying and washing three times with 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (PBS), the fixed sections were overlaid with a predetermined optimal concentration (see Results) of pooled anti-DAF mAbs (IA10, I1H6, and VIII A7) in PBS containing 10% goat serum or with corresponding amounts of pooled anti-CR1 mAbs (57F, 44D, 31D) or nonrelevant antimalaria mAbs (2D3, 2A10, and 3D11) of the same subclasses in the same buffer. The antibody-treated sections were incubated in a moist chamber at 20°C for 1 h, and after thorough washing three times in PBS, they were overlaid a second time with a 1:40 dilution in PBS of peroxidase-labeled goat anti-murine Ig antibody. After another incubation at 20°C for 30 min and a second thorough washing in PBS, the sections were stained by immersion for 10 min in 50 ml of PBS containing 500 µl of 3,3'-diaminobenzidine and 150 µl of 3% hydrogen peroxide. Stained sections were washed in PBS, counterstained for 90 s with hematoxylin, and after extensive washing with cold water and dehydration by baths in absolute ethanol and xylene, they were mounted in Permount.

*Affinity Purification of Urine DAF.* Fresh urine voidings were individually collected into 500-ml polystyrene bottles containing 5 ml of 100 mM PMSF, 100 µg/ml leupeptin, 1.2 mg/ml soybean trypsin inhibitor, 100 µg/ml aprotinin, 200 mM benzamidine, 500 mM EDTA, and 0.5% sodium azide, and after mixing were immediately frozen at -70°C. When sufficient numbers of bottles from a given individual (usually 8–10) were accumulated, they were simultaneously thawed, their contents pooled, and the pooled urine was concentrated to 10% of the original volume in an Amicon Corp. (Danvers, MA) DC-2 hollow fiber apparatus equipped with an HIP30 cartridge. After dialysis against 150 mM NaCl, 25 mM Tris, 20 mM EDTA, pH 7.4 and clarification by centrifugation at 10,000 g for 60 min, the urine concentrate was applied to an affinity column composed of anti-DAF mAb IA10 coupled to CNBr-Sepharose and crosslinked with glutaraldehyde (~100

ml concentrate/5 mg antibody) that was preequilibrated in the same buffer. After loading, the column was washed with 10 volumes of 0.5 M NaCl, 25 mM Tris, 20 mM EDTA, pH 7.4 and then eluted with 0.05 M diethylamine, 140 mM NaCl, pH 11.5 (20). Column fractions (usually 0.5 ml) were immediately neutralized by collection into tubes containing 0.1 ml of 1.0 M Tris, pH 6.0, saturated with glycine. Each fraction was assayed for DAF antigen by two-site radioimmunometric assay (see below) and positive fractions were examined by SDS-PAGE and silver staining. Urine DAF-containing fractions that were free of protein contaminants were pooled, dialyzed against PBS, and frozen in aliquots at  $-70^{\circ}\text{C}$ . Overall yield varied from 20–100  $\mu\text{g}$  urine DAF/L urine. The product gave a single band on Western blots developed with anti-DAF monoclonals and on autoradiographs after SDS-PAGE of the  $^{125}\text{I}$ -labeled product.

*Radioimmunometric and Hemolytic Assays.* Quantitations of DAF concentrations by two site radioimmunometric assay were performed as described in (6). Briefly, samples and DAF standards were added in duplicate 25- $\mu\text{l}$  aliquots to the wells of 96 well U-bottomed plastic microtiter plates (Becton Dickinson & Co., Oxnard, CA) precoated for 2 h at  $20^{\circ}\text{C}$  with 50  $\mu\text{l}$  of 20  $\mu\text{g}/\text{ml}$  anti-DAF mAb IA10 and the wells were blocked with PBS containing 1% BSA. After incubation at  $20^{\circ}\text{C}$  for 2 h and washing three times with the blocking buffer containing 0.05% Tween-20, 25  $\mu\text{l}$  of  $^{125}\text{I}$ -labeled anti-DAF mAb I1H6 (containing  $\sim 10^5$  cpm) was added, and after further incubation at  $20^{\circ}\text{C}$  for 1 h and extensive washing, bound DAF antigen in the samples was quantitated by comparison of counts in cut-out wells that received samples to those in wells that received DAF standards.

Hemolytic assays were performed as described (5) using complement intermediates prepared from sheep erythrocytes ( $\text{E}^{\text{sh}}$ ) sensitized with 300 site forming units (SFU) of rabbit hemolysin (A) per cell.  $\text{E}^{\text{sh}}\text{AC14}$  were prepared by incubating  $\text{E}^{\text{sh}}\text{A}$  sequentially with 300 SFU of guinea pig C1 and 10 SFU of human C4.  $\text{E}^{\text{sh}}\text{AC142}$  were prepared by incubating the resulting  $\text{E}^{\text{sh}}\text{AC14}$  with sufficient human C2 to yield cells bearing one hemolytic site of C4b2a after washing and decay for 15 min at  $30^{\circ}\text{C}$ . Incubations of  $\text{E}^{\text{sh}}\text{AC142}$  with  $\text{E}^{\text{hu}}$  DAF and urine DAF were performed at  $30^{\circ}\text{C}$  in 145 mM NaCl, 2.5 mM veronal, pH 7.3, containing 0.5 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{CaCl}_2$ , and 0.1% gelatin (GVB $^{2+}$ ). C4 hemolytic sites were quantitated by incubation of  $\text{E}^{\text{sh}}\text{AC14}$  with 300 SFU of C2 followed by 300 SFU of guinea pig C3-9 and C4b2a sites developed by direct incubation of  $\text{E}^{\text{sh}}\text{AC142}$  with 300 SFU of guinea pig C3-9.

*SDS-PAGE and Western Blotting.* SDS-PAGE was conducted on 7.5% linear slab gels according to the method of Laemmli (21). Gels of  $^{35}\text{S}$ -labeled samples were washed with DMSO and treated with 2,5-diphenyloxazole (PPO) followed by water before drying. Autoradiography and fluorography were performed at  $-70^{\circ}\text{C}$  on X-Omat XAR-5 film (Eastman Kodak Co., Rochester, NY).

For Western blots, proteins were subjected to SDS-PAGE on 7.5% gels under nonreducing conditions. The separated proteins were transferred to nitrocellulose using a Transblot Apparatus (Bio-Rad Laboratories, Richmond, CA). After blocking for 1 h at  $37^{\circ}\text{C}$  with PBS containing 5% BSA and 0.05% azide (AZ) (PBS-BSA-AZ), nitrocellulose strips were incubated at  $20^{\circ}\text{C}$  for 1 h with  $^{125}\text{I}$ -labeled anti-DAF mAb I1H6, washed three times with PBS-BSA-AZ, dried, and loaded into film cassettes.

*Preparation of Cell Extracts and Immunoprecipitation.* For radioimmunometric assays, cell monolayers were washed with PBS and extracted for 20 min at  $0^{\circ}\text{C}$  with 50  $\mu\text{l}/10^6$  cells of 0.5% NP-40 in PBS containing 1 mM PMSF and 5 mg/ml synthetic elastase inhibitor [Suc-(OMe)-Ala-Ala-Pro-Val-MCA] (Peninsula Laboratories, Inc., Belmont, CA). The NP-40 extracts were centrifuged at 12,000  $g$  for 15 min and the supernatant was transferred to new tubes. For SDS-PAGE and Western blot analyses, cell monolayers were scraped from culture dishes with 2% SDS in 20 mM Tris, pH 7.5, 100  $\mu\text{l}/\text{ml}$  trasyolol (250  $\mu\text{l}/10^6$  cells) using a rubber policeman. The SDS extracts were boiled for 10 min, diluted fivefold with 50 mM Tris, pH 7.4, containing 190 mM NaCl, 100  $\mu\text{l}/\text{ml}$  trasyolol, 6 mM EDTA, and 2.5% Triton X-100 (Tris-NaCl-EDTA), the mixture was centrifuged at 10,000  $g$  for 15 min, and the supernatant was transferred to a new tube.

Samples of SDS-cell extracts (usually 600  $\mu\text{l}$ ) or of extracellular fluids or culture supernatants (200–4,000  $\mu\text{l}$ ) were preabsorbed for 2 h at  $20^{\circ}\text{C}$  with 100  $\mu\text{l}$  of 10% Protein

A-Sepharose in Tris-NaCl-EDTA and were centrifuged. Supernatants were transferred to new tubes, pooled anti-DAF mAbs IA10, I1H6, and VIIIA7 (5  $\mu\text{g}/\text{ml}$  each) or nonrelevant antimalaria mAbs (of the same subclasses, 5  $\mu\text{g}/\text{ml}$  each) were added, and the mixtures were rotated for 2 h at 20°C. Immune complexes were precipitated by addition of 100  $\mu\text{l}$  of fresh 10% Protein A-Sepharose in Tris-NaCl-EDTA buffer, further rotation for 1 h at 20°C, and centrifugation. The beads were transferred to new tubes, washed twice with Tris-NaCl-EDTA buffer, and immunoprecipitated proteins were extracted by addition of 50  $\mu\text{l}$  of SDS-PAGE sample buffer followed by boiling for 3 min.

**Biosynthetic Studies.** Biosynthetic labeling was performed as described (22). Semiconfluent (60–70%) HeLa cell monolayers were prepared in 60-mm culture plates. The cells were preincubated for 1 h before labeling with cysteine (Cys)-free RPMI containing 10% dialyzed FCS. [ $^{35}\text{S}$ ]Cys (50  $\mu\text{Ci}$ ; New England Nuclear, Boston, MA) was added in 100- $\mu\text{l}$  aliquots to replicate plates. The plates were placed at 37°C, and after various times, the plates were transferred to ice, the medium was removed, and the cells were extracted with 2% SDS in Tris buffer as described above.

## Results

**Expression of DAF by Epithelial Cells.** To determine whether DAF is expressed by cell types other than those in the vascular space, immunohistochemical analyses of various tissues were performed with anti-DAF mAbs. Since B lymphocytes express  $7 \times 10^4$  DAF molecules per cell (6), initial studies were carried out with lymph node specimens to establish optimal conditions for the detection of DAF in tissue sections. Sections of the lymph nodes (fixed as described in Materials and Methods) were incubated with serial dilutions of pooled anti-DAF monoclonals, corresponding dilutions of pooled anti-CR1 mAbs, or with nonrelevant (antimalaria) mAbs as controls. After washing and development with peroxidase-labeled goat anti-mouse Ig (see Materials and Methods), the sections were examined by light microscopy. Bright anti-DAF staining of germinal center lymphocytes was observed after treatment of the sections with 250 ng/ml of pooled anti-DAF mAbs, while no staining was observed with 10  $\mu\text{g}/\text{ml}$  of nonrelevant mAbs. The anti-DAF staining was most intense in lymphocytes also stained by anti-CR1 mAbs (100 ng/ml) but was also apparent in lymphocytes that were anti-CR1-negative.

Samples of different tissues were next collected from surgical and autopsy specimens, frozen sections of the specimens were fixed and stained, and the sections were examined as described above for the lymph nodes. Selected photographs of the results are shown in Figs. 1 and 2. Unexpectedly, strong anti-DAF staining of epithelial cells in multiple locations was observed. The anti-DAF staining of epithelium was striking in cornea (Fig. 1A), conjunctiva (Fig. 1B), oral mucosa and salivary glands (Fig. 1C), esophagus (Fig. 2A), upper and lower GI tract (not shown), and in kidneys and urinary tract (not shown). The anti-DAF staining was also prominent in endocardium (not shown), synovial cells (Fig. 2B) lining joint spaces, and in endometrial and epithelial cells lining uterus (Fig. 2C) and cervix (not shown). In kidneys, epithelium was positive in proximal tubules, descending and ascending loops of Henle and in collecting ducts. In contrast to previously reported findings (23, 24) and control studies with anti-CR1 antibodies, anti-DAF staining in renal cortex was not localized to glomeruli (podocytes) but was diffuse. In the urinary tract, urethelium was strongly positive in medullary spaces, ureter, bladder, and urethra (not shown). In all sites with layered epithelium, intensity of anti-DAF staining increased with cell maturation

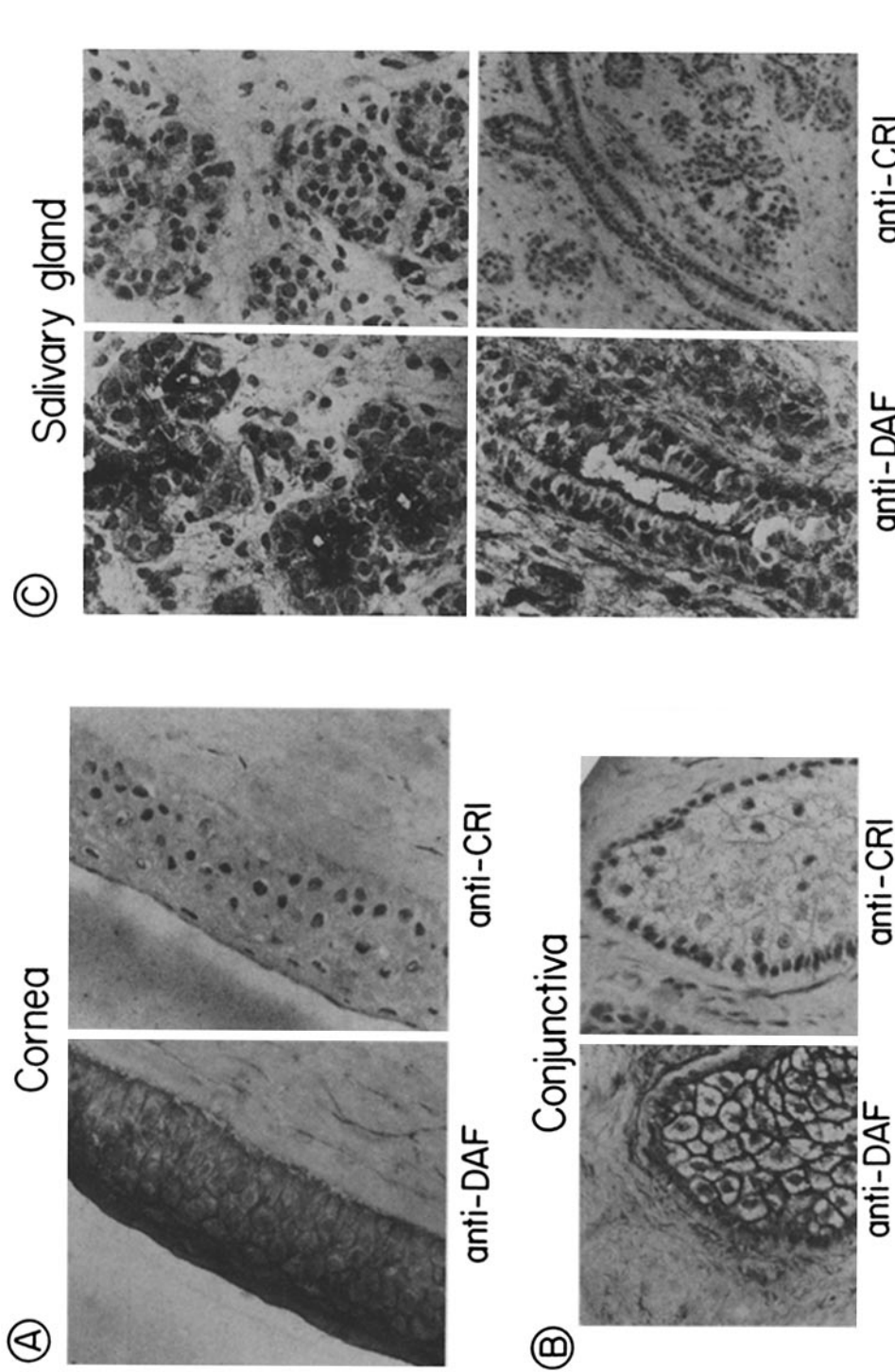


FIGURE 1. Various tissues were examined immunohistochemically for DAF using anti-DAF mAbs and the peroxidase method. Anti-DAF and control anti-CRI-stained sections of tissues are shown on the left and right hand sides of each panel, respectively. (A) Marked anti-DAF staining of corneal epithelium is apparent. The staining is localized to the epithelial cell membrane and increases in intensity in upper epithelial cell layers. (B) Sections of tarsal conjunctiva are shown. Prominent anti-DAF staining of glandular tissues is apparent. (C) Anti-DAF staining of salivary glands is observed. The staining is most intense on internal luminal surfaces.

and staining was greatest in epithelial margins and on surfaces.

In addition to the epithelial cell staining, intense anti-DAF positivity of exocrine gland cells in many sites (Figs. 1, *B* and *C*; 2*A*) was apparent and strong anti-DAF staining within extracellular matrix in several tissues (Figs. 1*B*; 2, *A* and *C*) was noted. As observed with epithelial cells, the intensity of anti-DAF staining of glandular cells increased with cell maturation. The anti-DAF staining within extracellular matrix consistently occurred in a fibrillar pattern. The fibrillar staining was prominent in fibrous sheaths surrounding myocardial muscle bundles, interstitium underlying endocardium, and connective tissue adjacent to synovium (Fig. 2*B*). The nature of the fibrils is unknown. Patterns of anti-DAF staining of epithelium, glandular cells, and connective tissue similar to those shown (in Figs. 1 and 2) were also observed in pleura, pericardium, and peritoneum.

*Soluble DAF Forms in Extracellular Fluids.* The possibility that DAF antigen might be present in extracellular fluids adjacent to epithelial cell surfaces surrounding various extracellular compartments was next investigated. Samples of different extracellular fluids were obtained and the specimens were assayed for DAF antigen by two-site radioimmunoassay using anti-DAF mAbs.

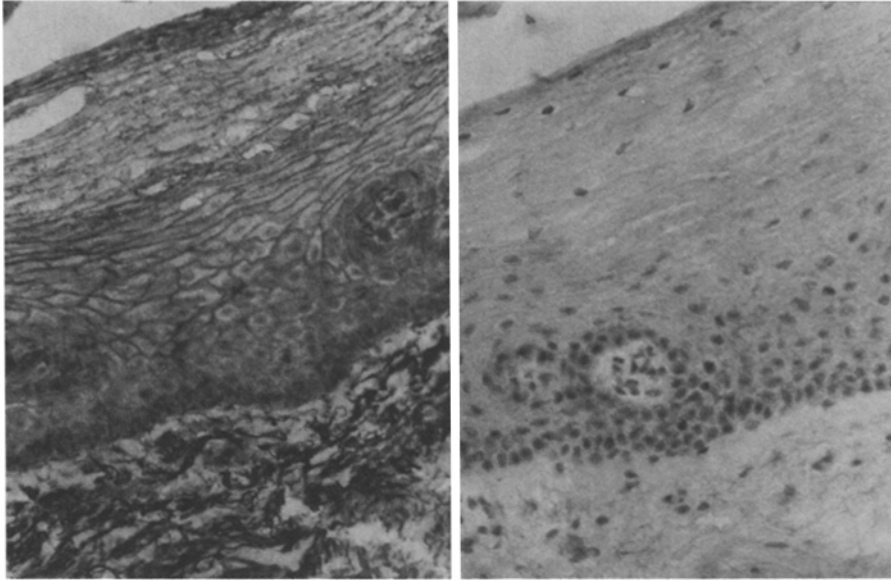
The results of analyses of tears, saliva, synovial fluid, cerebrospinal fluid, and of plasma are summarized in Table I. DAF antigen was present in all fluids tested, and the concentrations were in most instances greater than that in plasma. Measurements of DAF in urine samples collected from 10 normal individuals are given in Table II. 75–500 ng/ml of antigen was found in spot specimens. Quantitations of antigen in 24-h urine collections revealed outputs as high as 500  $\mu\text{g}$ , a value corresponding to ~3% of the total intravascular blood cell-associated membrane DAF.

To study the nature of DAF antigen present in the body fluids, the antigens detected in tears, saliva, and urine were precipitated with anti-DAF mAbs and examined on Western blots. The antigens in tears and saliva migrated with apparent  $M_r$  higher (>100,000) (not shown) than  $E^{\text{hu}}$  DAF, while that in urine migrated with a slightly lower  $M_r$  (~67,000) (see below). The more abundant urine antigen was isolated for further structural and functional investigations. Large volumes (~5 liters each) of urine from six normal individuals were collected into vessels containing multiple protease inhibitors. The six urine collections were separately concentrated, and the DAF antigen in each purified by affinity chromatography and compared by Western blot analysis (Fig. 3*A*). In all cases, the urine DAF variant was ~3 kD smaller than membrane DAF isolated from  $E^{\text{hu}}$ .

Urine DAF from one individual (Fig. 3*A*, lane 1) was next tested in complement assays for functional activity and its regulatory properties were compared to those of  $E^{\text{hu}}$  membrane DAF. Unlike purified  $E^{\text{hu}}$  DAF, which incorporated into  $E^{\text{sh}}$ AC14 and markedly inhibited C4 hemolytic activity on washed  $E^{\text{sh}}$ AC14 intermediates (Fig. 3*B*), the urine DAF had little activity in this assay. However, when the urine molecule was incubated in the presence of  $E^{\text{sh}}$ AC142 bearing preformed C4b2a, it accelerated C4b2a decay with an efficiency comparable to that of serum C4bp. The decay-accelerating effect was blocked by addition of pooled anti-DAF mAbs. A simple interpretation of these findings is that urine

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Esophagus

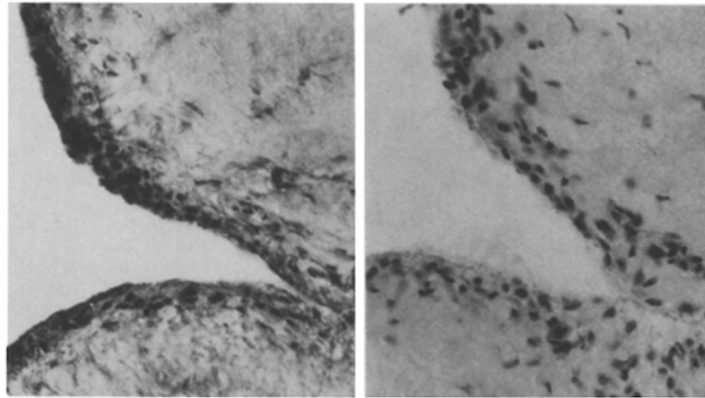


anti-DAF

anti-CRI

Ⓑ

Synovium

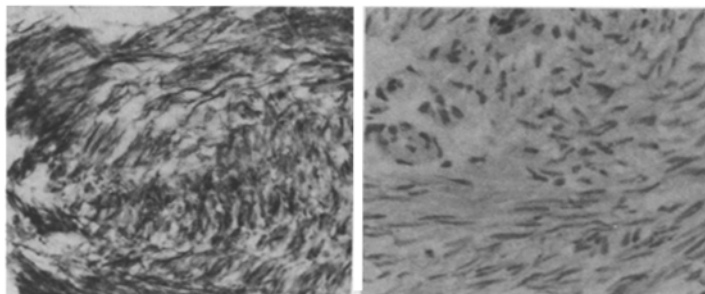


anti-DAF

anti-CRI

Ⓒ

Uterus



anti-DAF

anti-CRI

TABLE I  
DAF Levels in Body Fluids

Fluid	Number of samples	DAF concentration $\pm$ SD
		<i>ng/ml</i>
Plasma	12	64 $\pm$ 26
Saliva	9	112 $\pm$ 51
Tears	2	344, 68
Synovial fluid	8*	168 $\pm$ 99
Cerebrospinal fluid	1 <sup>‡</sup>	38

\* Osteoarthritis.

<sup>‡</sup> Evaluation for headache.

TABLE II  
Levels of Urine DAF in Normal Individuals

Donor	DAF	Volume	Total DAF
	<i>ng/ml</i>	<i>ml/24 h</i>	<i><math>\mu</math>g/24 h</i>
RW	150	1,000	150
AC	170	1,700	290
RC	127	1,600	200
RR	100	3,200	320
VN	200	2,550	510
AF	250	1,400	350
MD	350	1,200	420
RM	400	1,200	480
EW	75	1,700	128
SK	75	2,450	185
DE	200	1,200	240
Mean			298

DAF is unable to incorporate into cell membranes and therefore cannot exert its effect intrinsically. Indeed, urine DAF is less hydrophobic than membrane DAF. When subjected to chromatography on Phenyl-Sepharose beads, all of the membrane DAF bound tightly to the resin, while the urine molecule remained >90% in the fluid phase, indicating that it differs markedly with respect to hydrophobicity (not shown).

*Appearance of a Soluble DAF Form Resembling Urine DAF from HeLa Cell and Foreskin Epithelium Culture Supernatants.* In view of the concurrent findings of high levels of DAF expression by epithelial cells and the presence of soluble DAF forms in adjacent body fluids, *in vitro* studies were next performed to investigate the possible epithelial cell origin of the extracellular DAF species. For this purpose, human foreskin epithelium and the HeLa epithelial cell line were cultured.

FIGURE 2. (A) Anti-DAF staining of esophageal mucosa is evident. Prominent staining of glands and fibers in underlying submucosal connective tissue additionally can be appreciated. (B) Localized anti-DAF staining of synoviocytes and fibers is observed. Staining of fibers is again appreciated. (C) The staining of fibers underlying uterine endometrium is prominent.



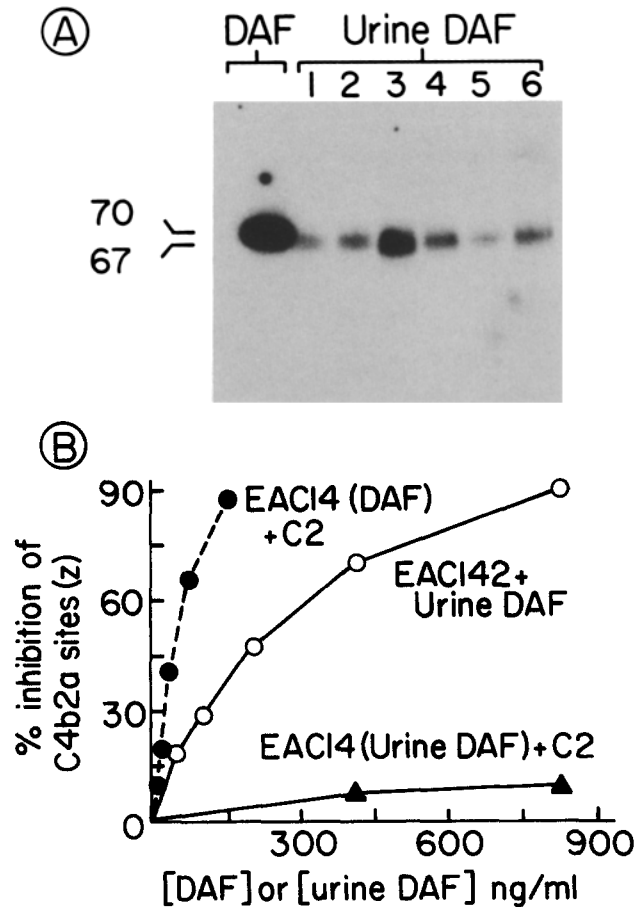


FIGURE 3. Comparison of the gel mobilities and functional properties of urine DAF and  $E^{hu}$  membrane DAF. (A) Urine DAF samples were immunoprecipitated from urine concentrates prepared from six normal individuals and compared with  $E^{hu}$  membrane DAF on Western blots after electrophoresis of proteins on 7.5% SDS-PAGE gels under nonreducing conditions. In the case of all six urine DAF isolates a single band  $\sim 3,000$  smaller in apparent  $M_r$  than  $E^{hu}$  membrane DAF was observed.  $M_r \times 10^{-3}$  shown at left. (B) Purified urine DAF and  $E^{hu}$  membrane DAF were incubated with  $E^{hu}$ AC14, and after washing, residual C4b sites were developed by addition of C2 and C3-9. Alternatively, the urine DAF was incubated for 15 min at  $30^\circ\text{C}$  with  $E^{hu}$ AC142 and remaining C4b2a sites were assayed by addition of C3-9. A control using urine DAF in the presence of anti-DAF mAbs was performed (not shown). Unlike  $E^{hu}$  membrane DAF, the urine DAF had no effect on  $E^{hu}$ AC14 but it inhibited hemolytic activity of preformed C4b2a with efficiency comparable to C4bp.

As shown in Fig. 4, immunohistochemical analyses of both cultured cell types showed strong anti-DAF staining. Immunoradiometric assays of the HeLa cell extracts revealed  $\sim 2 \times 10^5$  DAF molecules per cell, approximately fourfold more DAF than present in polymorphonuclear leukocytes (6). Purification of HeLa cell DAF by immunoprecipitation with anti-DAF mAbs and Western blot analyses of the immunoprecipitate demonstrated that the HeLa epithelial cell DAF was similar in apparent size ( $M_r \sim 72,000$ ) to blood mononuclear cell membrane DAF (see reference 6).

Ⓐ Foreskin epithelium    HeLa cells

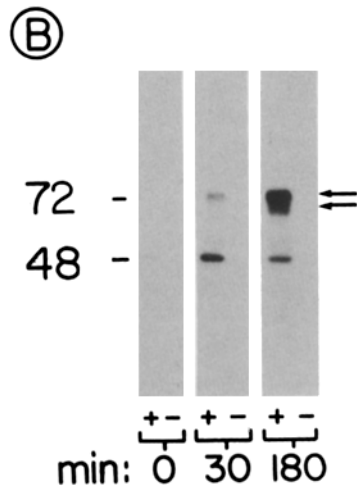
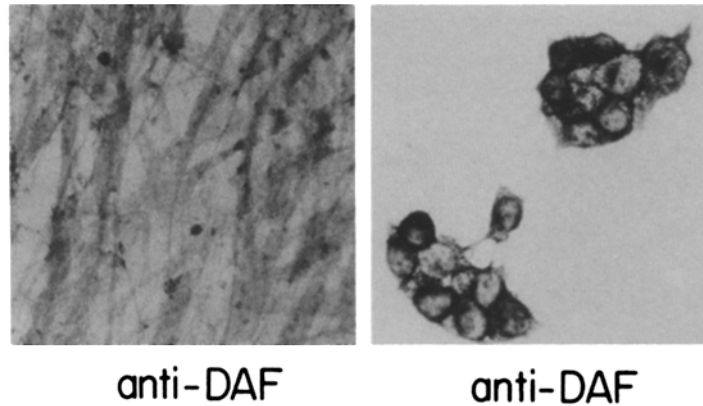


FIGURE 4. Human foreskin epithelium and the HeLa cell line were cultured. Immunohistochemical analyses of the cultured cells showed strong anti-DAF staining (A). Immunoradiometric assays of the HeLa cell extracts revealed  $\sim 2 \times 10^5$  membrane DAF molecules per cell. (B) Fluorographs of a continuous labeling study of DAF biosynthesis in HeLa cells. After a 1-h incubation with Cys-free RPMI and labeling with [ $^{35}$ S]Cys for the time periods shown, the monolayers were extracted with SDS and newly labeled DAF proteins were isolated by immunoprecipitation and analyzed by SDS-PAGE/fluorography. The lanes designated (-) show the results of control studies with nonrelevant mAbs.  $M_r \times 10^{-3}$  shown at left.

HeLa cells were next cultured, and during log phase growth, the culture supernatant was analyzed at different times by radioimmunometric assay for DAF antigen. The results showed a progressive accumulation of the antigen in the culture medium (Fig. 5). In contrast, no DAF antigen was detectable in the supernatant of K562 erythroleukemia cells that also contain DAF.

HeLa cells were next cultured for various times in the presence of [ $^{35}$ S]Cys and newly synthesized DAF in the membranes of the cells and in the culture

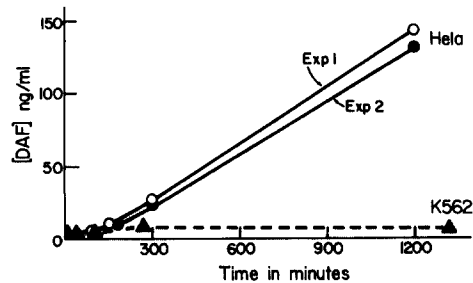


FIGURE 5. HeLa cells were cultured, and during log phase growth, the culture supernatant was assayed at different times for DAF antigen by radiometric assay. While no DAF antigen was detectable in the supernatant of DAF-containing ( $4 \times 10^6$  cells/ml) K562 erythroleukemia cells, progressively increasing levels of DAF antigen were measurable in the HeLa cell ( $2 \times 10^6$  cells/ml) supernatants.

supernatant were isolated by specific immunoprecipitation and analyzed by SDS-PAGE/radioautography. In addition to mature 72 kD HeLa membrane DAF and a 48 kD intracellular DAF precursor (22), a DAF species with apparent  $M_r$  5,000 smaller than the mature membrane molecule was isolated from the cells. The larger but not the smaller DAF species was detectable on autoradiographs of SDS-PAGE gels of anti-DAF immunoprecipitates of  $^{125}\text{I}$  surface-labeled HeLa cells. A DAF molecule of comparable size to the smaller DAF species was recovered from the culture supernatant (not shown). When examined next to urine DAF on Western blots, this 67 kD DAF molecule appeared similar in gel mobility.

### Discussion

In previous studies, we and others showed that DAF is present on the surface of blood cells (6, 7) and vascular endothelium (8) where its activity is essential to protect these cell types from serum complement proteins. In the present study, we investigated the distribution of DAF in tissues and body fluids outside the vascular space. Immunohistochemical analyses revealed large amounts of DAF antigen on the surface of epithelium in multiple sites and radioimmunometric assays identified soluble forms of the antigen in several extracellular fluids. Purification of the most abundant extracellular DAF form, urine DAF, and analyses of its properties demonstrated that, like  $\text{E}^{\text{hu}}$  membrane DAF, it possessed C4bp-like complement regulatory activity. Unlike  $\text{E}^{\text{hu}}$  DAF, however, it was unable to incorporate into membranes and on SDS-PAGE appeared  $\sim 3$  kD smaller in apparent size. Studies using primary cultures of foreskin epithelium and the epithelial cell line HeLa demonstrated *de novo* biosynthesis of DAF protein in amounts greatly exceeding those in blood cells. Analyses of the culture supernatants of these studies disclosed the release by these cell types of a soluble DAF form closely resembling urine DAF in SDS-PAGE gel mobility.

In our immunohistochemical analyses, epithelial cells stained brightly for DAF in all locations where surface epithelium is found. These included cornea, conjunctiva, oral and gastrointestinal mucosa, renal tubular epithelium and urinary tract urethelium, uterine endometrium and cervical epithelium, pleural and pericardial serosa, and synoviocytes lining diarthrodial joint spaces. In

addition to the anti-DAF staining of these surface cells, strong anti-DAF staining of exocrine glandular cells was observed throughout many tissues. In the case of both surface epithelium and glandular cells, intensity of anti-DAF staining increased in cells closest to the surface. Within glands, the most intense staining was observed in lumens. The increase could represent induction of DAF expression with cell differentiation/maturation. In vitro studies with epithelial cell lines (reviewed in reference 25) have shown that expression of certain membrane proteins is polarized to the nonadherent exterior cell surface.

The physiologic function that DAF serves on epithelial and glandular cell surfaces is not yet apparent. The principal secretory immunoglobulin is IgA and it can only activate the alternative pathway of complement. However, other immunoglobulins, e.g., IgM, are found in smaller amounts in secretions, as are complement components (26). The biological significance of complement activation in secretions is not understood. It is possible that the membrane DAF functions to prevent complement-mediated injury to the epithelial and glandular cells in a fashion similar to its postulated role in cells found in the vascular space. Alternatively, it is possible that the epithelial and glandular cell DAF could have some other, noncomplement-related activity.

Our finding that in skin, joints, and uterus, DAF is associated with fibrils raises a number of questions. No staining in blood vessel walls, in cartilage, or of reticular or elastic fibers occurred. The staining pattern followed that of type I collagen fibers on sections counter-stained with Giemsa or Toluidine Blue, raising the possibility that the DAF-positive fibrils may contain type I collagens, which in different tissues are related but not identical (27). We cannot exclude the possibility that the staining represents a spurious crossreaction, although this is unlikely because various mAbs reacting with different DAF epitopes gave identical results. Interestingly, asymmetric forms of acetylcholinesterase (AChE), a protein that is structurally related to DAF (see below), are associated with the extracellular matrix at neuromuscular junctions and in fish electric organs. In *Disopyge tchudii* electric organ, an asymmetric form of AChE consisting of 12 catalytic subunits attached to a triple helical collagen-like tail is anchored non-covalently to the extracellular matrix through a linkage that appears to involve heparin sulfate proteoglycan (29). It is tempting to speculate that the association of DAF with collagen fibers that contain hydroxyl or amino groups able to condense with nascent C4b or C3b could serve to reduce the potential for complement-mediated damage in connective tissues.

The identification of large amounts of DAF antigen in various extracellular fluids by two-site radioimmunoassay (Table I) was surprising. Since positivity in this assay depends on reactivity with two mAbs directed against different DAF epitopes, detection of the antigens by this method implied that they must contain a significant portion of the E<sup>hu</sup> membrane DAF structure. Immunoprecipitation of urine DAF with anti-DAF mAbs and Western blot analysis showed that the antigen migrated with apparent  $M_r$  3,000–8,000 smaller than membrane DAF of E<sup>hu</sup> (Fig. 3A) and other blood cells (6, 7). The small difference in apparent size between this extracellular DAF form and blood cell DAF molecules was associated with a marked difference in hydrophobicity as shown by its lack of adsorption to Phenyl-Sepharose and inability to incorporate

into membranes (Fig. 3C). The demonstration that urine DAF functioned (Fig. 3C) similarly to the serum regulatory protein, C4bp, indicated that urine DAF was able to interact with the classical pathway C3-convertase and therefore contained a DAF functional site. The dissociation of membrane incorporation and convertase inhibitory activity observed in these studies thus implies that membrane anchorage and complement regulatory activity in membrane DAF are mediated by different structures.

Structural studies of E<sup>hu</sup> membrane DAF have shown that it is an amphipathic protein (22, 30). Unlike most membrane proteins that contain polypeptide membrane anchors consisting of hydrophobic amino acids, E<sup>hu</sup> membrane DAF possesses a glycolipid membrane anchor. This unconventional non-amino acid anchoring structure is linked covalently to the COOH-terminus of DAF polypeptide and is similar to anchoring structures that recently have been described in (membrane form) variant surface glycoproteins (mfVSGs) of trypanosome (31, 32) of leishmania (33) parasites, murine thymocyte Thy-1 antigen (34, 35), and E<sup>hu</sup> AChE (36–38). It is composed of an oligosaccharide, containing ethanolamine(s) and nonacetylated-glucosamine, that is attached through the glucosamine glycosidic bond to a phospholipid composed of inositol and fatty acids (22). The fatty acids are inserted in the cell lipid bilayer and permit lateral mobility of the attached protein. The phospholipid can be partially cleaved by phosphatidylinositol-specific phospholipase-C (PI-PLC) (30, 22, 39). This cleavage releases membrane DAF from cells and generates a hydrophilic DAF derivative of slightly reduced apparent  $M_r$  similar to urine DAF.

Our studies with foreskin epithelium and HeLa cells (Fig. 4B) verified that epithelial cells synthesize membrane DAF and additionally showed that this cell type releases a DAF form into cell culture supernatants (Fig. 5). Analysis of the released DAF molecule showed that it was ~5,000 smaller in apparent  $M_r$  than epithelial cell membrane DAF and similar in mobility on gels to urine DAF. This finding suggests that urine DAF may be synthesized by the adjacent urethelium. Other extracellular DAF forms could likewise be generated by adjoining epithelia and/or glandular cells. The soluble DAF forms could be products of different genes than those that encode membrane DAF molecules, or could arise from alternative processing of DAF mRNA occurring after transcription resulting in secreted rather than membrane forms. Studies with VSGs and Thy-1 antigen have shown that the cDNAs that code for these proteins predict COOH-terminal extension peptides of 15–31 amino acids that are not found in the mature proteins (40, 41). There is evidence from biosynthetic studies with VSGs, that the missing COOH-terminal peptide is excised and replaced with the mfVSG glycolipid anchor immediately after formation of VSG polypeptide on ribosomes (42, 43). Consistent with this notion, studies of DAF biosynthesis (22) have shown that anchor components are associated with 48 kD pro-DAF before processing of pro-DAF in the Golgi. The lack of incorporation of the membrane DAF glycolipid or the attachment of a different moiety to the DAF COOH-terminus could result in a soluble DAF form. Alternatively, the soluble DAF species could derive from membrane DAF molecules themselves. Soluble forms of VSGs, termed sVSGs, can be isolated from trypanosome cultures (31). The sVSGs resemble urine DAF in that they are similar in  $M_r$  to the corresponding mfVSGs

and markedly less hydrophobic. They have been shown to arise via the action of an endogenous PI-PLC present in trypanosome membranes (reviewed in references 44 and 45). Structural analyses of the soluble DAF forms for components of the membrane DAF glycolipid anchor proximal to the known site of PI-PLC cleavage could clarify whether the soluble DAF forms arise by a similar mechanism.

### Summary

Decay-accelerating factor (DAF) is a 70 kD membrane regulatory protein that prevents the activation of autologous complement on cell surfaces. Using immunohistochemical methods and a radioimmunoassay based on mAbs to DAF, we found large amounts of membrane-associated DAF antigen on the epithelial surface of cornea, conjunctiva, oral and gastrointestinal mucosa, exocrine glands, renal tubules, ureter and bladder, cervical and uterine mucosa, and pleural, pericardial and synovial serosa. Additionally, we detected soluble DAF antigen in plasma, tears, saliva, and urine, as well as in synovial and cerebrospinal fluids. While plasma, tear, and saliva DAF are larger than erythrocyte ( $E^{hu}$ ) membrane DAF by Western blot analysis, urine DAF is slightly smaller (67,000) in  $M_r$ . Unlike purified  $E^{hu}$  DAF, however, urine DAF is unable to incorporate into the membrane of red cells. Although its inhibitory activity on the complement enzyme C3-convertase is lower than that of  $E^{hu}$  DAF, it is comparable to that of serum C4 binding protein (C4bp). Biosynthetic studies using cultured foreskin epithelium and HeLa cells disclosed DAF levels ( $\sim 2 \times 10^5$  molecules/cell) exceeding those on blood cells. In addition, these studies revealed the synthesis of two DAF species, one with apparent  $M_r$  corresponding to that of epithelial cell membrane DAF and the other to urine DAF, suggesting that the urine DAF variant arises from adjacent epithelium. The function of DAF in body fluids is unknown, but the observation that urine DAF has C4bp-(or factor H-)like activity shows that it could inhibit the fluid phase activation of the cascade.

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