DISTRIBUTION OF DECAY-ACCELERATING FACTOR IN THE PERIPHERAL BLOOD OF NORMAL INDIVIDUALS AND PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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Decay-accelerating factor (DAF),[1] which is an inhibitor of complement and is present on erythrocyte membranes, was first described by Hoffmann (1, 2) and later isolated and characterized as an M_r 70,000 protein by Nicholson-Weller et al. (3). DAF very effectively prevents the amplification steps of the cascade by interfering with assembly of the C3-convertases, C4b2a and C3bBb, and the C5-convertases, C4b2a3b and C3bBb3b (3–5). The precise mechanism of action of DAF is unknown but several lines of evidence indicate that it binds to C4b or C3b and competitively prevents the interaction with C2 and factor B. Erythrocyte DAF functions only in the membrane in which it is located, that is, DAF does not act on C3- or C5-convertases formed on neighboring cells or on foreign substrates, such as bacteria and immune complexes (5). For this reason it has been suggested that the physiological role of DAF is to protect cells from damage by autologous complement.

This hypothesis is in agreement with the finding that erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH), an acquired syndrome characterized by an unusual susceptibility of red cells to complement activation (6), are DAF deficient (4, 7–9). Platelet and leukocyte abnormalities are also found in PNH, but the role of DAF in these disorders is unclear (10–13).

The idea that DAF's role is to prevent damage to autologous cells by complement, receives further support from the findings reported here. In the present study we used monoclonal antibodies to measure the amounts of DAF on

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[1] *Abbreviations used in this paper:* AZ, sodium azide; BSA, bovine serum albumin; CR1, C3b/C4b receptor; DAF, decay-accelerating factor; DGVB^{++}, 2.47 mM sodium veronal buffer, pH 7.3, 72.7 mM NaCl, 2.5% dextrose, 0.1% gelatin, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$; E^{hu}, human erythrocytes; $EAC14_{lim}$, antibody-sensitized sheep erythrocyte bearing an excess amount of C1 and a limiting amount of C4; $EAC14_{lim}$(DAF) and $EAC14_{lim}$(buffer), $EAC14_{lim}$ cells sensitized with DAF or treated with buffer as a control; FACS, fluorescence-activated cell sorter; IRMA, immunoradiometric assay; NP-40, Nonidet P-40, a nonionic detergent; IA10, IIH6, and VIIIA7, three anti-DAF monoclonal antibodies; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocytes; PMSF, phenylmethylsulfonyl fluoride; PNH, paroxysmal nocturnal hemoglobinuria; SDS, sodium dodecyl sulfate; Z, number of hemolytic sites per cell.

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peripheral blood cells from normal individuals and from patients with PNH. We found that DAF is not only present on the membrane of red cells but also on other blood elements in contact with the complement system, i.e., platelets, neutrophils (polymorphonuclear leukocytes [PMN]), monocytes, B and T lymphocytes. In PNH patients, we found DAF deficiencies in platelets and in all types of leukocytes, in addition to the previously characterized red cell defect.

Materials and Methods

Buffers, Antibodies, Complement Components, and Red Cell Hemolytic Intermediates. We used as buffers Dulbecco's phosphate-buffered saline (PBS), and isotonic veronal buffer containing 2.47 mM sodium veronal buffer, pH 7.3, 72.7 mM NaCl, 2.5% dextrose, 0.1% gelatin, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$ ($DGVB^{++}$). Monoclonal anti–human CR1 (57F) (14), rabbit anti–glycophorin A (5), guinea pig C1 (15), and human C4 and C2 (16, 17) were prepared as described.

Antibody-sensitized sheep erythrocytes carrying 300 hemolytic sites of guinea pig C1 and one to two sites of human C4 ($EAC14_{lim}$) were prepared as described (5). $EAC14_{lim}$ cells were sensitized with DAF by incubating 5×10^8/ml $EAC14_{lim}$ in $DGVB^{++}$ with an equal volume of DAF (50 ng/ml) diluted in $DGVB^{++}$ for 30 min at 37°C. These cells, termed $EAC14_{lim}$(DAF), were washed twice with $DGVB^{++}$. As a control, $EAC14_{lim}$ cells were treated in the same way with buffer alone and designated as $EAC14_{lim}$(buffer).

Purification of DAF. DAF was purified from pooled human erythrocyte (E^{hu}) stroma. The fractionation sequence described by Nicholson-Weller et al. (3, 8) was initially used. E^{hu} ghosts from 4 U blood were extracted with butanol and the resulting butanol-saturated water phase was subjected to successive chromatographies using DEAE-Sephacel, phenyl-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), hydroxyapatite Bio-gel HT (Bio-Rad Laboratories, Richmond, CA) and lentil-lectin-Sepharose (Pharmacia Fine Chemicals). During purification, DAF was assayed by its ability to accelerate the decay of EAC142 (3). The preparation of DAF obtained by this procedure was treated with monoclonal antibodies to CR1 (57F) coupled to Sepharose beads to remove contaminating CR1. Analysis of this preparation by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (18) and silver staining (Bio-Rad Laboratories) showed the presence of several impurities, some of which were identified as glycophorins by Western blotting developed with specific rabbit antibodies.

To further purify DAF we used DEAE-Sephacel chromatography and high performance liquid chromatography through the TSKG 3000 SW gel filtration matrix (LKB Produkter, Bromma, Sweden), as follows. The pool of impure DAF was dialyzed against 0.01 M sodium phosphate buffer, pH 8.0, containing 0.05% Nonidet P-40 (NP-40), and applied to a 20 ml DEAE-Sephacel column equilibrated in the same buffer. The column was eluted with an NaCl and pH gradient formed with 50 ml of the starting buffer and 50 ml of 0.01 M sodium phosphate buffer, pH 6.8, containing 0.3 M NaCl and 0.05% NP-40. DAF eluted at 8–10 mS, slightly ahead of the main glycophorin peak. Fractions containing DAF and only trace amounts of glycophorin were pooled, concentrated over an Amicon PM30 (Amicon Corp., Lexington, MA), and applied to the TSKG 3000 SW equilibrated with 0.1% NP-40 in PBS. Four discrete peaks were obtained, but only the first (void volume) contained DAF activity and a band of M_r 70,000, as evaluated by SDS-PAGE and silver staining (See Fig. 1 of reference 5). No glycophorin was detected in this preparation by Western blotting.

Production of Monoclonal Antibodies Against DAF. BALB/c mice were immunized twice with 4 μg of purified DAF in complete Freund's adjuvant. The mice whose sera had antibody titers >1:200 by Western blotting were boosted intravenously with 20 μg of pure DAF in 0.1% NP-40 in PBS. After 3 d their spleen cells were fused with myeloma cells (X63Ag8.653). Culture supernatants of the resulting hybridomas were tested for anti-DAF activity as described below. Three positive monoclonal antibodies, IA10 (IgG2a), and IIH6 and VIIIA7 (IgG1), were obtained.

Monoclonal antibodies were purified from culture supernatants with protein A–Seph-

arose (Pharmacia Fine Chemicals) by the method described by Ey et al. (19) and labeled with ^{125}I with Iodogen (Pierce Chemical Co., Rockland, IL). Larger amounts of purified antibodies were obtained from ascites fluids of mice bearing the hybridoma by precipitation with 50%-saturated ammonium sulfate, DEAE-Sephacel chromatography, and Sephadex G-200 gel filtration.

Detection of Antibodies Against DAF in Hybridoma Supernatants. Purified DAF was subjected to SDS-PAGE under nonreducing conditions on a 7.5% gel and then transblotted onto nitrocellulose. The paper was blocked with 5% bovine serum albumin (BSA) in PBS containing 0.02% sodium azide (AZ) (5% BSA-PBS-AZ) for 1 h at 37°C, and cut into 5 × 50 mm strips. A piece of filter paper of the same size was placed onto each strip. 200 μl of hybridomas culture supernatants were applied to the filter paper. Controls contained culture supernatants from unrelated monoclonals, hypoxanthine, aminopterin, thymidine (HAT) medium (20), and immune or normal mouse serum. After 2 h incubation at room temperature in a humid chamber, the filter papers were removed and the strips washed individually, three times, with 5 ml each of 5% BSA-PBS-AZ. Groups of 10 washed strips were incubated with 10 ml of 5% BSA-PBS-AZ containing ^{125}I-labeled, affinity-purified goat anti–mouse IgG (10^7 cpm; Cappel Laboratories, Cochranville, PA) at room temperature for 1 h, and washed three times with 10 ml each of 5% BSA-PBS-AZ. Autoradiography was performed by exposing the dried strips to Kodak X-Omat XAR-5 film (Eastman Kodak Co., Rochester, NY).

Preparation of Peripheral Blood and Tonsil Cells. Citrated blood was centrifuged at 500 *g* for 10 min at 4°C. After removal of plasma and buffy coat, erythrocytes were washed three times with PBS. Blood samples from 35 normal individuals were obtained through the courtesy of Dr. Santiago Rodriguez de Cordoba (New York Blood Center). Red cells from three individuals were fractionated on the basis of their density by centrifugation into Percoll (Pharmacia Fine Chemicals) gradient (21). Platelets were collected from platelet-rich plasma by centrifugation for 10 min at 2,500 *g* and washed three times with PBS containing 10 mM EDTA. Contaminating erythrocytes and leukocytes were removed by centrifugation at 200 *g* for 10 min. PMN and mononuclear cells were separated from fresh citrated blood by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals) followed by dextran sedimentation (22). The contaminating erythrocytes were removed by hypotonic lysis. Monocytes and lymphocytes were obtained from a Percoll gradient as described by Wright and Silverstein (23). >95% of the cells from the upper band were positive by indirect immunofluorescent staining with monoclonal antibody OKM5, which is monocyte specific. Lymphocytes were obtained from the lower band and contained <3% contaminating monocytes (23).

Buffy coat cells were collected from the citrated blood and washed twice with PBS by centrifugation at 500 *g* for 5 min. Contaminating erythrocytes were removed by hypotonic lysis.

T lymphocytes were obtained by staining blood mononuclear cells with phycoerythrin-conjugated monoclonal anti-Leu-1 (Becton Dickinson Monoclonal Center, Mountain View, CA), followed by sorting the Leu-1^+ cells in a fluorescence-activated cell sorter (FACS) (Cytofluorograf 50-H; Ortho Instruments, Westwood, MA).

Tonsil lymphocytes were purified from tonsils by centrifugation on Ficoll-Paque. T and B lymphocytes were isolated as described (24).

Preparation of Cell Extracts. Erythrocytes: 5 × 10^8 packed, washed erythrocytes were lysed with 120 μl of 1% NP-40 in PBS containing 50 μg/ml of the synthetic elastase inhibitor, Suc (OMe)-Ala-Ala-Pro-Val-MCA (Peninsula Laboratories, Inc., Belmont, CA) and 1 mM phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Co., St. Louis, MO). After 1 h incubation at room temperature, 1 ml of 1% BSA in PBS containing the same protease inhibitors was added and the mixture centrifuged at 12,000 *g* for 15 min. The supernatant was used immediately. The concentration of erythrocytes was determined by measuring OD_{541} of the hemolysed sample. The DAF contents obtained by the two-site immunoradiometric assay were multiplied by 1.25 since this procedure solubilized only 80% of extractable DAF, as determined by the following experiment. Increasing amounts of packed E^{hu} (1.5 × 10^8 to 2.4 × 10^9) were extracted as above and the DAF contents of

the extracts were determined by two-site immunoradiometric assay. The amounts of extracted DAF per E^{hu} were plotted as a function of the number of E^{hu} initially treated with 1% NP-40. We found that this amount increased slightly but progressively as the number of detergent-solubilized E^{hu} decreased. The maximum amount of extractable DAF was obtained by extrapolation to zero E^{hu} (Fig. 1).

Platelets: The platelet suspension (2×10^9/ml in PBS) was mixed with the same volume of 1% NP-40 in PBS containing 50 μg/ml of the elastase inhibitor, 1 mM PMSF, 5 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.) and 100 U/ml Trasylol (Mobay Chemical Corp., Pittsburgh, PA). The mixture was incubated for 20 min on ice and centrifuged at 12,000 *g* for 15 min to remove the small amounts of remaining insoluble materials.

Leukocytes: The leukocyte suspension (1×10^8/ml in PBS) was mixed with the same volume of 1% NP-40 in PBS containing the protease inhibitors. The mixture was incubated for 20 min on ice and centrifuged at 1,500 *g* for 15 min to remove intact nuclei, and the supernatant was centrifuged at 12,000 *g* for 15 min to remove insoluble materials.

Immunoprecipitation of DAF from Cell Extracts. Protein A–Sepharose (100 μl) was incubated with 5 ml culture supernatant of monoclonal antibody IA10 for 1 h at room temperature. IA10–Protein A–Sepharose was washed twice with PBS and then incubated with NP-40 extracts of different kinds of cells (20-μl beads per 1 ml the extract) for 1 h at 4°C. The bound DAF molecules were eluted from the beads by incubation for 5 min at 80°C with 50 μl sample buffer consisting of 5% SDS, 125 mM Tris-HCl, pH 6.8, 10% glycerol, 0.01% bromphenol blue. The eluates were subjected to SDS-PAGE using 7.5% gels and transferred electrophoretically to a nitrocellulose paper. DAF was detected in the paper by ^{125}I-labeled IA10, IIH6, or VIIIA7, followed by autoradiography.

In other experiments, erythrocytes, mononuclear cells, and PMN were surface labeled with ^{125}I using Iodogen (1 mCi Na^{125}I per 2×10^8 E^{hu} or 1×10^7 leukocytes). NP-40 extracts were prepared and DAF was immunoprecipitated with IA10 or control nonrelevant antibodies. The DAF band was analyzed by SDS-PAGE under reducing and nonreducing conditions, followed by autoradiography.

Two-Site Immunoradiometric Assay (IRMA) for DAF in Cell Extracts. The wells of plastic

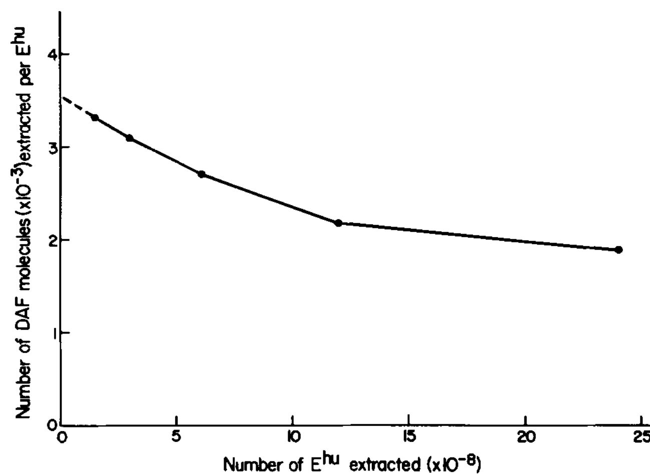

FIGURE 1. Estimation of efficiency of DAF extraction from E^{hu}. Various amounts of washed, packed normal E^{hu} (1.5×10^8 to 2.4×10^9) were extracted for 60 min at room temperature with 120 μl of 1% NP-40 in PBS containing 1 mM PMSF and 50 μg/ml synthetic elastase inhibitor. Each mixture was diluted to a concentration of 1.5×10^8 E^{hu}/ml with 1% BSA-PBS containing the same inhibitors, and centrifuged at 12,000 *g* for 15 min. The supernatants were recovered and DAF contents measured by two-site IRMA. As shown, the efficiency of extraction increased as the number of solubilized E^{hu} decreased. Under the conditions used for all the measurements (5×10^8 E^{hu}) included in the present paper, we calculate that only 80% of the total amount of DAF was extracted from the red cells.

plates (96 U-bottomed wells; Becton, Dickinson & Co., Oxnard, CA) were coated with anti-DAF monoclonal IA10 (capturing antibody) by incubation with 50 μl of 20 μg/ml IA10 in PBS containing 0.02% AZ, at room temperature for 2 h. The wells were filled with 1% BSA, PBS, 0.02% AZ and kept in the cold overnight to saturate excess binding sites. The wells were then washed three times with 1% BSA, 0.05% Tween 20, PBS, 0.02% AZ, and 25 μl cell extracts or serial dilutions of pure DAF, in 1% BSA, 0.05% Tween 20, PBS, 0.02% AZ, were added in duplicate. After incubation for 2 h at room temperature and three washes with the same buffer, 25 μl of ^{125}I-labeled anti-DAF monoclonal IIH6 (revealing antibody) (4 ng, 1.2×10^5 cpm) were added to each well. After incubation for 1 h at room temperature, the wells were washed four times with the same buffer, cut, and counted. The amount of DAF in the cell extracts was calculated from a standard curve obtained with purified DAF. This curve, in which counts bound of the revealing antibody were plotted as a function of DAF concentration, was linear up to 250 ng DAF/ml. The protein concentration of the pure DAF solution was measured by the method of Lowry et al. (25) using BSA as a reference protein.

FACS Analysis of Cell Surface DAF. Erythrocytes, platelets, or buffy coat cells from normal individuals and PNH patients were treated with anti-DAF monoclonal antibodies and fluorescein-conjugated goat $F(ab')_2$ anti–mouse IgG (heavy and light chains) (Cappel Laboratories), and then analyzed by FACS. Erythrocytes (10^6 cells in 25 μl of 1% BSA, PBS, 0.1% AZ) were treated with 25 μl of a mixture of three monoclonal antibodies against DAF (5 μg each per milliliter in 1% BSA, PBS, 0.1% AZ) or, as a control, with a mixture of nonrelevant monoclonal antibodies of the same subclass. After incubation for 30 min on ice, the red cells were washed twice, resuspended in 25 μl of the same buffer, incubated with 25 μl of a 1:50 dilution of the second antibody for 30 min on ice, and washed again with the same buffer. Buffy coat cells (10^6 cells in 25 μl of the same buffer) were incubated with 25 μl of 5 μg/ml IA10 or control monoclonal antibodies for 30 min on ice. This was followed by washing and incubation with the second antibody as above. Platelets were stained in the same way as the red cells, except that 10 mM EDTA was added to the buffer.

Isolation of DAF-positive and DAF-negative Erythrocytes from PNH Patients. DAF-positive erythrocytes: Washed erythrocytes from PNH patients were incubated with 60% acidified human serum (10^8 E^{hu}/ml) for 30 min at 37°C (7, 26). Unlysed erythrocytes were pelleted and washed twice with PBS. DAF-negative cells were selectively lysed by this procedure (see below).

DAF-negative erythrocytes: Erythrocytes from PNH patients (10^8 E^{hu}/ml in PBS, 0.02% AZ) were mixed with an equal volume of 10 μg/ml IA10 anti-DAF. After incubation for 60 min on ice, cells were washed once with PBS, 0.02% AZ, resuspended at 10^9 E^{hu}/ml, and then loaded into a protein A–Sepharose CL-4B column (2×10^8 E^{hu}/ml packed gel) equilibrated in the same buffer. The column was closed and kept for 15 min at room temperature. Unbound cells were collected by washing the column with five column volumes of the same buffer. >90% of the unbound cells were DAF-negative as assessed by FACS analysis.

Patients. Three patients with PNH at the New York University Medical Center were studied. Patient GC, a 46-yr-old Hispanic female, had severe disease of 17 yr duration. She was receiving norandralone, prednisone, and transfusions of frozen-thawed erythrocytes. Patient SB, a 47-yr-old Caucasian female, had milder disease of 4 yr duration. She was receiving no medication and maintained a stable hematocrit of ~38 vol %, which, during episodes of hemolysis, decreased to 24%. Patient VR is a 46-yr-old Hispanic female, with pancytopenia and persistent thrombocytopenia (platelets, 20,000/mm^3) and intermittent anemia. She is being treated with norandralone and prednisone. All patients had a positive acid serum (Ham) test and sucrose hemolysis test.

Results

Characterization of Anti-DAF Monoclonal Antibodies IA10, IIH6, and VIIIA7. The specificity of the anti-DAF monoclonal antibodies was shown by

Western blotting experiments using as antigen either purified DAF or the total extract of E^{hu} membranes (Fig. 2). Culture supernatants of the anti-DAF hybridomas were incubated with nitrocellulose strips previously blotted with pure DAF (Fig. 2*A*). All anti-DAF antibodies, but not HAT medium or monoclonal antibodies to CR1 (57F), detected the DAF band. When the nitrocellulose strips were blotted with crude membrane extracts of E^{hu}, the results were identical (Fig. 2*B*).

IA10, IIH6, and VIIIA7 recognized different epitopes on the DAF molecule, since the solid phase two-site IRMA described in Materials and Methods could

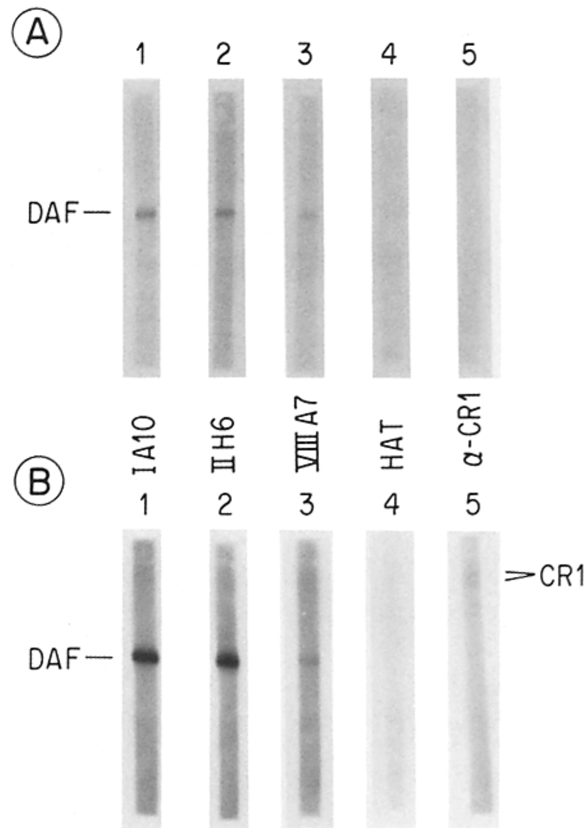

FIGURE 2. Detection of antibodies to DAF in culture supernatants of hybridomas by means of Western blotting. (*A*) Pure DAF was subjected to SDS-PAGE under nonreducing conditions and then transblotted onto a nitrocellulose paper. Each strip (containing 10 ng DAF) of the paper was incubated for 2 h at room temperature with 200 μl of culture supernatants from hybridomas IA10 (lane *1*), IIH6 (*2*), VIIIA7 (*3*), and 57F anti-CR1 (*5*); or with HAT medium (*4*). After washing, the strips were incubated with ^{125}I-labeled goat anti-mouse IgG, washed, and exposed to film. Anti-DAF antibodies, but not anti-CR1 or HAT medium, detected the DAF band. (*B*) Washed E^{hu} (5×10^8 cells) were lysed with 15 mM Tris-HCl buffer, pH 7.8. Ghosts were collected by centrifugation, solubilized with nonreducing SDS sample buffer by boiling, and subjected to SDS-PAGE on a 7.5% gel, followed by Western blotting. Each nitrocellulose strip (containing total membrane proteins derived from 3×10^7 E^{hu}) was incubated with culture supernatant from hybridomas IA10 (lane *1*), IIH6 (*2*), VIIIA7 (*3*), and 57F anti-CR1 (*5*); or with HAT medium (*4*). This was followed by incubation with the second antibody and autoradiography as described above. Anti-DAF antibodies detected one band with M_r 70,000. Control anti-CR1 detected two types of CR1.

be performed with any combination of two different monoclonal antibodies, while negative results were obtained if a single one was used as the capturing and revealing reagent.

The effect of monoclonal antibodies on the activity of DAF was tested using DAF-treated $EAC14_{lim}$ cells (Table I). IA10, IIH6, or VIIIA7 alone had no or only a slight effect on DAF activity. However, when combinations of these antibodies were used, stronger effects were observed, except for the combination of IA10 and VIIIA7. A mixture of three monoclonal antibodies at concentrations of 9 μg/ml each inhibited 98% of DAF activity. These results suggest that the binding sites of these antibodies do not coincide with the active site of the DAF molecule. Perhaps the inhibition of DAF activity by the combination of antibodies results from aggregation of the molecule and/or steric hindrance.

Demonstration of the Presence of DAF on Different Cell Populations by Immunopre-

TABLE I

Effect of the Monoclonal Antibodies to DAF on the Inhibitory Activity of DAF on C4b Hemolytic Sites

Concentration of monoclonal antibodies			Percent reversal of DAF activity*
IA10	IIH6	VIIIA7	
	μg/ml		
9	—	—	7.0
—	9	—	27.5
—	—	9	0
1	1	—	44.6
3	3	—	70.3
9	9	—	78.6
1	—	1	11.7
3	—	3	15.7
9	—	9	18.3
—	1	1	29.9
—	3	3	56.4
—	9	9	72.0
1	1	1	55.8
3	3	3	84.5
9	9	9	98.0

100 μl of 1×10^8/ml $EAC14_{lim}$(DAF) in $DGVB^{++}$ were incubated at 30°C for 15 min with 100 μl of various combinations of monoclonal antibodies to DAF, or $DGVB^{++}$ as controls. $EAC14_{lim}$(buffer) cells were treated with $DGVB^{++}$ in the same way to measure input C4b hemolytic sites. After incubation, the cells were washed once with $DGVB^{++}$ and resuspended in $DGVB^{++}$. Then the C4b hemolytic sites were developed with C2, followed by C3–9, as described (5). Degree of lysis was determined by OD_{412} of released hemoglobin in the supernatant.

* Percent reversal of DAF activity was calculated as follows: ([Z of anti-DAF–treated $EAC14_{lim}$(DAF) − Z of $EAC14_{lim}$(DAF)]/[Z of $EAC14_{lim}$(buffer) − Z of $EAC14_{lim}$(DAF)]) × 100, where Z is the number of hemolytic sites per cell. Z of $EAC14_{lim}$(buffer) and $EAC14_{lim}$(DAF) in this experiment were 2.480 and 0.700, respectively.

cipitation. NP-40 extracts of platelets, PMN, blood mononuclear cells, tonsil mononuclear cells, tonsil B and T lymphocyte fractions, and a solution of pure erythrocyte DAF, were incubated with IA10-bearing protein A–Sepharose beads. DAF was eluted from the beads and subjected to Western blotting. All the cell types contained DAF (Fig. 3). DAF from all cells shared at least three epitopes, since all bands could be detected by ^{125}I-labeled IIH6 or VIIIA7, as well as IA10. The M_r of the DAF band was different in different cell types (Fig. 3). DAF from platelets and PMN appeared larger (~5,000 M_r) than DAF from erythrocytes, while DAF from mononuclear cells was of intermediate size. The size difference was also shown by immunoprecipitation of DAF from surface-labeled cells, followed by SDS-PAGE under both reducing and nonreducing conditions (not shown).

Quantitation of DAF in Different Cell Types from Normal Individuals. DAF contents were measured in NP-40 extracts of cells using a two-site IRMA. As shown in Table II, erythrocytes from normal individuals had a mean of 3.3 ± 0.4 × 10^3 molecules per cell, ranging between 4.0 and 2.1 × 10^3. Platelets and PMN had 2.1 ± 0.3 × 10^3 and 85 ± 15 × 10^3 molecules per cell, respectively. The unseparated mononuclear cells, purified monocytes, and lymphocytes from peripheral blood had 35.7 ± 4.4, 67.9 ± 15, and 32.8 ± 9.7 × 10^3 molecules per cell, respectively. Leu-1$^+$ T lymphocytes purified by FACS from the peripheral blood of a normal individual had 9.0 × 10^3 molecules per cell. Unseparated

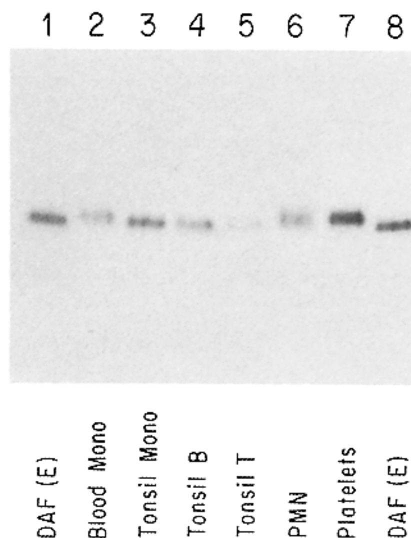

FIGURE 3. Detection of DAF on different cell types by immunoprecipitation. The NP-40 extracts (1 ml each) of different cell types were incubated with anti-DAF IA10–bearing protein A–Sepharose (20 μl of beads) for 1 h in the cold. The bound DAF molecules were eluted by boiling the beads in nonreducing SDS sample buffer, then subjected to SDS-PAGE and Western blotting as described above. DAF bands were detected by incubating the nitrocellulose paper with ^{125}I-labeled IA10 (10^7 cpm) for 1 h at room temperature, followed by washing and autoradiography. (Lanes *1* and *8*) Pure DAF from E^{hu}. (*2–7*) Extracts from cells: blood mononuclear cells (*2*), tonsil mononuclear cells (*3*), tonsil B lymphocytes (*4*), tonsil T lymphocytes (*5*), PMN (*6*), and platelets (*7*). All cell types showed the DAF band. The size of DAF was different among different cells.

TABLE II

Distribution of DAF Among Different Cell Types from the Peripheral Blood of Normal Individuals and PNH Patients

Cells	DAF Molecules ($\times 10^{-3}$) per cell ± standard deviation
Normal	
Erythrocytes	3.3 ± 0.4 (35)*
Platelets	2.1 ± 0.3 (4)
PMN	85.0 ± 15.0 (4)
Mononuclear, total	35.7 ± 4.4 (3)
Monocytes	67.9 ± 15.0 (3)
Lymphocytes	32.8 ± 9.7 (3)
PNH	
Erythrocytes	1.1 (SB), 1.0 (GC), 0.9 (VR)
Platelets	0.6 (SB), not detectable (GC)

* Number of samples examined.

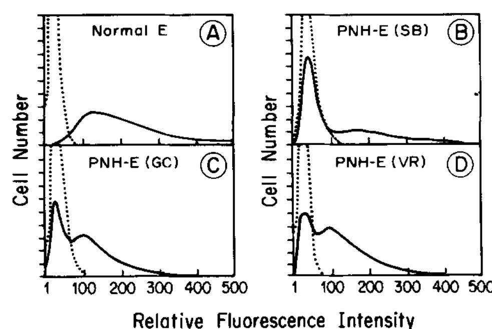

FIGURE 4. FACS analysis of normal and PNH erythrocytes using antibodies to DAF. Erythrocytes from a normal individual and three PNH patients were stained with a mixture of three monoclonal anti-DAF and fluorescein isothiocyanate-goat $F(ab')_2$ anti-mouse IgG (solid lines). Dashed lines represent control cells stained with a mixture of monoclonal antibodies of the same subclasses but directed to nonrelevant antigens. (*A*) Normal; (*B*) PNH (patient SB); (*C*) PNH (GC); (*D*) PNH (VR). The FACS patterns of erythrocytes from several other normal individuals were very similar to the one shown in this figure.

mononuclear cells from a tonsil had 42×10^3 molecules per cell. Purified B and T lymphocytes from this tonsil had 54×10^3 and 17×10^3 molecules per cell, respectively.

The surface expression of DAF on different cell types from peripheral blood was next studied by indirect immunofluorescent staining, followed by FACS analysis. Erythrocytes and platelets were stained with a mixture of the three monoclonal antibodies to increase the fluorescence intensity. Buffy coat cells were stained only with IA10 anti-DAF. PMN, monocytes, and lymphocytes were separated according to their light scattering properties and analyzed separately.

As shown in Figs. 4*A*, 5*A*, and 6*A*, all cell types expressed surface DAF. The fluorescence intensity appeared normally distributed in all cells, except in red cells, in which the distribution of DAF was very skewed in all individuals. For example, the mean relative fluorescence intensity of the sample in Fig. 4*A* was

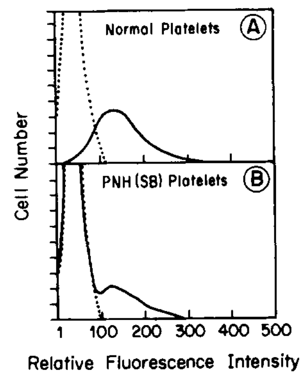

FIGURE 5. FACS analysis of normal and PNH platelets using antibodies to DAF. Platelets from a normal individual (*A*) and PNH patient SB (*B*) were stained with a mixture of three monoclonal anti-DAF and fluorescein isothiocyanate–goat F(ab′)$_2$ anti–mouse IgG (solid lines). Dashed lines represent control staining with nonrelevant monoclonal antibodies. PNH patient SB had two populations of circulating platelets.

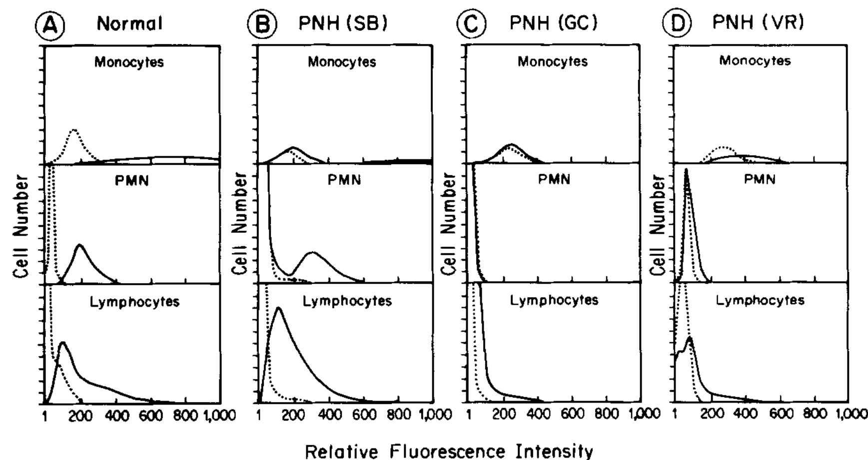

FIGURE 6. FACS analysis of leukocytes from a normal individual and three PNH patients using antibodies to DAF. Buffy coat cells from a normal individual (*A*) and three PNH patients, SB (*B*), GC (*C*), and VR (*D*) were stained with monoclonal antibody IA10 anti-DAF or with a nonrelevant antibody, followed by fluorescein isothiocyanate–goat F(ab′)$_2$ anti–mouse IgG. Results on monocytes, PMN, and lymphocytes are shown separately. (Solid lines) anti-DAF; (dashed lines) control antibody.

120, but a sizable proportion of the red cells showed intensities >300. In three individuals, the red cells were separated on the basis of density into four fractions, by centrifugation in a Percoll gradient. We found that DAF expression decreased with red cell density, and that the lightest cells had 19.4 ± 0.2% higher levels of DAF than the densest cells. However, the skewed DAF distribution was still observed in all fractions (not shown).

By FACS analysis, monocytes showed the highest DAF surface expression (mean fluorescent intensity, 756). Although, among extracts of blood cells, PMN had the highest amount of DAF (Table II), the surface expression of DAF in PMN (mean fluorescent intensity, 212) was much lower than that of monocytes.

Lymphocytes appeared as two distinct populations. The major population, ~65% of total lymphocytes, expressed less surface DAF (mean fluorescent intensity, 109) than the minor population (mean fluorescent intensity, 349) (Fig. 6*A*). When the buffy coat cells were simultaneously stained with phycoerythrin-conjugated anti-Leu-1 (a pan T reagent) and anti-DAF, >90% of the lymphocytes with high DAF expression were Leu-1$^-$ (not shown). This agrees with results of the IRMA, which showed that an extract of tonsil B cells had levels of DAF about three times higher than an extract of T cells, and with the finding that T lymphocytes purified by cell sorting had a very low DAF content (see above).

Quantitation of DAF in Erythrocytes from Patients With PNH. DAF levels in extracts of erythrocytes from three PNH patients (SB, GC, and VR) were measured by two-site IRMA and found to be much lower than normal (1,100, 1,000, and 900 molecules per cell, respectively) (Table II). To obtain information about the pattern of surface expression of DAF, erythrocytes from the same patients were studied by FACS. In patients who required no blood transfusions in the previous 6 mo (SB and VR, Fig. 4, *B* and *D*), two populations of red cells were detected. One was DAF deficient and constituted ~60% of the total in patient SB, ~30% in patient VR. The second population of red cells was positive and contained normal levels of DAF in patient SB but low levels in patient VR. Analysis of the red cells of the third patient (GC) was complicated by the fact that she had frequent hemolytic crises and received transfusions. The FACS pattern again revealed two populations of cells, one DAF negative (40%) and the other bearing lower than normal DAF levels (Fig. 4*C*). To examine the possibility that the DAF-containing cells originated at least in part from the blood transfusions, we separated the two populations by affinity chromatography and acid lysis (see Materials and Methods) and determined their reticulocyte contents. We expected the proportion of immature cells to be much higher among the patient's own erythrocytes than in the transfused cells. Indeed, we found that, while the unfractionated red cells contained 10% reticulocytes, the DAF-negative and -positive cells contained 17 and 2% reticulocytes, respectively. In contrast, when the red cells from the nontransfused patients were fractionated by the same method, the reticulocyte contents of the unseparated cells and the DAF-positive cells were not significantly different (2.1 vs. 1.6% in patient SB, and 0.9 vs. 0.6% in patient VR). It appears, therefore, that most or all of patient GC's own red cells were DAF negative. Further evidence to support this hypothesis was obtained by blood typing DAF-negative and -positive populations from patient GC, by Dr. Pablo Rubinstein (New York Blood Center). The DAF-negative population was devoid of antigens E, N, Kell, and Kp^a, while the same antigens were present in the population of DAF-positive cells.

When SB, GC, and VR erythrocytes were subjected to the Ham test, 54, 39, and 30%, respectively, were lysed by complement. To find out which cell population was complement sensitive, the patients' erythrocytes were first subjected to acidified serum lysis and the remaining cells then tested for DAF expression by FACS analysis. As shown in Fig. 7, the acidified serum preferentially lysed the DAF-negative cells.

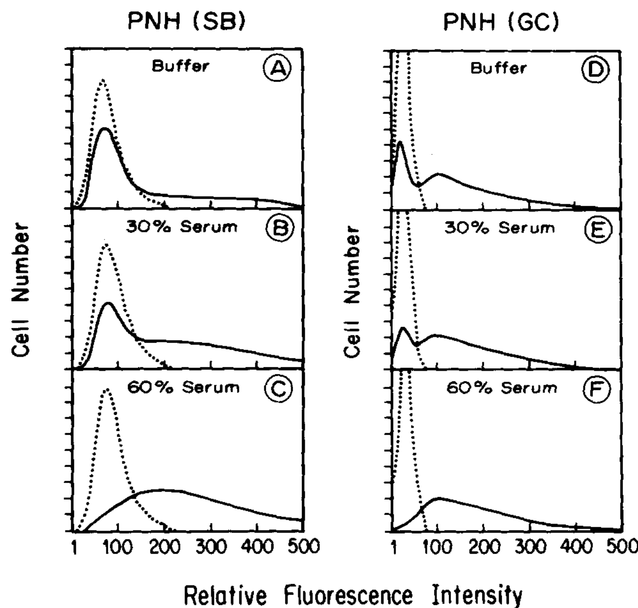

FIGURE 7. Acidified serum lysis of PNH E^{hu}. Erythrocytes from the patients SB (*A–C*) and GC (*D–F*) (10^7 cells) were incubated with 100 μl of buffer (*A, D*), 30% acidified serum (*B, E*), or 60% acidified serum (*C, F*) for 30 min at 37°C. Unlysed cells were collected by centrifugation, washed three times with PBS, stained with monoclonal antibodies to DAF or control antibodies as described in legend to Fig. 4, and then analyzed by FACS. (Solid lines) anti-DAF; (dashed lines) control antibodies. The DAF-deficient population was selectively lysed by acidified serum.

In short, the red cells from these patients differed with regard to DAF expression. In two of them (SB and VR), only some of the red cells were DAF negative and, in the third (GC), with the most severe hemolytic episodes, most or all endogenous cells were DAF negative. In every instance only the DAF-negative population was lysed by complement in the Ham test.

DAF Deficiency in Platelets and Leukocytes from PNH Patients. We next studied by FACS analysis the DAF surface expression in platelets and leukocytes from the same patients (Figs. 5*B*, 6, *B–D*). In patient SB (who had circulating DAF-negative and -positive red cells), neutrophils, monocytes, and platelets showed two distinct populations, one with normal amounts of DAF (23, 20, and 22% of each of these cell types) and the other DAF negative. DAF expression on lymphocytes appeared normal (Figs. 5*B*, 6*B*). The patients GC and VR differed from the first in two significant respects, that is, single populations of DAF-deficient neutrophils and monocytes were detected in their peripheral blood, and the lymphocytes were also DAF defective (Fig. 6, *C* and *D*). Small amounts of DAF were detected in the monocytes and neutrophils of patient VR, and in the lymphocytes from both VR and GC.

DAF levels in extracts of platelets from two PNH patients (SB and GC) were measured by IRMA (Table II). Consistent with the results of FACS analysis, the platelets from patient SB contained ~30% of the normal level of DAF. No DAF was detected in the platelets of patient GC.

Discussion

The present study reports quantitative information on the distribution of DAF in normal individuals and in PNH patients. The DAF measurements were performed by IRMA and FACS analysis using monoclonal antibodies whose specificity was confirmed both by immunoblotting and by their inhibitory activity on DAF function. These measurements revealed that DAF is present not only on red cells but on platelets, PMN, monocytes, and B and T lymphocytes.

The highest number of DAF molecules were found in neutrophils (8.5×10^4 per cell), followed by monocytes (6.8×10^4), and lymphocytes (3.3×10^4) (Table II). In agreement with the recent findings of Rosen et al. (27), a small number of DAF molecules were found in platelets (2.1×10^3 per cell). Although both B and T lymphocytes bear DAF, the results of FACS analysis and IRMA of tonsil lymphocytes showed that B lymphocytes had considerably more DAF molecules than T lymphocytes. Interestingly, the highest amounts of DAF were found among phagocytes and B lymphocytes, cells believed to interact frequently with immune complexes and complement.

The results of the IRMA and FACS analysis were in good agreement, with one exception: by FACS analysis neutrophils had three times less DAF than did monocytes (Fig. 6*A*), contrary to the results obtained by IRMA of cell extracts (Table II). Perhaps, as in the case of CR1 and CR3, this discrepancy reflects the presence of an internal pool of DAF in neutrophils (28–30).

We also found that the M_r of DAF varies among different cell types. DAF from platelets and PMN was slightly larger (~5,000 M_r) than erythrocyte DAF, while DAF from mononuclear cells was of an intermediate size. Small size differences have been also detected among CR1 molecules from different cells, but the significance of these structural variations in DAF and CR1 remains obscure (31, 32).

With regard to red cells, the distribution of DAF showed one intriguing feature. Although the total amount of DAF on red cells from normal adults varied only about twofold (between 2.1 and 4.0×10^3 molecules per cell; mean, 3.3×10^3), FACS analysis showed that in every individual its distribution varied as much as tenfold (Fig. 4*A*). This anomalous distribution could reflect heterogeneity in DAF biosynthesis or catabolism in the bone marrow precursor population, and/or secondary changes associated with red cell aging.

To study this question, we fractionated red cells from several individuals according to their density, and studied the DAF distribution by FACS analysis. Although the fluorescence intensity continued to be abnormally distributed among cells from all fractions, the mean amounts of DAF were smaller in the denser erythrocytes, which contain the older population of cells. It appears, therefore, that the aging of red cells in circulation is associated with DAF losses. This may be of interest because the removal of senescent red cells from circulation is enhanced by binding autoantibodies to a cryptic epitope of band 3 glycoprotein (33–35) or to clusters of this glycoprotein (36). It is conceivable that the decrease in DAF facilitates the deposition of C3b and subsequent interaction of the old erythrocytes, with scavenging cells of the reticuloendothelial system. In support of this idea, a prior study (37) showed that, although circulating young red cells were barely hemolysed when treated with antibody

and complement, lysis gradually increased in older cells, reaching a maximum of ~75% after 3–4 mo in circulation.

With regard to the three individuals with PNH, the most important new findings are that all circulating cells showed defects in surface expression of DAF antigen, that the DAF-negative and -positive cells could be present simultaneously in the circulation, and that the patterns of DAF deficiency were different in every patient (Table III).

In patient SB, two populations of red cells, monocytes, neutrophils, and platelets were found in the peripheral blood. One population was profoundly deficient in surface DAF while the other appeared normal. The lymphocytes from this patient had a normal level of surface DAF.

In the other two patients (GC, VR), the expression of DAF on the lymphocyte surface was also affected. In the patient with more severe disease (GC), PMN, monocytes, and most red cells and lymphocytes were DAF negative. Small amounts of DAF were detected in the blood elements of patient VR, suggesting that in this case the defect was caused by abnormal regulation of a gene rather than by a nonfunctional or deleted gene.

These observations offer additional support for the concept that PNH cells are of monoclonal origin and arise from the clonal expansion of an abnormal bone marrow progenitor (38–40). In patient SB, the erythroid, myeloid, as well as megakariocytic lineages were affected, suggesting that the mutation occurred in a common precursor. It is remarkable that in this patient, normal and abnormal elements from the three lineages were found in circulation, ~¾ of them DAF deficient. This proportion remained unchanged in blood samples collected 10 wk apart, reflecting perhaps a steady state between the proliferation of abnormal and normal bone marrow precursors and the removal from circulation of the end cells. Because in patients GC and VR the lymphocytes were also DAF deficient, a more primitive cell giving rise to all blood elements was probably

TABLE III
Distribution of DAF in Cells from Peripheral Blood of PNH Patients

Cell type	Expression of DAF in PNH patients		
	Patient SB	Patient GC	Patient VR
Erythrocytes	60% Undetectable 40% Normal	40% Undetectable 60% Below normal*	30% undetectable 70% low levels
Platelets	78% Undetectable 22% Normal	Undetectable (by IRMA)	ND‡
Monocytes	80% Undetectable 20% Normal	Undetectable	Very low
PMN	77% Undetectable 23% Normal	Undetectable	Very low
Lymphocytes	Normal	Very low or undetectable	Very low

* Most or all cells in this population represent transfused donor cells.
‡ Not determined.

affected. Such totipotent stem cells have not been isolated or morphologically identified, but there is compelling evidence for their presence in the postnatal bone marrow of mammals (41, 42).

Although the sequence of events leading to the multiple abnormalities found in the membranes of PNH cells remains to be explained, our studies are relevant to the pathogenesis of the disease. The DAF defect can explain some of its characteristic features, such as the large accumulation of C3 fragments on the surface of erythrocytes, PMN, and platelets and their abnormal susceptibility to lysis by complement (7, 10–12). Indeed, we have shown elsewhere (5) that DAF inhibits the assembly of C3 convertases and prevents C3b deposition on cell surfaces, and we present direct evidence (Fig. 7) that DAF-deficient cells are preferentially lysed when subjected to acidified fresh serum (Ham test). However, the relationship between the C3b accumulation on leukocytes and platelets and the observed thrombocytopenia, leukopenia, and enhanced propensity for thrombosis and infection (43) is not clear. Others have suggested that C3b deposition on platelets leads to their aggregation, release reaction, and enhanced clotting (11), and that C3b deposition on the PNH neutrophils results in a chemotactic defect (44).

However, on the basis of current knowledge of DAF function, it is difficult to explain the heightened susceptibility of cells from some PNH patients to the isolated C5b-9 attack complex (45). Further studies are required to verify whether this is secondary to the DAF defect or whether an unrelated abnormality of the cell membrane is involved.

Summary

Decay-accelerating factor (DAF) is a 70,000 M_r protein that has been isolated from the membrane of red cells. The function of DAF is to inhibit the assembly of amplifying enzymes of the complement cascade on the cell surface, thereby protecting them from damage by autologous complement. We raised monoclonal antibodies to DAF and used them to study its distribution in cells from the peripheral blood of normal individuals and of patients with paroxysmal nocturnal hemoglobinuria (PNH), a disease characterized by the unusual susceptibility of red cells to the hemolytic activity of complement.

The results of immunoradiometric assays and of fluorescence-activated cell sorter analysis showed that DAF was present not only on red cells but was widely distributed on the surface membrane of platelets, neutrophils, monocytes, and B and T lymphocytes. By Western blotting, we observed small but consistent differences in the M_r of DAF from the membranes of various cell types. Quantitative studies showed that phagocytes and B lymphocytes, which presumably enter more frequently in contact with immune complexes and other potential activators of complement, had the highest DAF levels.

As previously reported by others, the red cells from PNH patients were DAF deficient. When the patients' red cells were incubated in acidified serum (Ham test), only the DAF-deficient cells were lysed. In addition, we detected defects in DAF expression on platelets and all types of leukocytes. The observed patterns of DAF deficiency in these patients were consistent with the concept that the PNH cells were of monoclonal origin. In one patient, abnormal and normal cells

were found only in the erythroid, myeloid, and megakariocytic lineages. In two other patients, the lymphocytes were also DAF deficient, suggesting that a mutation occurred in a totipotent stem cell. It appears, therefore, that the lesion leading to PNH can occur at various stages in the differentiation of hematopoietic cells.

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