THE MECHANISM OF ACTION OF DECAY-ACCELERATING FACTOR (DAF)
DAF Inhibits the Assembly of C3 Convertases by Dissociating C2a and Bb

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Decay-accelerating factor (DAF)[1] is a 70,000-M_r membrane protein that regulates the C3 and C5 convertases of the classical and alternative pathways (1–5). It is a membrane constituent of normal human erythrocytes (E^{hu}) (3), neutrophils, lymphocytes, monocytes, platelets (6, 7), and endothelial cells (8), in which it is anchored by a glycolipid involving phosphatidylinositol (9). It is considered that DAF protects host cells from damage by autologous complement. DAF is partially or completely deficient in the membranes of erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH), an acquired hemolytic anemia characterized by an unusual susceptibility of red cells to complement-mediated lysis (4, 6, 10).

The precise mechanism by which DAF regulates complement C3 convertase is not entirely clear. DAF was first considered to accelerate the spontaneous decay of the classical and alternative C3 convertases (3, 4). Subsequently, it was reported that purified DAF, reincorporated into erythrocyte membranes, formed a complex with C4b and C3b deposited on the membrane, and inhibited the assembly of the C3 convertases (5, 11). It was proposed that DAF competitively inhibits the uptake of C2 or factor B (11). In the present study, we show that DAF acts on C2a and Bb, but not on intact C2 and B, and dissociates them rapidly from C4b and C3b, respectively, thereby preventing the assembly of the C3 convertases.

Materials and Methods

Buffers and Complement Components. Isotonic veronal-buffered saline with 2.5% dextrose, containing 0.1% gelatin, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$ (DGVB) and veronal-buffered saline, containing 0.1% gelatin and 0.01 M EDTA (EDTA-GVB), were used. Guinea pig C1 and C2 (12), human C4 (13), C2 (14), C3 (15), B (16), and D (17) were

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[1] *Abbreviations used in this paper:* C-EDTA, guinea pig serum diluted with EDTA-GVB; DAF, decay-accelerating factor; DGVB, isotonic veronal-buffered saline with dextrose, gelatin, $MgCl_2$, and $CaCl_2$; EDTA-GVB, veronal-buffered saline with gelatin and EDTA; E^{hu}, human erythrocytes; *p*-APMSF, *p*-amidinophenyl-methanesulfonyl fluoride hydrochloride; PNH, paroxysmal nocturnal hemoglobinuria.

purified as described previously. Guinea pig serum diluted 1:50 in EDTA-GVB (C-EDTA) was used as a source of C3-C9.

Cellular Intermediates. Antibody-sensitized sheep erythrocytes carrying guinea pig C1 and human C4 (EAC14b) were prepared as described (18). EAC4b were prepared by incubating EAC14b at 37°C for 30 min in EDTA-GVB and then treating them with 50 μM *p*-amidinophenyl-methanesulfonyl fluoride hydrochloride (*p*-APMSF; Wako Pure Chemicals Industries, Ltd., Osaka, Japan) to destroy the remaining C1. EAC4b3b cells were prepared as follows: EAC14b (10^8/ml) were incubated for 30 min at 37°C with 50 U/ml of guinea pig C2 and 300 U/ml of C3, and after washing, the cells were further incubated at 37°C for 3 h. Purified DAF (20 μg/ml) was incorporated into EAC14b, EAC4b, and EAC4b3b cells (10^9/ml) by incubating at 37°C for 1 h. These cells, E^{DAF}AC14b, E^{DAF}AC4b, and E^{DAF}AC4b3b were washed three times with DGVB. EAC14b2a were prepared by adding oxidized human C2 (19) to EAC14b to yield 1–1.5 hemolytic sites per cell. Human erythrocytes (E^{hu}) were obtained from healthy donors and sensitized with rabbit hemolysin (Cappel Laboratories, Cochranville, PA) in a subagglutinating concentration. E^{hu}AC4b cells were prepared by the same methods as EAC4b cells.

Assay. The activity of isolated DAF or DAF-containing preparations was assayed by its ability to accelerate the decay of EAC14b2a as originally described by Nicholson-Weller et al. (3). Briefly, the sample was mixed with 10^7 EAC14b2a in a total volume of 0.3 ml, and incubated for 30 min at 30°C. The residual hemolytic sites were developed by adding 1.2 ml of C-EDTA, followed by further incubation at 37°C for 1 h. Additional details of the assay are given in the legends to the Figures.

Purification of DAF. DAF was purified from pooled human erythrocyte stroma by the method described by Nicholson-Weller et al. (3, 10) with modifications. Since we obtained the mAbs to DAF, the fractions that showed DAF activity after anion-exchange column were subjected to an affinity chromatography of monoclonal anti-DAF-coupled Sepharose and eluted with 3 M KSCN, containing 0.1% Chaps (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; Dojindo Laboratories, Kumamoto, Japan). To remove the minor contaminants, we used reverse-phase HPLC through the TSK Phenyl 5PW-RP matrix (Toyosoda Manufacturing Co., Ltd., Tokyo, Japan). The main peak contained only DAF protein as evaluated by SDS-PAGE and silver staining. The protein concentration was calculated from its optical density at 280 nm, under the assumption that an absorption coefficient value of 1.0 equals 1 mg/ml.

mAbs to DAF. BALB/c mice were immunized twice with 5 μg of purified DAF in CFA. 3 wk later, the mice were boosted with 5 μg of DAF; the spleen was removed 3 d later and used for fusion with myeloma cells (NS-1). Culture supernatants of the resulting hybridoma were tested for anti-DAF activity as described previously (6) by immunoelectroblotting using an ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the instructions of the manufacturer. As the positive control, we used rabbit anti-DAF antibodies kindly supplied by Dr. V. Nussenzweig (New York University Medical Center, New York). Four positive mAbs were obtained and purified to homogeneity from ascites fluids of mice bearing the hybridoma by ammonium sulfate precipitation, followed by chromatography on DEAE Toyopearl (Toyosoda Manufacturing Co., Ltd.). All antibodies used in this study belonged to IgG1κ isotype.

Of the four mAbs (1C6, 4F11, 5B2, and 6F10), 1C6, 4F11, and 5B2 recognized the different epitopes on the DAF molecules, while 5B2 and 6F10 recognized the same or overlapping epitopes. 1C6 alone completely inhibited DAF activity, and although the other mAbs also inhibited DAF activity, their effects were weaker than that of 1C6. However, these mAbs alone and any combination of these antibodies did not inhibit the incorporation of radiolabeled DAF into sheep erythrocytes, showing that 1C6 is directed to the active site on DAF molecule, not to the site that is anchored to the cell membrane by phosphatidylinositol. In this study we used 1C6 to block the activity of DAF.

Radiolabeling. C2 was labeled with ^{125}I in the presence of iodoacetamide-treated monomeric human albumin (20) by using Iodogen (Pierce Chemical Co., Rockford, IL), and unbound ^{125}I was removed by gel filtration through Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ), followed by extensive dialysis. Since the hemolytic activity

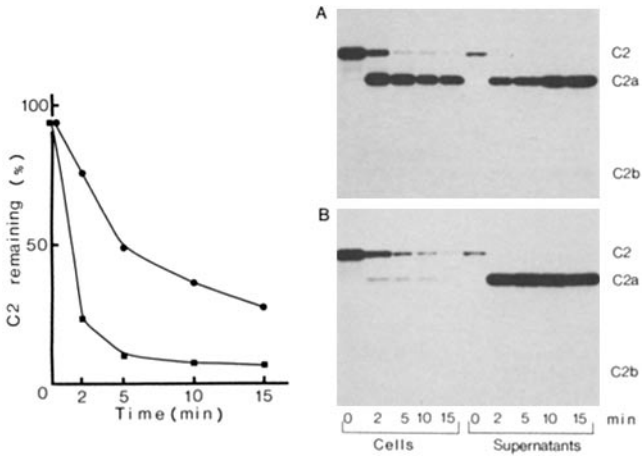

FIGURE 1. The effect of DAF on the decay and the structure of C2. EAC4b2 and E^{DAF}AC4b2 cells (10^8/ml) were prepared by incubating at 30°C for 15 min with ^{125}I-C2 (2.5 μg/ml), followed by washing. The cells were resuspended in 1 ml of DGVB and received 300 U of C1. At timed intervals, 100 μl of samples were removed from each tube and the reaction was stopped by adding 2 μl of 5 mM *p*-APMSF. After centrifugation, 80 μl of supernatants were removed. The cells were washed once with DGVB and solubilized with 100 μl of reducing sample buffer. The supernatants and the solubilized cells were counted for radioactivity and analyzed by SDS-PAGE and radioautography. From the radioactivities, the amounts of C2 bound to EAC4b2 (●) and E^{DAF}AC4b2 (■) cells were calculated and expressed as the percentage of the total counts (*left*). The radioautographs of EAC4b2 (*A*) and E^{DAF}AC4b2 (*B*) are shown in the right panel.

was fully preserved, we used ^{125}I-C2 without further purification. Factor B was also labeled with ^{125}I by using Iodogen.

Structural Analysis of Radiolabeled C2 and B. SDS-PAGE was performed according to Laemmli (21). After electrophoresis the gels were dried and analyzed by radioautography.

Results

Effect of DAF on the Classical Pathway C3 Convertase. To study the effect of DAF on the classical pathway C3 convertase, we prepared EAC4b2 and E^{DAF}AC4b2 cells with ^{125}I-C2. The same amounts of ^{125}I-C2 bound to EAC4b and E^{DAF}AC4b cells. After these cells received C1, the mixtures were incubated at 30°C and the samples were removed from each tube at time intervals to measure the radioactivity and to analyze the C2 structure by SDS-PAGE and radioautography. As seen in Fig. 1, the radioactivity of C2 dissociated more rapidly from E^{DAF}AC4b2a cells than from EAC4b2a cells. The rates of cleavage of the bound ^{125}I-C2 by C1 in both cells were the same. The C2b portion was not well labeled with ^{125}I. In EAC4b2 cells, C2a gradually dissociated into the supernatants, whereas in the DAF-treated cells, a large amount of converted ^{125}I-C2a rapidly appeared in the supernatants and only a small amount of C2a remained on the cells. It is clear that DAF does not prevent the binding of C2 zymogen to C4b, but acts on C2a to dissociate it from the binding site of C4b.

A similar experiment was performed using human red cells. E^{hu}AC4b cells were used as the cells having DAF, and E^{hu}AC4b pretreated with anti-DAF mAb 1C6 were used as the cells containing no DAF. As shown in Fig. 2, DAF on human erythrocyte membranes also dissociated the C2a from the cells, and the DAF activity was completely blocked by 1C6 (Fig. 2). The decay of C2a and its radioautograph in 1C6-treated E^{hu}AC4b2 and E^{hu}AC4b2 (Fig. 2) are comparable to those in EAC4b2 and E^{DAF}AC4b2 (Fig. 1), respectively. Under physiologic conditions, activated C1, which is present in the vicinity of C4b, cleaved C2

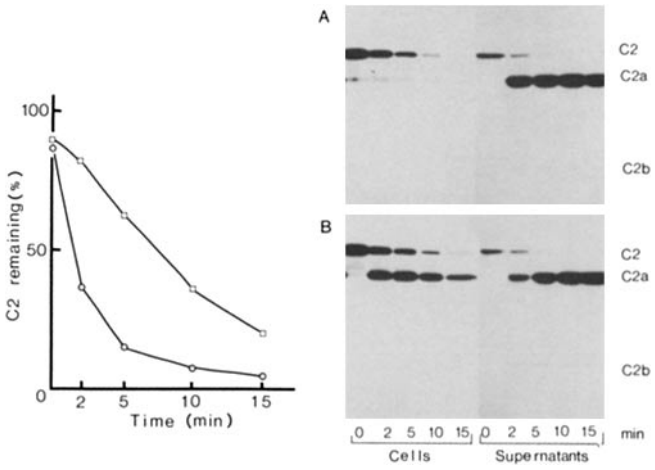

FIGURE 2. Activity of DAF on human erythrocyte membranes. $E^{hu}AC4b$ cells (10^8/ml) were treated at 0°C for 30 min with 1C6 (100 μg/ml) or buffer. The cells were washed and reacted with ^{125}I-C2 (2.5 μg/ml) at 30°C for 15 min. The same radioactivities were bound to $E^{hu}AC4b$ and 1C6-treated $E^{hu}AC4b$ cells. The decay of C2 and its structural change were analyzed by the same procedures as described in the legend of Fig. 1. C2 on $E^{hu}AC4b$ (○) decayed more rapidly than that on 1C6-treated $E^{hu}AC4b$ (□) (*left*). As shown in *A* of the right panel, only a small amount of C2a was observed on $E^{hu}AC4b$ cells at any time, while most of C2a appeared in the supernatants. (*B*) C2a on 1C6-treated E^{hu}-AC4b decayed gradually and appeared in the supernatants.

TABLE I

Binding of C2 to DAF-incorporated EAC14b Cells

Reaction mixture	Number of C4b2a sites
$E^{DAF}AC4b$/buffer/C1	0.03
$E^{DAF}AC4b2$/1C6 (50 μg/ml)/C1	2.00
$E^{DAF}AC4b2$/1C6 (10 μg/ml)/C1	1.74
$E^{DAF}AC4b2$/1C6 (2 μg/ml)/C1	1.00
EAC4b2/buffer/C1	2.28

EAC4b and $E^{DAF}AC4b$ cells were reacted for 15 min at 30°C with a limiting amount of oxidized C2. After centrifugation, 100 μl of $E^{DAF}AC4b2$ cells (10^8/ml) were reacted with 100 μl of varying amounts of 1C6 or buffer, and EAC4b2 cells were also reacted with buffer. After washing, the cells were incubated with 100 μl of C1 (1,000 U/ml) at 30°C for 15 min, and then further incubated with 1.2 ml of C-EDTA to quantitate the number of C4b2a sites.

bound to C4b into C2a and C2b. Simultaneously, DAF dissociates the generated C2a from C4b, thereby preventing the assembly of the classical pathway C3 convertase, C4b2a.

C2 Depletion by $E^{DAF}AC14b$. To confirm the above results, we determined whether the hemolytically active C2 binds to the DAF-incorporated cells. EAC4b and $E^{DAF}AC4b$ cells were reacted with a limiting amount of oxidized C2. DAF-incorporated cells were incubated at 30°C for 30 min with different amounts of 1C6. After adding C1 to the cells, the hemolytic activity of C4b2a was assessed (Table I). No hemolysis was observed in the DAF-incorporated EAC4b2a cells. However, treatment with anti-DAF mAb restored the C4b2a hemolytic activity on the DAF-incorporated cells, showing that hemolytically active C2 bound to the DAF-treated C4b sites. In addition, we hemolytically measured C2 depletion

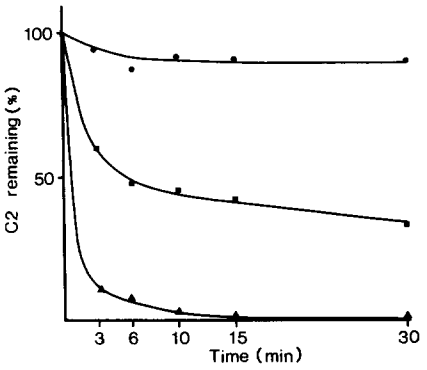

FIGURE 3. Depletion of C2 by EAC14b and E^{DAF}AC14b cells. EAC1 (●), EAC14b (■) and E^{DAF}AC14b (▲) cells (10^8/ml) were incubated at 30°C with 6,000 U/ml of oxidized C2 for varying periods of time and then the remaining C2 hemolytic activity was assayed. E^{DAF}AC14b consumed C2 more rapidly than EAC14b. There is a small amount of C2 utilization by EAC1 cells.

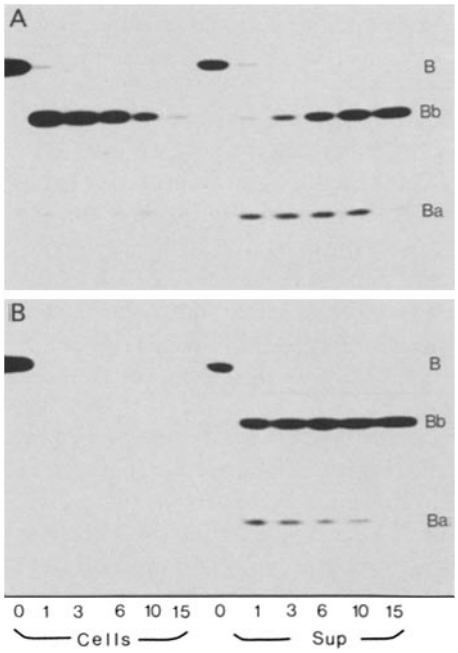

FIGURE 4. The effect of DAF on the alternative pathway C3 convertase, C3bBb. EAC4b3bB and E^{DAF}AC4b3bB cells (10^8/ml) were prepared by incubation at 30°C for 15 min with ^{125}I-B (1.5 μg/ml). After washing, 1 ml of these cells received 3.2 ng of factor D, and the mixtures were incubated at 30°C. At timed intervals, 100 μl of sample were removed from each tube and analyzed by SDS-PAGE and radioautography. As shown in *A*, in EAC4b3b cells, at 1 min, factor B was converted to Bb and Ba by the action of factor D, and Bb fragment remained on the cells, while Ba was observed in the supernatants. The bound Bb decayed gradually and appeared in the supernatants. In *B*, no Bb fragment was observed on E^{DAF}AC4b3b cells at any time, showing that DAF dissociates the Bb from the cells.

from the fluid phase by EAC14b and E^{DAF}AC14b cells. As shown in Fig. 3, compared with the moderate depletion of C2 by EAC14b, the rate of depletion of C2 by E^{DAF}AC14b was more rapid. These results indicate that the accelerated decay of C2a results in the rapid turnover of C2 on the DAF-incorporated EAC14b cells.

Effect of DAF on the Alternative Pathway C3 Convertase. Next, we investigated whether DAF acts on the alternative pathway C3 convertase, C3bBb, in the same manner. EAC4b3bB and E^{DAF}AC4b3bB cells were prepared with ^{125}I-B. Factor D was added to these cells and the mixtures were incubated at 30°C. The samples were removed from each tube at time intervals and analyzed by SDS-PAGE and radioautography (Fig. 4). At 1 min most of ^{125}I-B bound to EAC4b3b cells was converted to Bb and Ba by the action of D. Bb remained bound to the cells, while Ba appeared in the supernatant. The Bb decayed gradually and at 15 min most of Bb dissociated from the cells. In the DAF-incorporated cells, on the

other hand, Bb was not observed on the cells even at 1 min, although the same amount of B was bound to the cells. These results clearly show that in the alternative pathway intact B binds to C3b, and that DAF functions to release the Bb generated by D.

Discussion

DAF was first reported to accelerate the decay-dissociation of C2a and Bb from C4b2a and C3bBb, respectively (3, 4). Subsequently it was found that DAF exhibits the unique property of being able to reassociate with red cells and express its biological activity (5). It forms complexes with C4b and C3b deposited on the erythrocytes (11), and inhibits the formation of the C3 convertase of both the classical (C4b2a) and alternative (C3bBb) pathways (5). However, the precise mode of action of DAF is not fully understood. In this study, we demonstrated that DAF on the membranes of erythrocyte does not prevent the binding of C2 and factor B zymogens to C4b and C3b, respectively, but dissociates C2a and Bb from each binding site.

A key finding is that the same amount of the native C2 binds to C4b in the presence and absence of DAF, whereas C2a cannot remain on DAF-treated C4b sites (Figs. 1 and 2). These results were confirmed by the hemolytic assay (Table I). Therefore, it is possible that DAF competitively inhibits the binding of C2a generated by the action of C1s. Another possibility is that C4b molecule, allosterically modified by the binding of DAF, cannot hold the C2a molecule through the labile binding site. The latter possibility is more likely, because a limiting amount of DAF within cell membranes accelerated the natural decay of C2a (data not shown) and a small amount of C2a was observed on the cell membranes even in the presence of a large excess of DAF (Figs. 1 and 2). Whatever the mechanism, under physiologic conditions, DAF inhibits the assembly of the C4b2a enzyme by dissociating the C2a (Fig. 2). The alternative pathway C3 convertase, C3bBb, is regulated by DAF in the same manner (Fig. 4).

Recently, the molecular cloning of human DAF revealed that the deduced DAF sequence contains four repeating units homologous to a consensus repeat found in the complement proteins, factor B, C2, factor H, C4-binding protein, CR1, and C1r (22, 23). These complement components are all C3b- and/or C4b-binding proteins as is DAF. Each recognizes the activation-dependent sites in the structurally homologous C3b and C4b molecules, which deposit in clusters on targets. It is likely that the repeating units serve as the C3b/C4b-binding domains of DAF and these other molecules. The binding site of 1C6 is close to the active site of DAF molecule, not to the site that is anchored to the cell membrane by phosphatidylinositol (9). In this respect, it is possible that 1C6 recognizes the repeating units of DAF, thereby inhibiting the interaction of DAF with C3b/C4b.

In addition, our findings suggest that the whole molecule of C2 and its fragment C2a bind to different sites of the C4b molecules (Figs. 1 and 2). The previous reports show that the interaction of C4b and C2 appears to be mediated by the C2b part of the C2 molecule (14). Taken together, it is likely that the C2b region plays a role in the initial binding of C2 to C4b, and after the cleavage of C2 by C1s, the generated C2a binds to a different binding site on C4b molecule, resulting in the generation of proteolytic activity. This binding is labile and affected by DAF. Similarly, our results (Fig. 4) are consistent with the recent

report (24) that a binding site for C3b on intact B is located on the Ba portion of the molecule.

Summary

DAF is a 70,000-M_r membrane protein that inhibits the amplification of the complement cascade on the cell surface, and protects cells from damage by complement. The precise mechanism of action of DAF is not entirely clear. Purified DAF was incorporated into the membrane of EAC4b cells. EAC4b2 and E^{DAF}AC4b2 cells were prepared with radiolabeled C2. The same amount of labeled C2 bound to both cells, showing that DAF does not prevent the binding of C2 zymogen to C4b. After adding C1, the radioactivity of bound C2 dissociated more rapidly from E^{DAF}AC4b cells than from EAC4b cells. In EAC4b cells, bound C2 was converted to C2a, which gradually dissociated into the supernatants. In the DAF-treated cells, on the other hand, a large amount of C2a rapidly appeared in the supernatants and only a small amount of C2a remained on the cells. In a similar experiment using E^{hu}AC4b, DAF on human erythrocyte membrane also dissociated the C2a from the cells. These results were confirmed by hemolytic assay and the accelerated decay of C2a caused the rapid depletion of C2 from the fluid phase. In addition, we found that DAF functions on the alternative pathway C3 convertase, C3bBb in the same manner. Thus, DAF, which associates with C4b and C3b in the membrane, acts on C2a and Bb, but not on intact C2 and B, and dissociates them rapidly from the binding sites, thereby preventing the assembly of the classical and alternative pathways C3 convertases.

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