INHIBITION OF COMPLEMENT ACTIVATION ON THE SURFACE OF CELLS AFTER INCORPORATION OF DECAY-ACCELERATING FACTOR (DAF) INTO THEIR MEMBRANES

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A key step in complement activation on targets is the deposition of C4b and/or C3b fragments. These major cleavage products of C3 and C4 can associate covalently with the target surface (reviewed in 1) and then serve as anchors for the assembly of C3 and C5 convertases, the amplifying enzymes of the cascade.

This amplification must be focused only on the target and must not occur on host cells. Moreover, in the course of this focused amplification, large numbers of nascent C4b and C3b fragments are liberated into the fluid phase. Most react with water, but some by chance could bind to nearby host cells and lead to their damage. For this and possibly other reasons, the activities of bound, as well as free, C3b and C4b fragments are under strict control by a complex system of serum and membrane proteins.

Recent evidence (2, 3) suggests that regulation of the activities of substrate-bound C4b and C3b is distinct from control of the fluid phase fragments. The functions of the former are controlled mainly by two membrane proteins: the C3b/C4b receptor (CR1)[1] and the decay-accelerating factor (DAF). CR1 dissociates C2 and factor B from C4b and C3b in C3 and C5 convertase complexes (2–5) and promotes the cleavage of C3b (2, 4, 6, 7) and C4b (3, 5) by the serum enzyme C3b/C4b inactivator (I). DAF has been shown also to enhance the decay dissociation of C2 and factor B from C3 convertases (8, 9). The reason for the apparent redundancy in regulatory activities of the two membrane factors and their respective roles in convertase control has remained unclear. Abnormalities of CR1 have been found in systemic lupus erythematosus (SLE) (10–13), a condition associated with defective immune complex handling, and abnormalities of DAF have been found in paroxysmal nocturnal hemoglobinuria (PNH) (9, 14, 15), a condition associated with heightened susceptibility of blood cells to lysis.

In the present paper we purified DAF to homogeneity from the stroma of

This work was supported by grants from The Arthritis Foundation, The Systemic Lupus Erythematosus Society, The Kroc Foundation, and grant AI 13224 from the National Institutes of Health. M. E. Medof is an Investigator of The Arthritis Foundation. Some of the results of this paper were presented at the annual meeting of the ASBC/AAI, St. Louis, MO, June 1984.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CR1, C3b/C46 receptor; DAF, decay-accelerating factor; E, sheep erythrocytes; E^{hu}, human erythrocytes; H, β1H; HDL, high density lipoprotein; HPLC, high pressure liquid chromatography; LDL, low density lipoprotein; NP-40, Nonidet P-40; P, properdin; PNH, paroxysmal nocturnal hemoglobinuria; SFU, site-forming units; SLE, systemic lupus erythematosus, Z, number of hemolytic sites per cell.

human red cells and studied its functional properties. We found that purified DAF could be readily taken up into the membrane of red cells and that, once incorporated, its main function was not to accelerate the decay of the convertases but to prevent their assembly. Moreover, it appears that membrane-associated DAF, in contrast to CR1, can act only within the cell membrane, and not extrinsically on complement enzymes assembled on targets.

Materials and Methods

Buffers, Reagents, and Proteins. Isotonic veronal-buffered saline ($DGVB^{++}$), used in most studies, consisted of 2.5 mM veronal, pH 7.4, 73 mM NaCl, 2.5% dextrose, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% gelatin. Isoionic veronal buffer (GVB^{++}) lacked dextrose but contained 146 mM NaCl. The metal-chelating veronal buffer (GVB-EDTA) used in hemolytic assays was prepared without $CaCl_2$ and $MgCl_2$ and was supplemented with 10 mM EDTA. Phosphate-buffered saline (PBS) contained 0.02 M sodium phosphate, pH 7.4 and 146 mM NaCl.

C2 (16), C4 (17), C3 (17), C5 (17), factors B (18) and D (19), properdin (P) (20), β1H globulin (H) (17) and C3b/C4b inactivator (I) (21) were prepared by previously published techniques. I was also obtained commercially (Cordis Laboratories, Miami, FL). C2 was oxidized (22) (^{oxy}C2) for some studies. C1 (23), C6-9 (24), and C3-9 (C-EDTA) were prepared from guinea pig serum. The functional activity and purity of each component were assessed by specific hemolytic assays and, where appropriate, by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Glycophorin A was a gift of Dr. Olga Blumenthal, Albert Einstein College of Medicine.

Monoclonal anti-CR1 antibodies were obtained as previously described (11). Polyclonal anti-glycophorin antibodies were raised by a single subcutaneous injection of 100 μg of glycophorin A emulsified in complete Freund's adjuvant. Monoclonal antibodies to glycophorin A were a gift of Dr. Margaret Nichols, New York Blood Center. Polyclonal anti-DAF antibodies were raised by subcutaneous injection of rabbits or mice with 5 or 1 μg, respectively, of DAF emulsified in complete Freund's adjuvant. Rabbits were not further immunized. Mice were boosted intravenously at 6 wk with the same antigen dose. When these antibodies were used for developing Western blots of crude DAF preparations contaminated with glycophorin or blots of extracts of red cell ghosts, only the DAF band was revealed.

Radiolabeling. Proteins were labeled with ^{125}I by the Iodogen method (Pierce Chemical Co., Rockford, IL) according to the instructions of the manufacturer. Before the labeling of DAF, the protein was concentrated by chromatography on DEAE-Sephacel and the Nonidet P-40 (NP-40) concentration reduced to 0.001%. After labeling, unbound ^{125}I was removed by gel filtration through Sephadex G-25 QS-2A Quick-Sep columns (Isolab Inc., Akron, OH), followed by extensive dialysis.

Cellular Intermediates. Sheep erythrocytes (E) were sensitized with rabbit anti-sheep hemolysin (A) (Gibco Laboratories, Grand Island, NY). The resulting EA were washed, adjusted to 1×10^8/ml in $DGVB^{++}$, and, after incubation for 15 min at 30°C with 300 site-forming units (SFU) of C1, the intermediates (EAC1) were pelleted and resuspended to 1×10^8/ml. The EAC1 were then incubated for 20 min at 30°C with 100 SFU of C4 and the cells (EAC14) again pelleted and washed. ~1 SFU of C4 was used for the preparation of $EAC14_{lim}$ and ~10 SFU of C4 for the preparation of all intermediates bearing C3b. In the latter case, the EAC14 were incubated for 5 min at 30°C with 100 SFU of C2 and, after pelleting and resuspension, the intermediates (EAC142) were incubated for 15 min at 30°C with C3. After washing, C2 on the cells (EAC1423) was decayed at 30°C. The resulting intermediate EAC143 was used for titration of the classical pathway C5 convertase or for the preparation of cells bearing the alternative pathway C3 convertase (EAC143B or EAC143BP). For the preparation of $EAC143_{lim}$, EAC142 cells were treated with ~1 SFU of C3.

Assays. C4b hemolytic sites were quantitated by sequential incubation of EAC14 with 300 SFU of C2 (or ^{oxy}C2) for 5 min at 30°C and C-EDTA for 1 h at 37°C. Classical

pathway C3b hemolytic sites were determined by incubation of EAC1423 (or EAC14^{oxy}23) with 300 SFU of C5 for 5 min at 30°C and C6-9 for 1 h at 37°C. Alternative pathway C3b hemolytic sites were measured by incubation of EAC143 (in DGVB^{++}) with 20 μg/ml of factor B and excess factor D (in the absence or presence of 12 μg/ml of P) for 30 min at 30°C and further incubation of the washed cells with C-EDTA for 1 h at 37°C.

The inhibition of C4b2a functional activity by isolated DAF or DAF-containing preparations was quantitated as originally described by Nicholson-Weller et al. (8). Briefly, EAC14 were incubated for 5 min at 30°C with C2 at a concentration predetermined to yield EAC142 cells bearing ~1.5 C4b2a sites, after spontaneous decay of the washed cells in DGVB^{++} for 15 min at 30°C and incubation with C-EDTA for 1 h at 37°C. EAC142 were then incubated for 15 min at 30°C in the presence of DAF-containing samples and the residual C4b2a sites were developed with C-EDTA.

C2 (or ^{oxy}C2) and factor B titrations were performed by standard methods (25). The kinetics of C3 convertase decay was determined by removal at different times of 200-μl aliquots of the cell intermediates (5×10^7/ml in GVB^{++}) from a tube placed at 30°C and subsequent incubation of the aliquots with 1.3 ml C-EDTA for 1 h at 37°C. Additional details of the assays are given in the legends to the figures.

SDS-PAGE, Radioautography, and Western Blotting. SDS-PAGE was performed as described by Laemmli (26). Stacking gels of 3% and running gels of 7.5% were used. Samples were reduced by the addition of 20% 2-mercaptoethanol. Apparent molecular weights were estimated by comparison with standards (Bethesda Research Laboratories, Rockville, MD). After the run, gels were fixed by methanol/acetic acid and analyzed by Coomassie Blue or silver staining. Radioautographs were exposed at −70°C using X-OMAT XAR-5 film. Western blotting was done on nitrocellulose paper by standard methods.

Purification and Characterization of DAF. DAF was purified to homogeneity from a butanol extract of pooled human erythrocytes (E^{hu}) stroma. The modified fractionation sequence described by Nicholson-Weller et al. (15) was used in the initial phases of the isolation. Contaminating CR1 and glycophorin A were removed by treatment with Sepharose beads coupled to monoclonal antibodies. The resulting fraction was then subjected to additional chromatography on DEAE-Sephacel, followed by molecular sieving on high pressure liquid chromatography (HPLC), the details of which will be described elsewhere. The DAF preparation was analyzed by SDS-PAGE and silver staining, Western blotting, and by radiolabeling followed by HPLC. Upon silver staining, a single sharp band of 70,000 mol wt was observed when 30 μl of 28 μg/ml of protein were loaded in the gel (see inset of Fig. 1). By Western blotting, no glycophorin was detectable and, after HPLC purification of labeled DAF, a single 70,000 mol wt band was observed on radioautographs of SDS-PAGE gels.

Results

DAF Incorporates into Cell Membranes. Previous studies (8) have shown that *purified* DAF accelerates decay dissociation of C4b2a complexes preformed on red cell hemolytic intermediates. These findings have been interpreted to indicate that DAF functions similarly to C4-binding protein (C4bp), a serum protein that inhibits the classical pathway C3 convertase by competitively displacing C2a from C4b (27).

Initial experiments were designed to directly examine the mechanism of the DAF-mediated reaction. EAC14 were divided into aliquots that were incubated at 30°C for 15 min with increasing concentrations of DAF or with DGVB^{++} as control, and the cells thoroughly washed by centrifugation. The cells were then incubated with a predetermined limited amount of C2 (as described in the legend to Fig. 1), washed, decayed at 30°C for 15 min, and the residual C4b2a sites developed. The C4b2a hemolytic activity on the DAF-treated and washed cells was markedly reduced relative to the buffer-treated control cells (Fig. 1*A*).

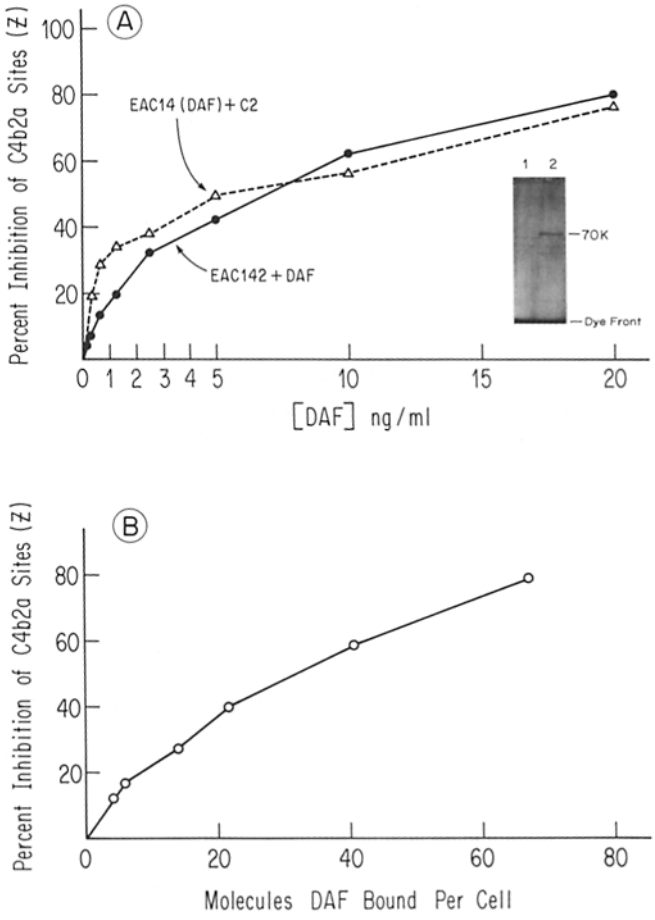

FIGURE 1. (*A*) Measurement of DAF inhibitory activity on C3 convertase by two independent assays, either after its incorporation into EAC14 intermediates (△), or directly on preformed EAC142 (●). For either titration, EAC14 were prepared with 300 SFU of C1, 100 SFU of C4, and an amount of C2 required to yield cells bearing 1.5 C4b2a sites after spontaneous decay for 15 min at 30°C, as determined in preliminary titrations. The measurement of DAF activity after its incorporation into EAC14 cells was performed as follows. The EAC14 cells were divided into aliquots which were incubated for 15 min at 30°C with increasing concentrations of DAF. After thorough washing, the DAF-sensitized cells were incubated for 5 min at 30°C with the predetermined amount of C2, washed, and placed at 30°C for 15 min, and C4b2a sites were developed by addition of C3-C9. The measurement of DAF activity on EC142 cells (conventional assay) was performed as follows: Another portion of the same EAC14 cells, treated for 15 min at 30°C with $DGVB^{++}$ instead of DAF, was incubated for 5 min at 30°C with the same amount of C2. The resulting EAC142 cells were divided into aliquots, which were decayed for 15 min at 30°C in the presence of increasing concentrations of DAF. The C4b2a sites were developed as above. For comparison, the results of both titrations are expressed as the percent inhibition of the C4b2a hemolytic activity and are plotted against the DAF concentration. (*B*) EAC14 cells were presensitized with ^{125}I-labeled DAF and, after washing, incubated with C2, decayed, and C4b2a sites developed in an identical fashion. The percent inhibition of C4b2a hemolytic activity is shown as a function of molecules of DAF incorporated per cell. A silver stain of purified DAF (28 μg/ml) after SDS-PAGE is shown in lane *2* of the inset in *A*. Buffer alone is included (lane *1*) to show the background.

Significant inhibition of hemolysis was observed on cells incubated with DAF at concentrations of <1 ng/ml.

In a parallel experiment, DAF titration was performed as described by Nicholson-Weller et al. (8). EAC14 were incubated with the same concentration of C2. After washing, the conventionally prepared EAC142 were treated with increasing concentrations of DAF and the inhibitory effect on C4b2a hemolytic sites measured. As also shown in Fig. 1*A*, the treatment of EAC142 with DAF inhibited C4b2a hemolytic activity in a dose-dependent fashion. The dose-response curve was similar to that obtained after sensitization of EAC14 with DAF, but, at low DAF concentrations, less inhibition was consistently observed than with the presensitized cells. This suggests that, in the conventional assay, DAF activity may have been incorporated into the red cells before exerting its inhibitory effect on EAC142.

To demonstrate that DAF molecules were in fact associated with the red cell membrane, the sensitization experiment was repeated with ^{125}I-labeled DAF. When the inhibition of the C4b2a hemolytic activity on the sensitized cells was plotted against cell-bound ^{125}I-DAF counts, a similar dose-response curve was obtained (Fig. 1*B*). A small number of incorporated DAF molecules (<100) had a striking effect on the hemolytic activity. SDS-PAGE analysis and radioautography of an NP-40 extract of the stroma of DAF-sensitized EAC14 cells revealed the presence of the radiolabeled 70,000 mol wt DAF band (not shown).

Interestingly, the incorporation of DAF into red cells is a selective process. When an impure radiolabeled DAF preparation was used to sensitize EAC14, DAF was taken up but the contaminants remained in the incubation medium (Fig. 2). In this experiment we used a DAF preparation in which only 14% of the ^{125}I counts were associated with the DAF polypeptide (Fig. 2, lane *1*). When EA were sensitized with this preparation, >95% of the counts that remained associated with the stroma of the red cells were identified as DAF (lane *3*). In addition, as will be discussed below, the incorporation of DAF was totally inhibited when the incubation medium contained 100 μg/ml of high density lipoprotein (HDL) from human serum (Fig. 2, lane *2*). The incorporated DAF could not be removed from the stroma of the red cells by repeated washing in the presence of high salt concentrations (1 M NaCl), but was solubilized when the stroma were extracted with 0.1% NP-40.

Studies were next performed to establish some of the characteristics of DAF sensitization. As shown in Fig. 3, this process is temperature dependent. The incorporation of DAF activity into the erythrocytes was substantially greater at 37°C than at 20°C and could not be detected at 0°C. When kinetics studies were repeated using labeled DAF, it was found that although a maximum functional effect was achieved within 20 min at 37°C incubation, uptake of label continued to increase even after 3 h.

We also found that the incorporation of DAF is not dependent on the presence of C4b on the red cells. Indeed, when EA were first sensitized with DAF, and C1 and C4 added, the inhibitory effect on C4b2a hemolytic activity was similar to that observed when EAC14 were treated with DAF under the same conditions. Uptake of DAF also occurred if the erythrocytes had been pretreated with trypsin (1 mg/ml, 30 min at 37°C) or pronase (100 μg/ml, 30 min at 37°C)

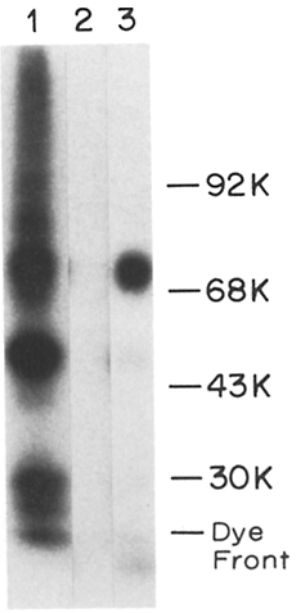

FIGURE 2. Selectivity of DAF incorporation into erythrocytes. An incompletely purified DAF preparation obtained prior to HPLC (see Materials and Methods) was labeled with ^{125}I. When the preparation was subjected to SDS-PAGE and radioautography (lane *1*), the 70,000 mol wt DAF band was apparent but other non-DAF contaminants were also present. Some of these contaminants were identified as glycophorin by Western blotting using polyclonal antibodies to glycophorin (not shown). The ^{125}I-labeled mixture was incubated for 30 min at 30°C with EA, the cells thoroughly washed, and the stroma dissolved with SDS. When the dissolved stroma was subjected to SDS-PAGE and radioautography (lane *3*), only the DAF band was detected. If, however, 100 μg/ml of HDL was added to the incubation medium during sensitization, DAF was not taken up by EA (lane *2*).

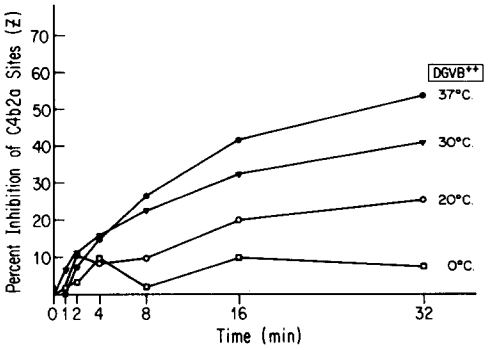

FIGURE 3. Temperature dependence and kinetics of expression of DAF activity by erythrocytes after DAF exposure. EAC14 (prepared with 300 SFU of C1 and 100 SFU of C4) were incubated at 0°, 20°, 30°, or 37°C for different times with 14 ng/ml DAF in $DGVB^{++}$. After thorough washing, the DAF-treated cells were incubated with C2, washed, decayed, and C4b2a sites developed as described in the legend to Fig. 1. As shown, DAF inhibitory activity increased with both time and temperature. In other experiments, using ^{125}I-labeled DAF, the incorporation continued to increase for >3 h of incubation at 30°C.

before the deposition of antibody, C1, and C4. In this same series of experiments we confirmed the previous observations (8, 28) that DAF sensitization had no discernible effect on the assembly of EAC1 or EAC14.

Interestingly, DAF incorporation was blocked by certain serum proteins. Sensitization of red cells (or incorporation of radiolabeled DAF; see Fig. 2) was totally inhibited if human serum lipoproteins (HDL or LDL) were present in the incubation medium at concentrations of 50 μg/ml. Bovine serum albumin (BSA) and orosomucoid were also inhibitory, but only at higher concentrations, and ovalbumin had no effect even at a concentration of 4 mg/ml (Fig. 4). In sharp contrast with these observations, we found that LDL or HDL, even at concentrations 50 times higher than those which abolished the effect of isolated DAF, had no effect on DAF activity that had been previously incorporated into EAC14 cells. The simplest interpretation of these findings is that the incorporation of DAF into the pseudomicelles of the lipoproteins or its interaction with hydrophobic areas of serum albumin or orosomucoid, prevents it from entering the red cell membrane and exerting its activity. However, once DAF (or one of the DAF domains) inserts into the nonpolar environment of the lipid bilayer of the erythrocyte surface membrane, it cannot interact with those inhibitory serum proteins.

DAF Functions to Block C3 and C5 Convertase Assembly. The ability to incorporate DAF into erythrocytes permitted us to study its effect, independently of other regulatory factors, on the formation of complement enzymes involved in the early stages of complement activation. To determine the effect of DAF on the assembly of classical pathway C3 convertase, EAC14 prepared with 100 SFU of C4 were sensitized with increasing concentrations of DAF and the cells were washed. As controls, other cell samples were incubated with buffer or with a 10^3–10^4-fold greater concentration of glycophorin A. C2 hemolytic titrations were then performed with the various cell types by standard methods; that is, by incubating them with increasing amounts of C2, followed by treatment with C3-9. The amount of C2 required for lysis increased in a dose-dependent fashion as

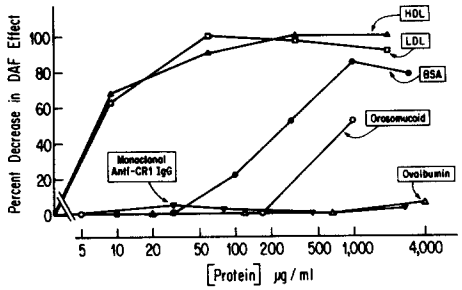

FIGURE 4. Inhibition of incorporation of DAF into erythrocytes by serum proteins. EAC14 (300 SFU of C1 and 100 SFU of C4) were incubated at 30°C for 30 min with 14 ng/ml DAF in $DGVB^{++}$ or in the presence of increasing concentrations of serum lipoproteins (HDL or LDL), BSA, orosomucoid, ovalbumin, or monoclonal antibodies (57F) to CR1. The cells were thoroughly washed and assayed for DAF activity as described in the legends to Figs. 1 and 3. HDL and LDL inhibited incorporation of DAF at low concentration. BSA and orosomucoid were inhibitory at higher concentrations, and ovalbumin and IgG antibodies to CR1 had no effect.

a function of the DAF concentration used for sensitization of EAC14, whereas glycophorin A had no effect (Fig. 5*A*).

The effect of DAF sensitization on formation of the alternative pathway C3 convertase (C3bBbP) was similarly studied. In the experiment illustrated in Fig. 5*B*, EAC143 were sensitized with three concentrations of DAF or $DGVB^{++}$. The DAF- and buffer-treated cells were then incubated at 30°C for 30 min with increasing amounts of factor B in the presence of excess factor D and P (12 μg/ml) and, after washing, C3bBbP sites were developed by incubation for 1 h at 37°C with C-EDTA. Results parallel to those observed for the C4b2a enzyme were obtained; that is, the amount of factor B required for C3 convertase assembly increased in proportion to the sensitizing dose of DAF, even when P was present.

Similar experiments were done to study the DAF effect on assembly of C5 convertase. DAF- and buffer-treated EAC143 cells, prepared with 30 SFU of C3, were incubated with different amounts of C2 and the cells incubated

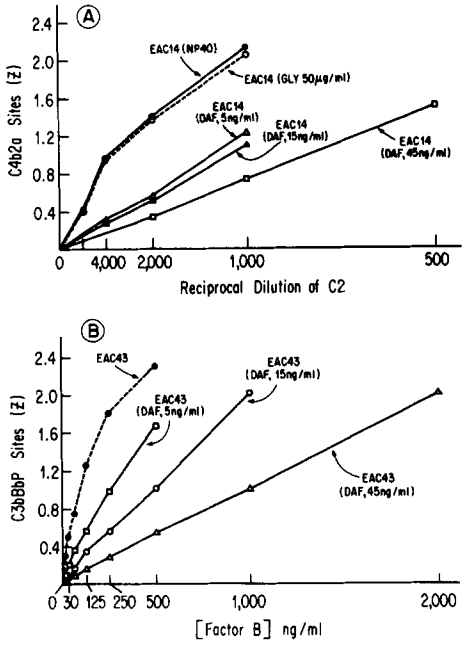

FIGURE 5. Inhibition by DAF of assembly of C3 convertases of the classical (*A*) and alternative (*B*) pathways. In *A*, EAC14 (prepared as in the legend to Fig. 1) were incubated for 30 min at 30°C with 5, 15, or 45 ng/ml of DAF, or as controls with glycophorin A (50 μg/ml) or $DGVB^{++}$ containing 0.003% NP-40, which was the highest concentration of detergent present in the DAF-containing mixtures. The variously treated EAC14 were then incubated for 5 min at 30°C with increasing concentrations of C2, as in a standard C2 titration. Without a decay step, the C4b2a sites were developed by addition of C3-9. DAF sensitization inhibited C4b2a formation in a dose-dependent fashion, while glycophorin A or NP-40 had no effect. In *B*, EAC143 (prepared as described in Materials and Methods) were similarly incubated with 5, 15, or 45 ng/ml of DAF or with buffer as control. After thorough washing, the treated cells in $DGVB^{++}$ were incubated at 30°C for 30 min with increasing amounts of factor B in the presence of excess factor D and 12 μg/ml P. The cells were washed and C3bBbP sites developed by addition of C3-9. DAF sensitization inhibited the assembly of the alternative pathway enzyme in a dose-dependent fashion.

sequentially with 300 SFU of C5 and C6-9. As shown in Fig. 6, DAF sensitization markedly reduced the efficiency of assembly of C5 convertase activity on the EAC143 cells.

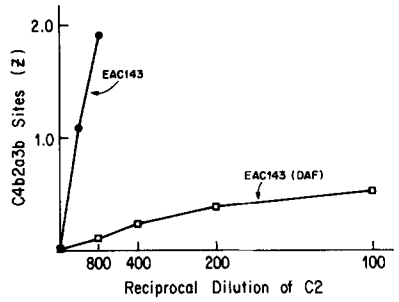

FIGURE 6. Inhibitory effect of DAF on C5 convertase assembly. EAC143 were sensitized at 30°C for 30 min with DAF (45 ng/ml) in $DGVB^{++}$ or with buffer as in the legend to Fig. 5. The DAF-treated and control cells were then incubated with increasing concentrations of C2 and, after washing, the resulting C4b2a3b sites on the two cell types developed by incubation for 5 min at 30°C with C5 and 60 min at 37°C with C6-C9. Generation of the C4b2a3b enzyme was markedly reduced on the DAF-treated cells.

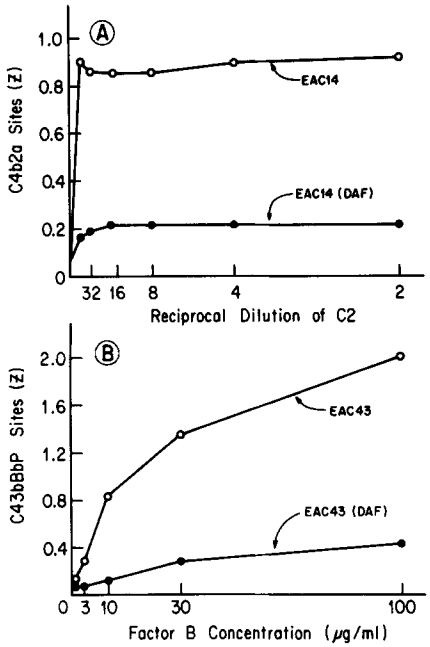

FIGURE 7. Blockage by membrane-associated DAF of C4b (*A*) and C3b (*B*) hemolytic activity on $EAC14_{lim}$ and $EAC143_{lim}$ cells. In *A*, $EAC14_{lim}$ were incubated at 30°C for 30 min with 14 ng/ml DAF or buffer. The cells were incubated at 30°C for 5 min with C2 over an extended range of concentrations and C4b2a sites developed by addition of C3-9. In *B*, $EAC143_{lim}$ sensitized with DAF or treated with buffer in the same way were incubated at 30°C for 30 min with factor B (in $DGVB^{++}$) over an extended range of concentrations, in the presence of excess D and 12 μg/ml P, and C3bBbP sites were developed by addition of C3-9. The DAF-mediated inhibition of classical or alternative pathway C3 convertase formation was not overcome by augmenting the C2 or factor B concentrations.

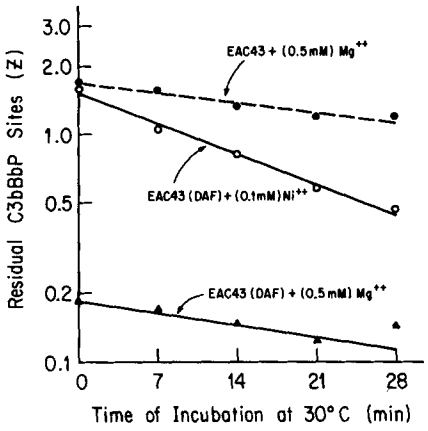

FIGURE 8. Effect of metals on assembly and decay of alternative pathway C3 convertase on DAF-containing erythrocytes. $EAC143_{lim}$ were incubated at 30°C for 30 min with DAF (14 ng/ml) or with $DGVB^{++}$ as a control. The washed DAF-treated and control cells were then incubated at 30°C for 30 min with 100 μg/ml factor B, excess factor D, and 12 μg/ml P in buffer containing 0.5 mM Mg^{++} or buffer containing 0.1 mM Ni^{++}. The different cell types were then placed at 30°C, and C3bBbP sites developed after progressively longer incubation times as described in Materials and Methods. In the presence of Ni^{++}, the initial C3bBbP hemolytic activity on the DAF-treated cells approached that on the control cells, but the enzyme subsequently decayed at a faster rate.

The above studies were performed using EAC14 or EAC143 bearing excess C4b or C3b. When they were repeated using cell intermediates bearing limited C4b or C3b, the effects of DAF were even more striking. After incubation of the DAF-treated $EAC14_{lim}$ with up to 3,000 SFU of C2 or $^{oxy}C2$ (Fig. 7*A*), the number of hemolytic sites per cell (Z) was only 0.2, while after incubation of the buffer-treated $EAC14_{lim}$ with 30 SFU of C2, Z was 0.9. Similarly, when DAF-treated $EAC43_{lim}$ were incubated with factors B (100 μg/ml), D, and P, the C3b hemolytic activity was reduced >80% relative to the control cells (Fig. 7*B*).

Because of the reported enhancing activity of Ni^{++} or high Mg^{++} concentrations on the assembly of alternative pathway C3 convertase (29, 30), next we studied the effects of these ions on DAF activity. We found that raising the Mg^{++} concentration in the incubation medium from 0.5 to 5 mM or substituting 0.1 mM Ni^{++} for Mg^{++} partially overcame the inhibitory activity of DAF. In the series of experiments shown in Fig. 8, DAF-treated $EAC43_{lim}$ or control cells were treated with factor B (100 μg/ml), D, and P in the presence of physiological Mg^{++} concentrations (0.5 mM) or in the presence of Ni^{++} (0.1 mM). After washing, the cells were placed at 30°C and the kinetics of dissociation of the C3bBbP enzyme on the different cell types was compared. As expected, when a physiological Mg^{++} concentration was used at the start of the experiment, substantially lower C3b hemolytic activity was detectable on the DAF-treated cells relative to the control cells, but the decay rates of the enzymes on both cells were comparable. When Ni^{++} was substituted for Mg^{++}, the effect of DAF was overcome; that is, the initial C3b hemolytic activity on the DAF-treated cells increased to that on the nontreated cells. However, the rate of decay of the Ni^{++}-stabilized enzyme generated on the DAF cells was substantially faster than that

of the Mg^{++} (or Ni^{++})-dependent enzyme generated on the control cells. It is known that raising the Mg^{++} concentration or substituting Ni^{++} for Mg^{++} increases the binding affinity of factor B for C3b (29, 30). The present results indicate that this maneuver has facilitated the assembly of alternative pathway convertase experimentally in vitro on DAF-containing E^{hu} and explain previous observations by others (9, 14).

Mechanism of Action of DAF. In view of the above findings that DAF can render C4b and C3b sites functionally inert, it was important to ascertain whether DAF alters the C4b/C3b structure and how it blocks convertase assembly. In one series of experiments, buffer-treated and DAF-treated EAC14 were either incubated with anti-DAF polyclonal IgG antibodies or with preimmunization IgG, and the cells analyzed for C4b hemolytic activity. As seen in Fig. 9, pretreatment with rabbit anti-DAF antibodies completely restored the C4b functional activity on the DAF-treated cells, while pretreatment with normal rabbit IgG had no effect. The DAF effect was fully reversible even when the DAF-treated EAC14 were preincubated at 37°C for 2 h before treatment with antibody. Additional studies were done in which the DAF-treated EAC14 were incubated with C2 and the cells allowed to decay before the addition of anti-DAF antibodies. Complete restoration of the C4b functional sites was again observed. Anti-DAF antibodies also fully restored the C3b hemolytic activity of $EAC43_{lim}$ cells sensitized with DAF.

To study directly the effect of DAF on C3b and C4b structure, we prepared $EAC43_{lim}$ cells with ^{125}I-C3. After sensitization of the labeled intermediate with DAF (10 ng/ml) and titrations to confirm that the C3b hemolytic activity was diminished by ~80%, the cells were lysed and the stroma subjected to SDS-PAGE and radioautography. No changes in the apparent molecular weights of the α and β chains of C3b were observed. In further studies, the DAF-treated $EAC43_{lim}$ prepared with ^{125}I-C3 were mixed with excess factors B, D, and P and, after 30 min incubation at 30°C, subjected to SDS-PAGE and radioautography. As before, the C3b gel patterns remained unchanged (not shown).

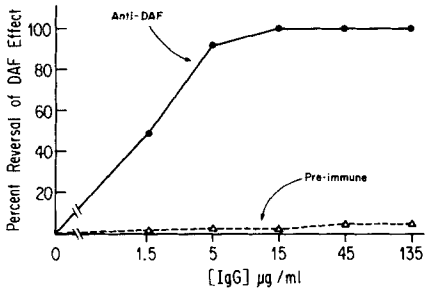

FIGURE 9. Reversibility of the DAF effect on EAC14 by anti-DAF antibodies. EAC14 (300 SFU C1 and 100 SFU C4) were incubated with 14 ng/ml of DAF or buffer as a control. After thorough washing, the cells were then incubated for 15 min at 30°C and 15 min at 0°C with increasing concentrations of IgG from a rabbit anti-DAF antiserum or with preimmunization IgG. After another thorough washing, C4b sites on the cells were developed by incubation at 30°C for 5 min with 300 SFU of C2 and C4b2a sites developed by addition of C3-9. The percent reversal of DAF-mediated inhibition of C4b hemolytic activity is shown as a function of IgG concentration. The anti-DAF antibodies restored the hemolytic activity of DAF-sensitized cells, while the preimmunization IgG had no effect.

We also performed experiments to verify whether, after its incorporation, the DAF inhibitory effect could be enhanced by further incubation of the sensitized cells at 37°C. EAC14 cells prepared with 300 SFU of C4 were sensitized with suboptimal concentrations of DAF (1 ng/ml) for 30 min at 30°C. The cells were washed and reincubated at 37°C. At various times thereafter, up to 2 h, samples were removed and C2 titrations performed. The DAF inhibitory activity was identical in all samples, suggesting that once incorporated into the cell membrane, DAF inhibits C4b function stoichiometrically, not enzymatically.

DAF could act to prevent C3 convertase formation either by blocking uptake of C2 and factor B or preventing conversion of C4b-bound C2 or C3b-bound factor B zymogens to enzymatically active C2a or Bb fragments. This question was examined using the alternative pathway enzyme. Portions of $EC143_{lim}$ were sensitized with DAF, or, alternatively, incubated with buffer as control. After washing, the two cell types were assayed in parallel studies for C3b hemolytic activity and for uptake of ^{125}I-labeled factor B. Diminished C3b hemolytic activity on the DAF cells relative to the buffer control cells was associated with reduced uptake of ^{125}I-factor B (not shown). In a further experiment, uptake of ^{125}I factor B on DAF-treated $EAC43_{lim}$ cells was induced by adding 0.1 mM Ni^{++} instead of Mg^{++} and, after decay at 30°C for 30 min, the supernatant and stroma of the cells were subjected to SDS-PAGE. No unusual factor B fragments were detected either still associated with the DAF cells or released into the supernatant (not shown).

DAF Can Only Function Intrinsically Within the Cell Membrane. Studies were next undertaken to determine whether membrane-associated DAF only functions intrinsically or also can act extrinsically, that is inhibit C3 convertase on other cells. In the first series of experiments we evaluated the influence of cell dilution on the activity of membrane-associated DAF. We reasoned that if DAF acted extrinsically, its effect should be highly dependent on the cell concentration. EA were sensitized with DAF or incubated with $DGVB^{++}$ as control and then reacted stepwise with C1, C4, and limiting C2. After washing, aliquots of the cells were suspended into progressively larger volumes of ice-cold $DGVB^{++}$, to concentrations of 10^6–10^8/ml. The cell suspensions were placed at 30°C for 15 min to decay and the residual C4b2a hemolytic activity was measured. We found that although the hemolytic activity of DAF-sensitized EAC142 was lower than that of EAC142 ($Z = 0.090$ and 0.330, respectively), for each cell type the degree of hemolysis was constant and independent of cell concentration.

Next, we studied directly the effect of DAF-containing cells on the C4b2a enzyme assembled on non–DAF-containing cells. In one set of experiments (Fig. 10*A*), aliquots of EAC142 were mixed with equal numbers of EA, DAF-treated EA, or buffer. The mixtures were then placed at 30°C and the kinetics of decay of C4b2a measured. The rate of decay was identical in all samples. In another set of experiments, we used intact E^{hu} as a source of DAF. EAC142 were incubated with increasing numbers of intact E^{hu} for 30 min at 30°C. As shown in Fig. 10*B*, E^{hu} inhibited C4b2a enzymatic activity in a dose-dependent fashion, but this effect was mediated by the complement receptor CR1 also present on E^{hu}, and not by DAF. Indeed, polyclonal anti-DAF antibodies had no effect on the E^{hu}-mediated inhibition of C4b2a, while anti-CR1 completely reversed it.

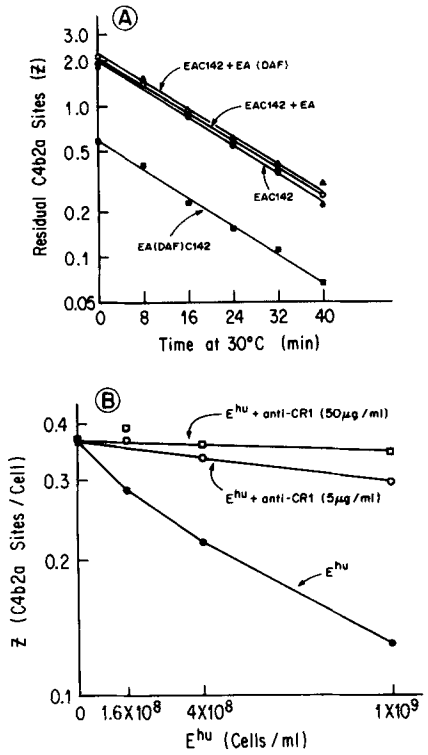

FIGURE 10. Inability of membrane-incorporated DAF to inhibit C3 convertase extrinsically on substrates. The effect of DAF-containing cells on the decay rate of C4b2a assembled on non–DAF-containing cells is shown in *A*. EAC142 (5×10^7/ml) prepared as described in the legend to Fig. 1, were mixed with an equal number of DAF-sensitized EA, which had been prepared with 14 ng/ml DAF and thoroughly washed. HDL (50 μg/ml) was added to the buffer and the mixtures placed at 30°C. The decay of C4b2a on EAC142 was then measured as a function of time as described in Materials and Methods. The decay of C4b2a on EAC142 prepared in an identical fashion and mixed with nontreated EA was also measured in parallel as a control. As shown, the presence of DAF in the DAF-treated EA inhibited the formation of C4b2a on these cells. However, the addition of DAF-treated EA to the incubation mixture had no effect on the decay rate of C4b2a on non–DAF-treated cells. (*B*) The ability of monoclonal anti-CR1 antibodies to reverse the extrinsic C3 convertase inhibition mediated by intact E^{hu}. $EAC142_{lim}$ (5×10^7/ml) prepared with 300 SFU of C1, 100 SFU of C4, and ~1.5 SFU of C2 were incubated for 30 min at 30°C with increasing numbers of intact E^{hu} in the absence or presence of pooled monoclonal anti-CR1 antibodies (57F, 44D, and 31D). The cells were washed and C4b2a hemolytic activity developed by addition of C3-9. Control tubes containing E^{hu} alone were included, and the optical density of the hemoglobin released was subtracted from the optical density in the experimental tubes. The anti-CR1 antibodies completely blocked the extrinsic inhibitory effect of the E^{hu} on C4b2a assembled on the sheep cells. In several other experiments, we found that polyclonal mouse or rabbit anti-DAF had no effect at concentrations that reversed the DAF effect on DAF-sensitized sheep cells.

To establish whether the intrinsic inhibitory effect of DAF on complement activation can in fact diminish lysis of cells by complement, we performed studies with rabbit erythrocytes, known to be strong activators of the alternative pathway in human serum and not to be subject to regulation by factors I and H (31, 32). Rabbit erythrocytes were sensitized with increasing concentrations of human DAF or incubated with buffer as control. After thorough washing, aliquots of

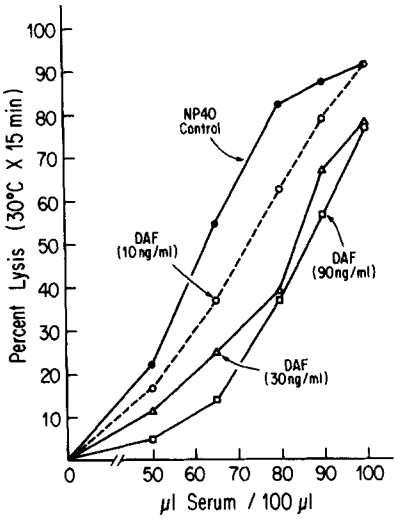

FIGURE 11. Effects of membrane incorporation of DAF on the sensitivity of rabbit erythrocytes to hemolysis by human complement. The rabbit cells were pretreated with different amounts of DAF, as in Fig. 5, and washed. Aliquots of 100 μl of the DAF-treated or nontreated control cells (1×10^8/ml) in GVB containing 1 mM EGTA and 1 mM $MgCl_2$ were incubated with 100 μl of various dilutions of human serum. After 15 min at 30°C, 1.8 ml of GVB-EDTA were added, the cells pelleted, and the percent lysis determined.

the DAF-treated and control cells were incubated at 30°C for 10 min with decreasing dilutions of Mg^{++}-EGTA human serum. The cells were then diluted with ice-cold buffer and centrifuged, and lysis was measured (Fig. 11). The amount of human serum required for lysis increased as a function of the concentration of DAF used to sensitize the cells. In control studies, the rabbit erythrocytes were incubated with 100 μg/ml of purified glycophorin A instead of DAF, and no effect was observed. Moreover, similar to the results in Fig. 2, only the 70,000 mol wt DAF band could be detected in the washed rabbit erythrocyte stroma when these cells were sensitized with partially purified DAF. As an additional control, we found that the DAF-mediated inhibition of lysis was reversed when the DAF-containing rabbit cells were exposed to anti-DAF but not to anti-glycophorin antibodies.

Discussion

In the present study, we purified DAF to homogeneity from human red cell stroma and found that it exhibits the unique property of being able to reassociate with red cells and express its biological activity. Association with the cells does not depend upon the prior presence of deposited complement components, since the uptake of DAF proceeds efficiently on EA or untreated E. After membrane binding, the incorporated DAF has some properties of an integral membrane protein. It cannot be removed by repeated washing of the cells, even in the presence of high salt concentrations, but can be extracted with buffers containing detergents. The conditions under which membrane incorporation of DAF occurs, and the efficiency with which the reincorporated molecule exerts its

biological effect (Fig. 1), suggest that the accelerated decay dissociation of EAC142, previously observed upon exposure to DAF (8), involves its prior membrane incorporation into the cellular intermediate.

The precise mechanism of DAF incorporation into the cell membrane is not clear. The temperature dependency of the uptake (Fig. 3) is compatible with a process requiring membrane mobility. One possibility is that DAF binds to a specific membrane acceptor molecule. The incorporation was not abolished, however, by pretreatment of the cells with trypsin or pronase, indicating that, if specific acceptor sites exist, they are either not accessible or not susceptible to proteolysis by these enzymes. An alternative possibility is that DAF is inserted into the lipid bilayer nonspecifically via a hydrophobic domain. The observation that DAF binds tightly to phenyl-Sepharose (8) and can be removed only with high concentrations of detergent supports the existence of such a domain in the molecule. The strong inhibitory effect of LDL and HDL on red cell incorporation (Fig. 4) may be due to the tendency of DAF to alternatively insert into the pseudomicelles of these lipoproteins. Hydrophobicity of a DAF domain may also explain the finding that, in purified form, the protein appears to be a multimer. Indeed, isolated DAF has an apparent molecular weight of $\geq$150,000 by molecular sieve chromatography performed in the presence of different amounts of detergents, below or above the critical micellar concentration (manuscript in preparation).

The finding that incorporation of DAF into membranes is a highly selective process may have relevance to the interesting prior reports of Okada et al. (33, 34). They observed that erythrocytes were not lysed by heterologous complement if they were pretreated with a glycophorin preparation homologous to the complement source, and suggested that the bound glycophorin prevented complement activation. Our studies with sheep cells, using a partially purified DAF preparation containing glycophorin, showed that only DAF was incorporated (Fig. 2). Moreover, we performed studies similar to theirs using rabbit erythrocytes, except that we treated the cells with either purified DAF or glycophorin. Only DAF was incorporated, and it enhanced the resistance of the red cells to lysis by human complement (Fig. 11). It therefore seems possible that Okada's glycophorin preparation contained DAF.

The ability to incorporate DAF into cell membranes is of great interest for at least two reasons. In the first place, it permits the study of DAF function on the early steps of complement activation in selected microenvironments, as well as its potential effect at other stages of the complement cascade, for example, formation of terminal pathway complement complexes. In addition, it raises the intriguing possibility of correcting in vitro the defect in the membranes of cells from patients with PNH, which would demonstrate directly that DAF deficiency is the cause of the clinical abnormality.

Once integrated with the cell membrane, DAF acts primarily to prevent convertase assembly on the cell surface. Formation of C3 and C5 convertases of both the classical and alternative pathways is blocked. When added to EA, DAF does not interfere with the deposition of C1 or C4. The inhibition of the classical pathway enzymes occurs at the level of C2 and cannot be bypassed by augmenting the C2 concentration. The presence of as few as 70 DAF molecules in the

membrane markedly inhibited C4b2a assembly (Fig. 1 *B*). This striking effect of DAF in preventing C2 activation is the most likely explanation of previous observations by Brown et al. (28) that, after sensitization with antibody, E^{hu} consume and take up C1 and C4 as efficiently as sheep E, but do not consume C2.

By acting primarily to prevent formation of convertases, the membrane factor can function to block the hemolytic activity of C4b or C3b fragments inadvertently deposited on host membranes, while simultaneously preventing turnover of C2 and/or factor B. Although the membrane factor can also accelerate the decay of preformed convertase complexes (8, 9, 14), and therefore can additionally act to immediately dissociate any complexes that escape the block of convertase formation, it could be argued that the present nomenclature, i.e., decay-accelerating factor, does not accurately reflect its main function.

Several lines of evidence indicate that DAF prevents the binding of C2 and factor B zymogens to C4b and C3b, respectively. Pangburn et al. (14) previously found that DAF accelerates the dissociation of C3bBb assembled on zymosan particles. We demonstrated here that when EAC14 cells were sensitized with a limiting amount of DAF, the extent of inhibition of the convertases did not increase regardless of the time of incubation, indicating that the DAF effect was stoichiometric, not enzymatic. Gel patterns of C3b after DAF sensitization of EAC43 were unchanged. More important, reduced C4b and C3b hemolytic activities on DAF-treated EAC14 and EAC43, respectively, were fully restored after incubation of the cells with anti-DAF antibodies, indicating that DAF does not alter C4b or C3b structure in some unusual way not apparent in the gel patterns. Finally, decreased hemolytic activity on EAC43 cells exposed to DAF correlated with decreased uptake of ^{125}I-labeled factor B. The simplest interpretation for all these findings is that DAF associates within the cell membrane directly with C4b and C3b, thereby preventing uptake of C2 and factor B.

When the regulatory factor is membrane incorporated, and presumably is in its native state, it inhibits convertase formation on the same membrane, but does not influence the formation or stability of convertases assembled on other nearby cells or substrates. Indeed, the rate of decay of C4b2a hemolytic activity on DAF-treated cellular intermediates was not influenced by dilution of the cells, indicating that DAF in the membrane of neighboring cells did not contribute to the decay. The addition of DAF-sensitized EA to nontreated EAC142 did not alter the decay rate of the C4b2a enzyme on the neighboring cells. The inhibitory effect of intact E^{hu} on EAC142 was not diminished by antibodies to DAF but instead was totally abolished with monoclonal anti-CR1 antibodies. Finally, serum proteins inhibited the function of purified DAF before it was taken up by the red cells, but did not affect the function of membrane-incorporated DAF. The ability of DAF to function only within the membrane may relate to the manner in which it is oriented spatially and/or the way in which it interacts with the covalently bound C4b or C3b.

In Fig. 12 we represent schematically our current hypothesis explaining the results described in this and previous papers. We postulate that DAF contains a hydrophobic domain in close proximity to its active site. In the purified state, DAF is aggregated and inactive, or poorly active, because its binding site is not

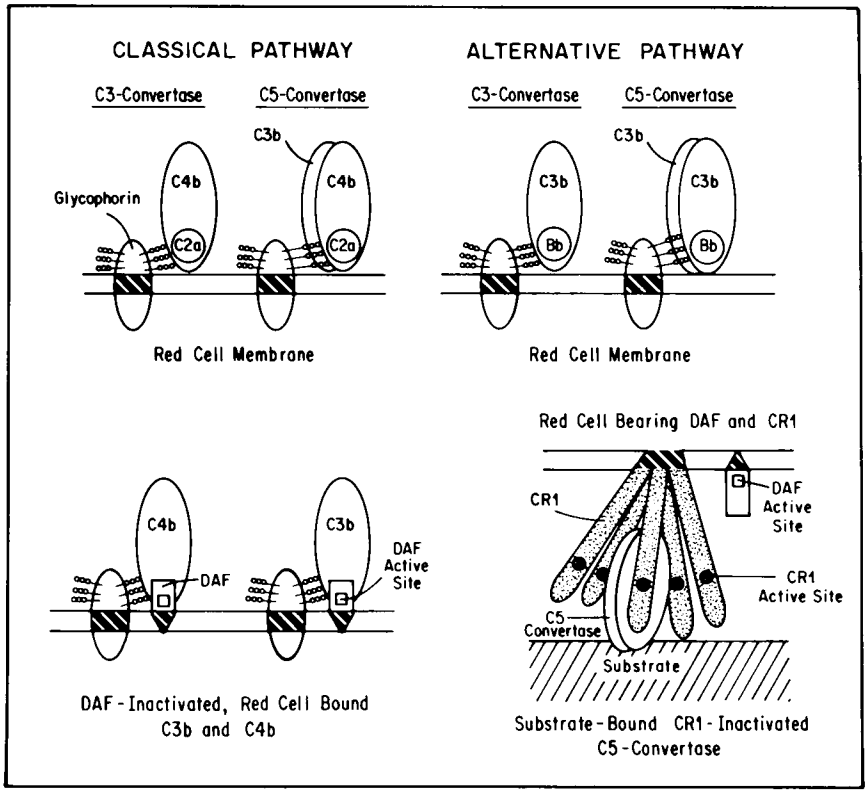

FIGURE 12. Schematic representation of the proposed mechanism of action of DAF and CR1 on C3 and C5 convertases assembled on host cells or on substrates. Both DAF and CR1 can prevent the assembly of the enzymes by competing with factor B and C2 for sites on C3b and C4b. However, DAF can only act intrinsically, while CR1 can reach C3b or C4b clusters on neighboring cells or substrates such as immune complexes. CR1 is represented in a clustered form (41).

fully available to the exterior, or because of allosteric changes in the molecule. When DAF is incorporated into the cell membrane, through its hydrophobic domain, its original configuration is reestablished and it can now interact with C3b or C4b, mostly associated with glycophorin molecules (35, 36). DAF must be fully mobile in the plane of the membrane in order to come rapidly into contact with the scarce C4b or C3b ligand deposited elsewhere on the surface of the cell. Also, according to this concept, the active site on CR1, which is a much larger molecule, can reach C3b and C4b on neighboring cells (or in immune complexes) (2, 3, 37) but the active site of DAF is unavailable to the exterior.

The molecular mechanisms responsible for the protection of host cells from the deleterious effects of activation of autologous complement on their surface have been the subject of extensive investigations and speculations. Although sialic acid has been shown to play an important role in controlling activation of the alternative pathway (32, 38, 39), E^{hu} still resist lysis by human complement after desialation. In view of this finding and of the observation that C3 convertase can be inhibited by CR1, it has been proposed (4) that the CR1 molecule also

plays a role in the intrinsic control of complement activation in cell membranes. The results of the present paper suggest that this intrinsic control is the main function of DAF rather than of CR1. This notion is in agreement with the observations of Okada et al. (40) who found that nucleated cells lacking CR1 resist lysis by complement even after their desialation. Also, the complement-sensitive red cells of patients with PHN are DAF deficient but can have normal levels of CR1 (14).

In short, it appears that the 70,000 mol wt DAF molecule may not only safeguard autologous cells against the results of spontaneous activation via the alternative pathway, but could also protect them from the classical pathway cascade if they were to be recognized by autoantibodies. The strict restriction of DAF functional activity to the cell membrane would prevent progression of the cascade on host cells but not impair the initiation and amplification of the cascade on foreign substrates.

Summary

Decay-accelerating factor (DAF), extracted from the stroma of human erythrocytes, was purified to homogeneity and incorporated into the membrane of sheep red cell complement intermediates, where its functional properties were analyzed. Incorporation of DAF into the cell membranes was temperature dependent, took place on pronase- or trypsin-treated erythrocytes, and did not depend on prior deposition of antibody, C1 or C4. Serum lipoproteins (high and low density) effectively inhibited DAF incorporation, but had no effect on the activity of DAF after its association with the cell membrane. The incorporated DAF could not be removed from the red cell surface by repeated washings in the presence of high salt concentration but was solubilized when the stroma were extracted with 0.1% Nonidet P-40.

The presence of DAF in the membrane of EA did not affect the deposition of C1 and C4, but as few as 10^2 DAF molecules per cell profoundly inhibited the assembly of C3 and C5 convertases of both the classical and alternative pathways. The DAF inhibitory effect on EAC14 or EAC43 was not overcome by supplying an excess of C2 or factor B, but the alternative pathway C3 convertase could be assembled in the presence of Ni^{++}, or nonphysiological concentrations of Mg^{++}, which enhances the binding affinity of factor B for C3b. The DAF effect on EAC14 or EAC143 was entirely reversed by treating the cells with specific anti-DAF antibodies, showing that DAF did not alter the structure of C4b or C3b. Taken together, the experimental evidence suggests that DAF interacts directly with membrane-bound C3b or C4b and prevents subsequent uptake of C2 and factor B.

DAF can function only within the cell membrane. Indeed, the decay dissociation of the C4b2a enzyme on DAF-containing sheep intermediates was not changed by varying the cell concentration. DAF-treated EA had no influence on the decay of nontreated EAC142 present in the same mixture. Moreover, the inhibitory activity of intact human erythrocytes on C4b2a was not blocked by antibodies to DAF, but was abolished by antibodies to the C3b/C4b receptor (CR1). When incorporated into the membrane of rabbit erythrocytes, human DAF inhibited their lysis by human complement.

In conclusion, on the basis of these and previous results, it appears that DAF plays a central role in preventing the amplification of the complement cascade on host cell surfaces. While DAF can act only intrinsically to protect the host cells, CR1 inhibits the assembly of the convertases extrinsically on substrates, such as immune complexes, which may come in contact with host cells.

We thank Elizabeth Walter, Keiko Kinoshita, and Richard Melton for technical assistance, and Joanne Joseph, Randi Klein, and Roger Rose for manuscript preparation.

Received for publication 8 August 1984.

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