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Purification and characterization of decay-accelerating factor (DAF) from Raji cells

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SUMMARY

Decay-accelerating factor (DAF), a membrane protein that regulates the complement system, was purified to homogeneity from lymphoblastoid (Raji) cells (DAF-R). It exhibited almost the same molecular weight as DAF from stroma of erythrocytes (DAF-S). Purified DAF-R, which could be reincorporated into cell membranes, accelerated the decay of the C3 convertases, in both the classical (C4b2a) and the alternative (C3bBb) pathways. This activity was completely inhibited by a monoclonal anti-DAF antibody, 1C6. From these results, DAF-R and DAF-S can not be distinguished; however, the decay-accelerating activity of DAF-R was much higher than that of DAF-S. 1C6 enhanced the binding of C3 to Raji cells by incubating with six purified components of the alternative pathway, whereas it did not induce the killing of Raji cells after a short incubation period. When antibodies against Raji cells were added to the above system, the blocking of DAF activity by 1C6 resulted in efficient killing of Raji cells by autologous complement. From these results, it is clear that DAF on nucleated cells plays an important role in protecting these cells from the damage caused by autologous complement.

INTRODUCTION

Decay-accelerating factor (DAF) was first described by Hoffman (1969a,b) as an inhibitor of erythrocytes against complement, later isolated and characterized as a 70,000 MW protein by Nicholson-Weller *et al.* (1982). The function of DAF is to prevent the assembly of the classical and alternative pathway C3 convertases on the cell surface by dissociating C2a and Bb from the binding sites (Fujita *et al.*, 1987). It is considered that DAF is partially or completely deficient in the membrane of erythrocytes of patients with paroxysmal nocturnal haemoglobinuria (PNH), an acquired haemolytic anaemia characterized by an abnormal susceptibility of red cells to the lytic action of complement (Nicholson-Weller *et al.*, 1983; Pangburn, Schreiber & Müller-Eberhard, 1983).

It has been shown that DAF is present not only on red cells, but also on the surface membrane of neutrophils, lymphocytes, monocytes, platelets (Nicholson-Weller *et al.*, 1985; Kinoshita *et al.*, 1985) and endothelial cells (Ash *et al.*, 1986). However, DAF has not yet been purified nor characterized from cells other than erythrocytes. It is well known that nucleated cells are more resistant against damage by complement activation than erythrocytes, although the mechanism is not clear. In this paper, we purified DAF from Raji cells, a lymphoblastoid cell line, and clarified the function of DAF on nucleated blood cells.

Abbreviations: C-EDTA, guinea-pig serum diluted 1:50 with EDTA-GVB; DAF, decay-accelerating factor; DAF-S, DAF from stroma of human erythrocytes; DAF-R, DAF from Raji cells.

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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 (Nelson *et al.*, 1966), human C4 (Bolston *et al.*, 1977), C2 (Polly & Müller-Eberhard, 1967), C3 (Tack & Prahl, 1976), B (Boenisch & Alper, 1970), D (Lesavre *et al.*, 1979), H (Whaley & Ruddy, 1976), I (Fearon, 1977) and P (Medicus *et al.*, 1980) were purified as described previously. Guinea-pig serum diluted 1:50 in EDTA–GVB (C-EDTA) was used as a source of C3–C9.

Cells

Raji cells were cultured in RPMI-1640 containing 10% fetal calf serum, penicillin and streptomycin at 37° in 5% CO_2.

Cellular intermediates

Antibody-sensitized sheep erythrocytes carrying guinea-pig C1 and human C4 (EAC1 4b) were prepared as described elsewhere (Fujita & Tamura, 1983). EAC14b2a were prepared by adding oxidized human C2 (Polly & Müller-Eberhard, 1967) to

EAC14b to yield 1–1·5 haemolytic sites per cell. EAC4b3b cells were prepared as follows: EAC14b (1×10^8/ml) were incubated for 30 min at 37° with 50 U/ml of guinea-pig C2 and 300 U/ml of C3 and, after washing, these cells were further incubated at 37° for 3 hr. EAC3bBbP were obtained by adding a limiting amount of B and excess of D and P to EAC4b3b to yield 1–1·5 haemolytic sites per cell.

Assay

The activity of isolated DAF or DAF-containing preparations was assayed by its ability to accelerate the decay of EAC14b2a, as described by Nicholson-Weller *et al.* (1982). Briefly, 100 μl of sample were incubated for 30 min at 30° with an equal volume of EAC14b2a (1×10^8/ml). The residual haemolytic sites were developed by adding 1·3 ml of C-EDTA, followed by further incubation at 37° for 1 hr. Additional details of the assay are given in the legends to the figures.

Antibodies

Monoclonal antibodies against DAF were prepared as described previously (Fujita *et al.*, 1987). Of four monoclonal antibodies, 1C6 (IgG1 k isotype), which completely blocked the activity of DAF, was used in this study. This antibody was purified to homogeneity from ascites fluids of mice bearing the hybridoma by ammonium sulphate precipitation, followed by chromatography on a DEAE Toyopearl column (Toyosoda Manufacturing Co. Ltd, Tokyo). Fab fragment of 1C6 was prepared as described previously (Parham *et al.*, 1982). Anti-lymphocyte serum obtained from a patient with systemic lupus erythematosus was heated at 56° for 30 min and used as antibodies against Raji cells.

Purification of DAF

DAF from Raji cells (DAF-R) was purified as follows: harvested Raji cells (about 5×10^9 cells) were suspended in 50 ml of homogenizing buffer (10 mM Tris, pH 7·2 containing 40 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 100 μg/ml leupeptin, 0·1 μg/ml pepstatin, 20 μg/ml soybean trypsin inhibitor and 50 μM *p*-amidinophenyl-methanesulphonyl fluoride hydrochloride; *p*-APMSF; Wako Pure Chemicals Industries Ltd, Osaka) and disrupted by N_2 cavitation at 25 kg/cm^2. Nuclei and debris were removed by centrifugation for 15 min at 600 ***g*** and the supernatants were centrifuged for 1 hr at 100,000 ***g***. The pellets were suspended in 10 mM Tris buffer, pH 7·5, containing 40 mM NaCl and 50 μM *p*-APMSF, and extracted twice with *n*-butanol. The resulting butanol-saturated aqueous phase was applied to a DEAE–Toyopearl column equilibrated with 10 mM Tris buffer, pH 7·5, containing 40 mM NaCl and 0·1% Chaps (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Dojindo Laboratories, Kumamoto). The column was then washed with the same buffer and eluted with a linear NaCl gradient. Fractions with DAF activity were applied to monoclonal anti-DAF-bound Sepharose 4B column (Pharmacia, Uppsala, Sweden) and eluted with 3 M KSCN, containing 0·1% Chaps. SDS–PAGE analysis of purified DAF-R showed a single-stained band of a 70,000 MW.

DAF was also purified from pooled human erythrocytes. According to the method described by Nicholson-Weller *et al.* (1983), ghosts were prepared and extracted with *n*-butanol. The resulting butanol-saturated water phase was applied to a DEAE–Toyopearl column as described above. The active fractions were applied to an affinity chromatography of monoclonal anti-DAF-coupled Sepharose and eluted with 3 M KSCN. 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.

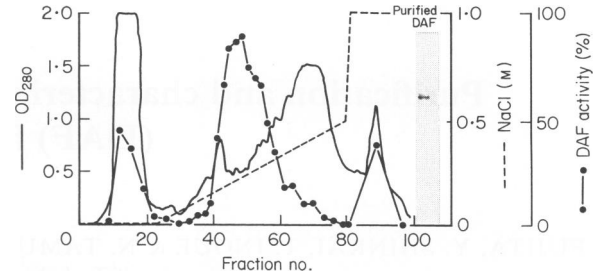

Figure 1. Chromatography on DEAE–Toyopearl of butanol extracts of Raji cells. The fractions were diluted 1:40 and 100 μl were incubated for 30 min at 30° with an equal volume of EAC14b2a (1×10^8/ml). Then, 1·3 ml of C-EDTA were added to the cell and after incubation at 37° for 1 hr, the degree of lysis was measured. Fractions 39–61 were collected and subjected to an affinity chromatography, as described in the Materials and Methods. The inset shows the results of the 7·5% SDS–PAGE, under non-reducing conditions, of the finally purified DAF-R.

SDS–PAGE and immunoelectroblotting

SDS–PAGE was performed according to Laemmli (1970). Purified DAF was subjected to SDS–PAGE under non-reducing conditions, and then transblotted onto hydrophobic Durapore sheets (Millipore ltd Corp., Bedford, MA). The sheet was blocked with 5% BSA in phosphate-buffered saline (PBS) for 1 hr at 37°, and incubated for 1 hr with monoclonal anti-DAF (1 μg/ml in PBS containing 1% horse serum). After washing, the sheet was treated with Vectastain ABC kit (Vector Laboratories, Sunnyvale, CA) according to the instruction of the manufacturer.

Mixture of the six isolated alternative pathway components

Alternative complement pathway was reconstructed with one-fourth the physiological concentrations of purified C3, B, D, P, I, and H in DGVB as described by Fujita, Takata & Tamura (1981).

Radiolabelling

C3 was labelled with ^{125}I by the Iodogen method (Pierce Chemical Co., Rockford, IL) and unbound ^{125}I was removed by gel filtration through Sephadex G-25 (Pharmacia Fine Chemicals), followed by extensive dialysis.

Killing assay

Fifty microlitres of Raji cells (5×10^6/ml) were first incubated with 50 μl of anti-lymphocyte serum (1:50 dilution) for 30 min at 0°C, and then with 50 μl of monoclonal anti-DAF antibody, 1C6 or Fab of 1C6 (100 μg/ml) for 30 min at 0°. The mixtures were incubated further with 50 μl of normal human serum as a source of complement for 40 min at 37°. Viabilities were measured by dye exclusion with trypan blue and the percentage killing was calculated.

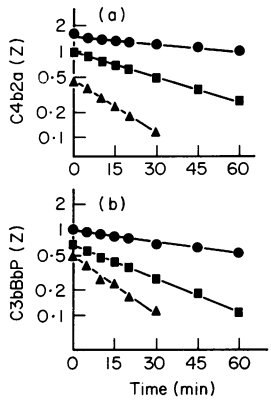

Figure 2. Decay-accelerating activity of DAF-R on C3 convertases. (a) EAC14b cells (1×10^8/ml) were reacted with an equal volume of DAF-R 50 ng/ml (▲), 25 ng/ml (■) or DGVB (●) for 30 min at 37°. The cells were washed twice with DGVB and incubated at 30° for 15 min with a limiting amount of oxidized C2. After centrifugation, the cells were resuspended in DGVB and incubated at 30°. At time intervals, 200 μl of samples were withdrawn and 1·3 ml of C-EDTA were added. These mixtures were incubated further for 1 hr at 37°. Haemolysis was evaluated by measuring the OD of the supernatants at 414 nm, and the remaining haemolytic sites were calculated. (b) EAC4b3b cells (1×10^8/ml) were reacted with an equal volume of DAF-R 12·5 ng/ml (▲) 6·25 ng/ml (■) or DGVB (●) for 30 min at 37°. After washing twice, a limiting amount of B and excess amounts of D and P were added to the cells and the mixtures were incubated for 10 min at 30°. After centrifugation, the cells were resuspended in DGVB and then incubated at 30°. As described above, 200 μl of samples were withdrawn at timed intervals and the remaining C3 convertase activities were measured.

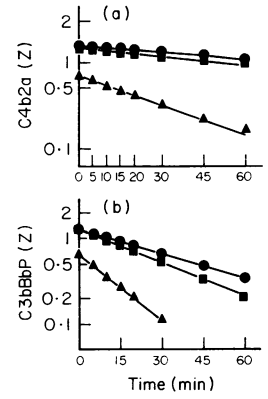

Figure 3. Effects of the anti-DAF monoclonal antibody on DAF-R activity. (a) EAC14b (1×10^8/ml) were reacted for 30 min at 37° with an equal volume of DAF-R (30 ng/ml) or DGVB (●) as a control. After washing twice, DAF-R-incorporated cells were dividied into two portions; one was reacted with anti-DAF monoclonal antibody, 1C6, 50 μg/ml (■) and the other with DGVB (▲). These mixtures and the cells treated with DGVB were incubated for 1 hr at 0°. After centrifugation, the cells were reacted with C2 as described in the legend of Fig. 2, and the remaining C3 convertase activities were measured at various times. (b) EAC4b3b (1×10^8/ml) were reacted for 30 min at 37° with an equal volume of DAF-R (12·5 ng/ml) or DGVB (●) as a control. After washing, 1C6 50 μg/ml (■) or DGVB (▲) were added to the DAF-R-incorporated cells and incubated for 1 hr at 0°. After centrifugation, the cells were reacted with B, D and P, as described above, and the C3 convertase activities were measured at various time intervals at 30°.

RESULTS

Purification and characterization of DAF-R

Since butanol extraction, compared to NP-40 extraction, showed a greater recovery of DAF activity, membrane fractions of Raji cells were extracted with butanol, as described in the Materials and Methods. The supernatants were applied to DEAE–Toyopeal (Fig. 1). Decay-accelerating activity was eluted ahead of the main protein peak. The active fractions were subjected to affinity chromatography of monoclonal anti-DAF-coupled to Sepharose and eluted with 3 M KSCN. Analysis of purified DAF-R by SDS–PAGE and silver staining exhibited the single-stained band of 70,000 MW.

Next, we examined the decay-accelerating activity of DAF-R against C3 convertases, C4b2a and C3bBb. Different amounts of DAF-R were incorporated into EAC14b and EAC4b3b cells. After washing, these cells were incubated with oxidized C2 or factor B, D and P, and the samples were removed from each tube at time intervals to measure the residual C3 convertase activity. As shown in Fig. 2, DAF-R diminished the C4b2a and C3bBb site formation and accelerated their decay. From these results, it seems likely that DAF-R, which may be anchored by a glycolipid, is able to reincorporate into cell membranes and to dissociate the C2a and Bb. These effects were completely blocked by pretreatment of DAF-R-incorporated cells with monoclonal anti-DAF antibody, 1C6, and the C3 convertases activities were restored (Fig. 3).

We then compared the ability of DAF-R and DAF-S to accelerate the decay the C3 convertase. EAC14b cells were incubated with increasing concentrations of DAF-R or DAF-S. After incubation at 37° for 1 hr, the cells were washed and reacted with a limiting amount of C2. The residual C4b2a sites were developed with C-EDTA. As shown in Fig. 4, DAF-R inhibited C4b2a sites in a dose-dependent fashion, and the activity was about four-fold higher than that of DAF-S, judging from the concentrations of DAF-R and DAF-S causing 50% inhibition of C4b2a sites. In addition, both DAFs had an apparent MW of 70,000, when compared by immunoblotting.

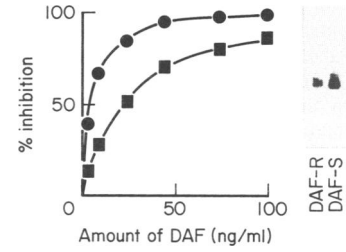

Figure 4. Dose–response curves of inactivation of C4b2a by DAF-R and DAF-S. A hundred microlitre of EAC14b cells (1×10^8/ml) were incubated with 100 μl of serially diluted DAF-R (●——●) or DAF-S (■——■) at 37° for 1 hr. After washing, the cells were resuspended in 0·1 ml of DGVB and incubated at 30° for 30 min with 0·1 ml of a limiting amount of oxidized C2. Then 1·3 ml of C-EDTA were added to each tube and, after incubation for 1 hr at 37°, the residual C4b2a sites were measured. The number of C4b2a sites in control EAC14b2a cells incubated with DGVB was 1·8, and the percent inhibition was then calculated. It is apparent that DAF-R is more effective than DAF-S in the inactivation of C4b2a sites. The inset shows the analysis of DAF-R and DAF-S by 7·5% SDS–PAGE and immunoblotting.

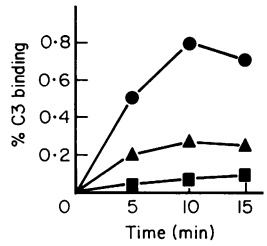

Figure 5. Enhancement of binding of C3 to Raji cells by anti-DAF monoclonal antibody, 1C6. A hundred microlitre of Raji cells (5×10^6/ml) were reacted with an equal volume of Fab of 1C6 50 μg/ml (●) or PBS (▲) for 30 min at 0°. After centrifugation, a mixture of six isolated components of the alternative pathway, containing ^{125}I-C3 were added to the cells and incubated at 37°. After incubation for various periods, the cells were washed three times, and counted for radioactivity. As a control, the cells were incubated with a mixture of six components in EDTA–GVB (■).

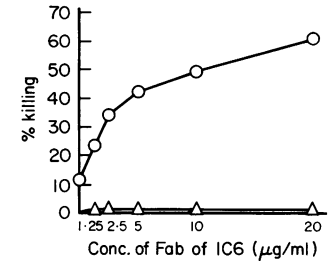

Figure 7. Enhancement of the killing of Raji cells by anti-DAF. Fifty microlitres of Raji cells (5×10^6/ml) were reacted at 0° for 30 min with 50 μl of anti-lymphocyte serum (1:50 dilution) and then with 50 μl of a varying amount of Fab fragment of 1C6, monoclonal anti-DAF antibody. After incubation for 30 min at 0°, the mixtures were further incubated at 37° for 40 min with 50 μl of 1:2 diluted normal human serum (○——○) or EDTA-serum (△——△). Viabilities were measured by dye exclusion with Trypan blue and percentage killing was calculated.

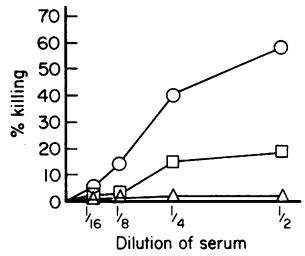

Figure 6. The role of DAF in the killing of Raji cells by autologous complement. Fifty microlitre of Raji cells (5×10^6/ml) were reacted at 0° for 30 min with 50 μl of anti-lymphocyte serum (1:50 dilution) or buffer and then with 50 μl of Fab of 1C6, monoclonal anti-DAF antibody (100 μg/ml) or buffer for 30 min at 0°. The mixtures were further incubated with 50 μl of serially diluted normal human serum as a source of complement for 40 min at 37°. Viabilities were measured by dye exclusion with Trypan blue and percent killing was calculated. In the absence of anti-DAF, 10–20% of Raji cells treated with anti-lymphocyte serum were killed (□——□), while in the presence of anti-DAF killing of Raji cells was markedly enhanced (○——○). When Raji cells were not treated with anti-lymphocyte serum, no killing was observed even in the presence of anti-DAF (△——△). As the controls, Raji cells were treated with normal human serum alone or incubated with EDTA-treated human serum instead of normal human serum. The lysis was less than 2% (not shown).

Effect of monoclonal anti-DAF antibody on complement-mediated cytolysis of Raji cells

It is known that Raji cells are activators of the alternative pathway. In order to study whether DAF on Raji cells prevent the complement-mediated cytolysis, Raji cells were incubated with normal human serum in the presence and absence of anti-DAF monoclonal antibody, 1C6. No killing was observed during 1 hr incubation even in the presence of 1C6. However, when Raji cells were incubated with six isolated components of the alternative pathway, including radiolabelled C3, in the presence and absence of Fab of 1C6, the binding of C3 to the cells was increased by the treatment with 1C6 (Fig. 5).

The above results suggested that mechanisms other than the action of DAF are involved in the prevention of cytolysis of nucleated cells by the alternative pathway complement activation. In order to overcome these mechanisms, antibodies to Raji cells were introduced to the system. As antibodies against Raji cells, we used autoantibodies to lymphocytes detected in serum from a patient with systemic lupus erythematosus. As shown in Fig. 6, a Fab fragment of 1C6 enhanced the lysis of Raji cells by antibodies and complement. Even in the presence of IC6, however, no lysis was observed in the absence of antibodies to Raji cells after short incubation period. The effect of 1C6 on the killing of Raji cells by autologous antibodies and complement was dose-dependent (Fig. 7).

DISCUSSION

In this report, we purified and characterized DAF from Raji cells. By SDS–PAGE and immunoblotting, it was observed that DAF-R as well as DAF-S is composed of a single protein of 70,000 MW (Figs 1 and 4). DAF-R was easily reincorporated into cell membranes, and it accelerated the decay of the classical and alternative C3 convertases (Fig. 2). As reported previously (Davitz, Low & Nussenweig, 1986), DAF-R may also be anchored by a glycolipid involving phosphatidylinositol. From these results, DAF-R and DAF-S can not be distinguished immunochemically and functionally; however, the ability of DAF-R to inactivate the C4b2a sites was about four-fold higher than that of DAF-S (Fig. 4). The reason for this difference is not known. According to the previous report (Davitz *et al.*, 1986), only 10–20% of the surface DAF on erythrocytes was susceptible to release by phosphatidylinositol-specific phospholipase C (PIPLC), while the amount of DAF released by PIPLC corresponded to 80% of the total DAF on mononuclear cells. In this respect, it is possible that some differences in the anchor portion of DAF molecules changes the ability to reincorporate into cell membranes or to accelerate the decay.

Recently, we have reported that DAF acts on C2a and Bb, but not on intact C2 and B, to accelerate the natural decay of the C2a and Bb (Fujita *et al.*, 1987). We show here that the rate of decay of the C4b2a and C3bBb enzymes on the DAF-R-incorporated cells was substantially faster than that of the enzyme generated on the control cells (Figs 2 and 3). This seems inconsistent with the prior report of Medof, Kinoshita & Nussenzweig (1984) that shows that DAF did not accelerate the

decay of C2. The conditions of the experiments were different; our kinetic experiments were started after the binding of C2a to the cells, while in their experiments an additional incubation period of 15 min at 30° was performed. During this incubation, C2a bound to DAF-reacted C4b seemed to be dissociated already. Although DAF inhibited the assembly of C4b2a in both experiments (Figs 2 and 3), this could be explained by the assumption that DAF dissociated C2a that was generated during reaction of DAF-incorporated EAC14b with C2. Thus, it appears that the decay-acceleration is the only basis for the inhibitory effects of DAF on the C3 convertases.

It is well known that nucleated cells are much more difficult to be killed with complement, although the mechanisms are not well understood. One possible explanation is that membrane control proteins, such as DAF, inhibit the complement activation at the stage of C3 and C5 convertase formation. However, whether DAF influences the capacity of nucleated cells to resist complement lysis has not been evaluated critically. The results of this study indicate that DAF on Raji cells indeed prevents the cytolysis mediated by autologous antibodies and complement (Figs 6 and 7). Budzko, Lachmann & McConnell (1976) reported that Raji cells activate the alternative pathway. However, alternative pathway activation resulted in cell lysis only when the cells were incubated over a prolonged period of time, reaching completion at 24 hr (Theofilopoulos & Perrin, 1977). In our experiments, Raji cells were not killed by normal human serum after a short time incubation even in the presence of anti-DAF, although the blocking of DAF activity enhanced the binding of C3 (Fig. 5). These results suggest that other membrane proteins, for instance C8 binding protein (Schoenermark *et al.*, 1986) or homologous restriction factor (Zalman, Wood & Müller-Eberhard, 1986), also prevent the complement-mediated lysis of nucleated cells, or that nucleated cells can recover from complement attack even after formation of the potentially cytolytic membrane attack complex on the cell surface (Morgan, Dankert & Esser, 1987). Further study is necessary to clarify the precise molecular and biochemical events underlying the resistance of complement-mediated lysis of nucleated cells.

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