Isolation and characterization of the complement-inhibiting protein CD59 antigen from platelet membranes

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Several groups have recently described the isolation of a 20 kDa membrane-attack-complex (MAC)-inhibiting protein, termed 'CD59 antigen', from human erythrocyte membranes. Antibodies raised against erythrocyte CD59 antigen detect antigen on the surface of many other cell types, and in some of these cells the antigen has been shown to have a molecular mass similar to that of the erythrocyte protein and to confer resistance to lysis by the MAC. A platelet-membrane form of CD59 antigen has been described and reported to be much larger than the erythrocyte protein. Here I report the isolation of CD59 antigen from platelet membranes and its molecular and functional characterization. The platelet protein is not significantly larger than the erythrocyte form and possesses similar MAC-inhibiting activity. Platelet CD59 antigen is anchored to the membrane via a glycosyl-phosphatidylinositol link, and consequently it is suggested that deficiency of this protein might be responsible for the increased thrombotic tendency observed in paroxysmal nocturnal haemoglobinuria.

INTRODUCTION

Within the past 5 years it has become clear that human cells express membrane proteins which confer resistance to lysis by the membrane attack complex (MAC) of homologous complement. At least two such proteins exist, a 70 kDa molecule termed 'homologous restriction factor' (HRF; Zalman *et al.*, 1986), C8-binding protein (C8bp; Schonermark *et al.*, 1986) or MAC-inhibiting protein (MIP; Watts *et al.*, 1987, 1990) and a 20 kDa protein, CD59 antigen, which possesses a bewildering array of alternative names (Sugita *et al.*, 1988; Davies *et al.*, 1989; Holguin *et al.*, 1989; Okada *et al.*, 1989). CD59 antigen has been purified from erythrocyte membranes, and the purified protein, by virtue of its retention of an intact glycosyl-phosphatidylinositol (GPI) anchor, can be incorporated into the membranes of heterologous target cells or liposomes, rendering them resistant to lysis by the homologous MAC. The protein acts at the final stage of assembly of the MAC, incorporating into the complex, thereby preventing recruitment of multiple C9 molecules and subsequent pore expression (Meri *et al.*, 1990; Rollins & Sims, 1990). Specific antibodies raised to the cell-associated or purified protein have identified the presence of CD59 antigen on many cell types and in tissues (Hideshima *et al.*, 1990; Nose *et al.*, 1990; Sayama *et al.*, 1990). The molecular and functional characteristics of the protein have, however, been examined on only a few cells. Neutralization of CD59 antigen with antibody rendered human glomerular epithelial cells, amniotic epithelial cells and thyroid follicular cells more susceptible to lysis by the MAC (Rooney & Morgan, 1990; Rooney *et al.*, 1991; Weetman & Morgan, 1991) and Western blotting of cell membranes demonstrated a band with a molecular mass similar to that of the erythrocyte protein. In contrast, anti-CD59 antibodies have been reported to recognize a 37 kDa protein in Western blots of platelet membranes, and neutralization of platelet CD59 antigen with antibody caused little enhancement of lysis (Sims *et al.*, 1989). Nevertheless, a functional role of platelet CD59 antigen was suggested by the observation that antibody neutralization enhanced MAC-induced platelet procoagulant activity.

The presence of a markedly different form of the CD59 antigen was intriguing, particularly in view of the similarity between the erythrocyte protein and that on diverse nucleated cell types. The purpose of the present study was therefore to isolate and characterize platelet CD59 antigen in order to elucidate the reasons for the reported differences.

MATERIALS AND METHODS

Materials

Phosphate-buffered saline (0.01 M-sodium phosphate/0.14 M-NaCl), pH 7.4 (PBS) was supplied in tablet form by Oxoid Ltd. Alternative pathway buffer (APB) was Veronal-buffered saline (0.005 M-sodium Veronal/0.14 M-NaCl), pH 7.4, containing 0.007 M-MgSO₄ and 0.01 M-EGTA. Phosphatidylinositol-specific phospholipase C (PI-PLC) was obtained from Fumakoshi Pharmaceutical Co. (Sapporo, Japan). All other chemicals were from Sigma (Poole, Dorset, U.K.) or from BDH (Poole, Dorset, U.K.) and were of the best grade available. Normal human serum (NHS) was obtained from volunteers and stored in aliquots at -70 °C until use. Cobra venom factor (CVF) was purified from venom by published methods (Vogel & Muller-Eberhard, 1984). Guinea-pig blood was obtained by cardiac puncture and erythrocytes (GPE) washed and stored in Alsever's solution at 4 °C. BRIC-229 anti-CD59 monoclonal antibody was kindly given by Dr. D. J. Anstee, International Blood Group Reference Laboratories, South-Western Regional Transfusion Centre, Bristol, U.K. YTH-53.1 anti-CD59 antibody was kindly given by Dr. Herman Waldmann, Department of Pathology, University of Cambridge, Cambridge, U.K. Monoclonal anti-(human platelet glycoprotein IIIa) (CD61; Leung *et al.*, 1981; Tetteroo *et al.*, 1983) was purchased from Dakopatts (Glostrup, Denmark). Horseradish peroxidase (HRP) and fluorescein isothiocyanate (FITC)-labelled second antibodies were from Bio-Rad (Hemel Hempstead, Herts., U.K.).

Western blotting of platelet membranes

Platelets were purified from outdated platelet concentrate as described below. Washed platelets (2 ml of packed cells) were

Abbreviations used: CVF, cobra venom factor; FACS, fluorescence-activated cell sorter; GPE, guinea-pig erythrocytes; PBS, phosphate-buffered saline, pH 7.4; PI-PLC, phosphatidylinositol-specific phospholipase C; APB, alternative-pathway buffer; NHS, normal human serum; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase; MAC, membrane-attack complex.

Fig. 1. Analysis of platelet membrane CD59 antigen

(a) Western blotting of solubilized platelet membranes and purified platelet CD59 antigen. Platelet membranes from three donors (lanes 1–3) and purified platelet CD59 antigen (1 μ g; lane 4) were separated on an SDS/15%–PAGE gel under non-reducing conditions, Western-blotted and probed with BRIC-229 anti-CD59 antibody. Lane M shows molecular-mass (M) markers run on the same gel and blotted but cut off the blot for staining with Amido Black. (b) Silver-stained SDS/PAGE gel (15% acrylamide; reducing conditions) of purified platelet CD59 antigen (2 μ g, lane 1) and erythrocyte CD59 antigen (5 μ g, lane 2). Lane 3 contains the same amount of platelet CD59 antigen preadsorbed on solid-phase anti-CD59. Molecular-mass (M) markers were run in lane M.

solubilized in PBS containing 2% (w/v) CHAPS, insoluble material removed by centrifugation and the supernatant mixed with an equal volume of SDS/PAGE solubilizing solution (Tris, 1.4 g; sucrose, 20 g; SDS, 4 g; Bromophenol Blue 20 mg in 100 ml of water, pH 6.8). Portions of this mixture were then subjected to SDS/15%–PAGE and electroblotted on to nitrocellulose. The nitrocellulose was then blocked in PBS/2% BSA and incubated with BRIC-229 monoclonal anti-CD59 antibody (0.15 μ g/ml in PBS/0.1% Tween) overnight at room temperature. After washing (three times in PBS/Tween), the nitrocellulose was incubated with rabbit anti-mouse IgG–HRP conjugate diluted 1:500 in PBS/Tween for 4 h at room temperature. The blot was then washed and incubated with HRP colour-development reagent (Bio-Rad) to develop the antibody staining.

Effects of anti-CD59 antibody on MAC lysis of platelets

Platelets (about 10^9 /ml in PBS) were incubated with BRIC-229 anti-CD59 monoclonal antibody or with DAKO-GpIIa (anti-platelet glycoprotein IIIa), both tissue-culture supernatants of IgG2b antibodies, at a 1:10 final dilution, for 30 min at 37 °C. Control cells were incubated without antibody. The cells were then washed and resuspended in APB (2 ml) containing 2 μ g of CVF, divided into 100 μ l portions, and triplicate portions were incubated with 100 μ l of dilutions of NHS between 0 and 1:2 for 1 h at 37 °C. Intact cells and debris were removed by centrifugation and the lactate dehydrogenase (LDH) content of the

supernatants measured by a micromodification of standard methods (Decker & Lohmann-Matthes, 1988).

Release of platelet CD59 antigen by PI-PLC

Washed platelets in PBS (10^9 /ml) were incubated in either the absence or presence of PI-PLC (2 μ l/ml; about 0.1 unit) at 37 °C for 1 h. The cells were then washed once and resuspended in PBS containing 1% BSA and 0.1% NaN_3 and incubated with BRIC-229 or DAKO-GpIIa (each at 1:10 final dilution of culture supernatant) for 30 min on ice. After washing twice in the above buffer the cells were incubated with rabbit anti-mouse IgG–FITC conjugate diluted 1:100 in the above buffer for 30 min on ice. The cells were then washed and fixed in 2% paraformaldehyde before fluorescence-activated-cell-sorter (FACS) analysis. Analysis was performed on a FACS 440 instrument (Beckton–Dickinson). In some experiments, lymphocytes and erythrocytes were similarly PI-PLC-treated, stained and analysed on the FACS to provide evidence of the efficacy of the techniques used.

Purification of platelet CD59 antigen

Outdated platelet concentrate (25 packs; total vol. 1200 ml) was obtained from the blood bank at the University Hospital of Wales. Platelet concentrate contains at least 1.1×10^9 platelets/ml. The platelets were pooled and centrifuged at low speed (650 g in the GSA rotor of a Sorvall Superspeed centrifuge) for 20 min to pellet contaminating erythrocytes and leucocytes. The supernatant platelet-rich plasma was then spun at 24000 g to pellet platelets. Platelets were then resuspended in 500 ml of PBS, pelleted by centrifugation, and this wash step was repeated twice more. Contamination of the platelet preparation with erythrocytes and leucocytes was assessed by microscopic examination in a haemocytometer. At least ten separate fields of the suspension were counted. Erythrocyte contamination was less than 10^4 /ml, and leucocyte contamination was less than 10^5 /ml (platelet concentration 2.5×10^9 /ml in this suspension).

The platelets were again pelleted (50 ml packed cell volume), resuspended in 200 ml of water and stirred for 30 min to lyse cells. Membranes were collected by centrifugation at 27000 g in the SS34 rotor for 30 min, washed once in PBS, suspended in 50 ml of PBS containing 4% CHAPS and stirred overnight at 4 °C. Insoluble material was removed by centrifugation (27000 g in a SS34 rotor) for 30 min at 4 °C and the clear supernatant extensively dialysed against PBS containing 0.05% CHAPS. The dialysed extract (total volume 90 ml) was tested for MAC-inhibitory activity as described below and then applied to an anti-CD59 immunoaffinity column (YTH53.1 immobilized on Sepharose 4B) equilibrated in PBS/0.05% CHAPS. After extensive washing in equilibration buffer and in equilibration buffer containing 0.5 M-NaCl, the bound protein was eluted using 0.02 M-diethylamine, pH 11.5, containing 0.05% CHAPS. Fractions (1 ml) were collected into Tris (1 M, pH 7.0, 0.1 ml) to restore the pH value to neutrality. Eluted protein was dialysed against PBS/0.05% CHAPS overnight and then concentrated to 4 ml in an Amicon ultrafiltration cell using a PM10 membrane. The eluted protein was further purified by gel filtration on an f.p.l.c. system (Pharmacia) using a Superose 6 column equilibrated and run in PBS/0.05% CHAPS.

Assessment of MAC-inhibitory activity of isolated platelet CD59

Portions (1 ml) of GPE (5% v/v in PBS) were incubated with various amounts of dialysed platelet membrane extract or affinity-purified platelet CD59 antigen (all diluted to 0.1 ml in PBS/0.05% CHAPS) for 30 min at 37 °C. The cells were washed twice in APB and resuspended to 1 ml in APB containing CVF at 2 μ g/ml. Portions (100 μ l in triplicate) were then incubated

with 100 μ l of NHS at a final dilution of 1:10, 1:20 or 1:40 for 30 min at 37 °C. Cells were centrifuged and the percentage haemolysis calculated from haemoglobin release into the supernatant by measuring absorbance at 540 nm in a Titertek Multiscan plate reader (Flow Laboratories, Paisley, Renfrewshire, Scotland, U.K.).

In one experiment, erythrocyte CD59 antigen (purified as described previously; Meri *et al.*, 1990) and platelet CD59 antigen at the same apparent concentration were incubated with GPE and the resultant effects on MAC lysis measured as described above.

Fig. 2. Anti-CD59 antibodies enhance lysis of platelets

Platelets were pre-incubated with BRIC-229 anti-CD59 antibody (○) or with anti-GPIIIa (●) as described in the Materials and methods section, and then attacked in a CVF-activated 'reactive lysis' system using different dilutions of serum. Lysis was assessed after 1 h by measurement of LDH release. Control cells pre-incubated without antibody were lysed to the same extent as those incubated with anti-GPIIIa (results not shown). Results are the means $\pm$ S.D. of triplicate determinations.

RESULTS

Western blotting of platelet membranes

Platelet membranes prepared from several donors were separately fractionated by SDS/PAGE and electroblotted on to nitrocellulose. Probing of the nitrocellulose with BRIC-229 monoclonal anti-CD59 antibody detected a single broad band in the molecular-mass range 18–21 kDa in all samples (Fig. 1a). No specific bands were detected at higher molecular masses. Within the broad band a doublet or triplet of sharper bands was discernible in most blots. The appearance was identical with that previously observed on blotting of nucleated cell CD59 antigen and differed only slightly from blots of erythrocyte CD59 antigen, which ran as a broad band between 18 and 22 kDa without discernible sharp bands. Similar results were obtained using three different monoclonal anti-CD59 antibodies (YTH-53.1, BRIC 229 and a new antibody, A35, made 'in house' by Dr. A. Davies of this Department) and a polyclonal rabbit antiserum against CD59 antigen (the gift of Dr. A. Davies).

Anti-CD59 antibody enhances MAC lysis of platelets

Platelets were incubated with BRIC-229 anti-CD59 or with an anti-platelet antibody (anti-GPIIIa) of the same subclass and were then exposed to MAC attack in a CVF-activated 'reactive lysis' system. Control cells and those pre-incubated with anti-GPIIIa were only significantly lysed at the highest dose of serum, and lysis never exceeded 15%. By contrast, anti-CD59-treated cells were lysed at lower serum doses and the highest serum dose caused more than 35% lysis (Fig. 2).

No lysis of antibody-treated platelets occurred if CVF was omitted, confirming that classical-pathway activation was not a contributory factor.

FACS analysis of CD59 expression and PI-PLC release

Platelets were incubated in the presence or absence of PI-PLC, stained with BRIC-229 anti-CD59 or with anti-GPIIIa, followed by fluorescent second antibody, and analysed on the FACS instrument. Platelets not treated with PI-PLC were stained by the

Fig. 3. Release of platelet CD59 antigen by PI-PLC

Platelets were incubated in either the presence or absence of PI-PLC as described in the Materials and methods section. The cells were then washed and stained using anti-CD59 antibody (BRIC 229) or anti-GPIIIa (glycoprotein IIIa, CD61) antibody, followed by fluorescent second antibody and analysed by flow cytometry. Control cells were incubated with second antibody alone (—). PI-PLC treatment markedly reduced staining of platelets with BRIC-229 (..... no PI-PLC; ---, PI-PLC-treated), but had no effect on staining with anti-GPIIIa (—; two traces superimposable)

positive control antibody anti-GPIIIa and with the anti-CD59 antibody. The fluorescence with both antibodies was unimodal, but displayed considerable overlap with the negative controls (Fig. 3). For GPIIIa, 69 % of the cells were positive with respect to the gate set using the negative control population, and the modal fluorescence was 105.6 units (modal fluorescence of controls 32 units). For CD59 antigen, 52 % of the cells were positive with respect to the control gate and the modal fluorescence was 91.4 units. PI-PLC treatment caused a downward shift in the fluorescence pattern for CD59 antigen expression (27 % positive with respect to control gate; modal fluorescence 59.2 units), but did not alter the expression of GpIIIa (Fig. 3). Lymphocytes treated in an identical manner were 89 % positive for CD59 antigen before incubation with PI-PLC, but only 19 % positive afterwards, whereas erythrocyte expression was only slightly decreased (92 % before, 81 % after) (results not included).

Purification of platelet CD59 antigen

Platelet membrane CD59 antigen was purified from solubilized membranes by immunoaffinity chromatography and gel filtration. A second passage of the solubilized membranes over the affinity column extracted no more protein, implying that all the available antigen bound in the first pass. The protein eluted from the Superose 6 column as a single peak with an apparent molecular mass of approx. 500 kDa, indicating that CD59 antigen was present as a detergent-protein complex. On SDS/PAGE the purified protein gave a single major band with an apparent molecular mass of between 18 and 20 kDa (Fig. 1b). Only this major band was detected by anti-CD59 antibody in Western blots of the purified protein (Fig. 1a). The yield from two purifications was 85 μ g and 65 μ g respectively by a Coomassie Blue-based protein assay kit (Pierce).

MAC-inhibitory activity of platelet CD59 antigen

Incubation of GPE with solubilized dialysed platelet membranes or with purified platelet CD59 antigen rendered the cells resistant to subsequent lysis by the MAC. The purified antigen was much more active than the membrane extract, and the effects were dependent on the dose of antigen added to the cells, reaching a maximum at 1.5 μ g/ml at the highest serum dose used (Fig. 4a). At the lowest serum dose used, 50 % inhibition of GPE lysis was achieved by preincubation with purified antigen at 60 ng/ml, whereas a 1:5 dilution of membrane extract (protein concn. approx. 100 μ g/ml) was required to achieve this degree of inhibition.

The protective effect of preincubation with CD59 antigen or membrane extract could be neutralized by incubation of protected cells with anti-CD59 antibody (results not shown). Comparison of the protective effects of erythrocyte CD59 antigen, purified as described previously (Meri *et al.*, 1990), and platelet CD59 antigen on GPE revealed that the two proteins had similar inhibitory activities (Fig. 4b).

DISCUSSION

The first aim of the present study was to characterize the 38 kDa variant of CD59 antigen which had been described in platelet membranes (Sims *et al.*, 1989). However, in the initial platelet blotting studies, anti-CD59 antibodies identified only a band of apparent molecular mass 18–22 kDa. The detected protein gave an appearance identical with that obtained in this laboratory upon Western blotting of several human nucleated cell types, including neutrophils, lymphocytes, K562 cells, am-

Fig. 4. Inhibitory activity of platelet CD59 antigen

(a) GPE were pre incubated with dialysed platelet membrane extract (PM) or with various concentrations of platelet CD59 antigen (10–2000 ng/ml). Control cells were pre-incubated in buffer alone. The cells were then incubated with CVF plus serum at a final dilution of 1:10 ($\square$), 1:20 ($\square$) or 1:40 ($\square$) as described in the Materials and methods section. Haemolysis was measured after 30 min. Results are means $\pm$ s.d. of triplicate determinations. (b) GPE were pre-incubated with platelet CD59 ($\square$) or erythrocyte CD59 antigen ($\blacksquare$) at the same apparent concentrations. The cells were washed and incubated with CVF plus serum (final dilution 1:10) and haemolysis was measured as described above. Results are the means $\pm$ s.d. of triplicate measurements.

niotic epithelial cells and thyroid follicular cells. The band detected in platelets and these other cells had a marginally higher molecular mass than that obtained from erythrocyte membranes and appeared to contain a doublet or triplet of sharp bands not discernible in blots of erythrocyte membranes. No specific bands were seen at higher molecular masses. Blotting was performed with four different anti-CD59 reagents with similar results, eliminating the possibility that the antibody chosen was to a 'missing' epitope in the reported 38 kDa protein. The differences between the findings reported here and those of the original study may reflect differences in the way that platelets or membranes were stored before purification. The outdated platelets used in the present study were obtained 5 or 6 days after venesection and had been stored at 4 °C in acid-citrate/dextrose plasma during this period. Membranes were prepared and CD59 antigen purified immediately upon receipt of platelets. No details of platelet age or storage conditions were given in the original

study, but it is possible that long-term storage or freezing may have caused aggregation of CD59 antigen with the formation of stable dimers.

By using a CVF-activated 'reactive lysis' system, which results in the efficient deposition of MACs on target cells, it was possible to show that neutralization of CD59 antigen on the platelet surface markedly enhanced MAC-mediated lysis, implicating this protein in the protection of platelets against MAC attack. The MAC-inhibiting activity of platelet CD59 antigen was confirmed by using the purified protein, which protected GPE from MAC lysis as efficiently as did erythrocyte CD59 antigen. Regulation by CD59 antigen of the non-lethal stimulatory effects of the MAC, which include the induction of procoagulant platelet responses, are likely to be of more relevance *in vivo* than protection against lysis (Wiedmer *et al.*, 1986; Sims *et al.*, 1988; Morgan, 1989).

The specific inhibitory activity of the purified protein was approx. 1500-fold that of the membrane extract from which it was derived. However, the final protein yield represented 0.14 % of the protein in the initial detergent extract, from which it can be calculated that the inhibitory activity had increased at least 2-fold over the purification. It is likely that this anomaly is the result of other factors in the platelet membrane extract which enhance lysis of GPEs in the assays used. Assuming that the yield of CD59 antigen was close to 100 %, the average number of molecules of this antigen per platelet can be calculated to be about 1500. This compares with an estimate for erythrocytes of 25000 molecules per cell (Davies *et al.*, 1989). The average platelet volume is less than 10 % of that of erythrocytes (8 fl as against 90 fl); the surface density of CD59 antigen is thus likely to be similar on the two cell types.

Flow cytometry of platelets stained with anti-CD59 revealed a single population of cells, indicating that, despite the large overlap with the negative-control population, all platelets were positive for this antigen. A similar large overlap with the control was observed when cells were stained with antibodies to the platelet membrane protein GPIIIa, suggesting that this is a feature of the cell, perhaps because of its small size. Treatment of platelets with PI-PLC removed a large percentage of the CD59 antigen (approx. 50 % as estimated by the decrease in modal fluorescence), demonstrating that the protein is linked to the membrane by a GPI anchor. The platelet GPI anchor appears to resemble that of neutrophils and lymphocytes in that it is efficiently cleaved by PI-PLC. The erythrocyte anchor is much less susceptible to PI-PLC cleavage by virtue of the inclusion of an additional ester-linked lipid which confers upon the anchor resistance to cleavage (Low, 1987; Holguin *et al.*, 1990; Walter *et al.*, 1990). Paroxysmal nocturnal haemoglobinuria is a clonal disorder of haemopoietic stem cells which results in episodes of haemolysis and thrombosis (Rosse, 1990). The underlying biochemical abnormality is a failure to form stable GPI anchors. As a result, the products of the abnormal clone express markedly decreased amounts (or none) of GPI-anchored proteins, including the complement-inhibitory proteins decay accelerating factor ('DAF'), HRF/MIP and CD59 antigen. Absence of these inhibitors on erythrocytes renders them highly susceptible to haemolysis by complement. Deficiency of DAF on platelets in paroxysmal nocturnal haemoglobinuria has previously been

described (Kinoshita *et al.*, 1985), and this, together with the demonstration of GPI anchoring reported here, make it likely that paroxysmal-nocturnal-haemoglobinuria platelets are also deficient in CD59 antigen. Absence of this protein on platelets could, by rendering platelets more susceptible to MAC-induced injury and activation, contribute to the thrombotic tendency, which is the commonest cause of death in this condition.

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