Biochem. J. (1992) 284, 169–176 (Printed in Great Britain)

Isolation and characterization of a membrane protein from rat erythrocytes which inhibits lysis by the membrane attack complex of rat complement

Timothy R. HUGHES*, Sara J. PIDDLESDEN,* John D. WILLIAMS,† Richard A. HARRISON‡ and B. Paul MORGAN*§

* Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN,
† Institute of Nephrology, Cardiff Royal Infirmary, Cardiff CF2 1SZ,
and ‡ MIP Unit, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.

The membrane attack complex (MAC) of complement in humans is regulated by several membrane-bound proteins; however, no such proteins have so far been described in other species. Here we report the isolation and characterization of a rat erythrocyte membrane glycoprotein of molecular mass 21 kDa which inserts into cell membranes and is a potent inhibitor of the rat MAC. This protein, here called rat inhibitory protein (RIP), was first partially purified by column chromatography from a butanol extract of rat erythrocyte membranes. Monoclonal antibodies (Mabs) were raised against RIP and used for its affinity purification. Affinity-purified RIP was shown to inhibit in a dose-dependent manner the cobra venom factor (CVF)-mediated 'reactive' lysis of guinea pig erythrocytes by rat complement. Conversely, the anti-RIP MAbs 6D1 and TH9 were shown to markedly enhance the CVF-mediated lysis of rat erythrocytes by rat complement. RIP acted late in the assembly of the MAC (at or after the C5b–8 stage) and was releasable from the membranes of rat erythrocytes by phosphatidylinositol-specific phospholipase C. These features, together with its size, deglycosylation pattern and *N*-terminal amino acid sequence, lead us to conclude that RIP is the rat homologue of the human MAC-inhibitory protein CD59 antigen.

INTRODUCTION

Several membrane proteins have now been described which regulate the activation of complement on human cells. These regulators act either on the C3/C5 convertase enzymes of the classical and alternative pathways or on the membrane attack complex (MAC). Two proteins which inhibit the lytic activity of the MAC have been identified. The first to be described was a 65 kDa protein isolated from erythrocyte membranes and known variously as homologous restriction factor (HRF), C8-binding protein (C8bp) or MAC-inhibiting protein (MIP) (Zalman *et al.*, 1986; Schonermarck *et al.*, 1986; Watts *et al.*, 1987). MIP is also present in serum and other biological fluids and may be functionally relevant in these situations (Zalman *et al.*, 1989; Watts *et al.*, 1990). The second MAC-inhibitory protein to be identified was a 20 kDa protein, independently isolated from erythrocyte membranes by several groups and thus possessing many names: P-18 (Sugita *et al.*, 1988), HRF-20 (Okada *et al.*, 1989*a*), MIRL (Holguin *et al.*, 1989) and CD59 antigen (Davies *et al.*, 1989). This last name has gained widespread acceptance and is used here to describe this protein. Both CD59 antigen and MIP are linked to the membrane via a glycosyl-phosphatidyl-inositol (GPI) anchor, and appear to act at the same stages of MAC assembly by incorporating into the forming complex at the C5b–8 stage and blocking the uptake and insertion of multiple C9 molecules (Schonermark *et al.*, 1988; Meri *et al.*, 1990; Rollins & Sims, 1990).

The CD59 antigen has been sequenced and shown to have no sequence similarities with any of the other complement inhibitory proteins or components (Davies *et al.*, 1989; Okada *et al.*, 1989*b*; Sugita *et al.*, 1989). The only significant similarity identified was with the murine LY-6 antigens, a family of leucocyte surface molecules involved in T cell activation (Shevach & Korty, 1989; Philbrick *et al.*, 1990). It has been suggested that CD59 antigen is similarly involved in cell activation (Groux *et al.*, 1989).

To date, no MAC-inhibitory proteins have been isolated from species other than human. Isolation of these proteins would enable comparisons of structure and function and should aid the elucidation of the mechanisms by which homologous restriction of the MAC is mediated. Antibodies against these inhibitors might also prove useful in determining the roles both of the MAC and of the regulatory proteins in animal models of human diseases. The rat is particularly attractive in this respect, as many excellent disease models exist in which the MAC has been implicated, e.g. Heymann nephritis (Salant *et al.*, 1989) and collagen-induced arthritis (Stuart *et al.*, 1982).

Here we report the purification of a potent MAC-inhibitory protein from rat erythrocyte membranes. The purified protein, termed rat inhibitory protein (RIP), resembled the human CD59 antigen in terms of molecular mass, *N*-terminal sequence and other structural and functional parameters. A number of monoclonal antibodies (MAbs) have been raised against this protein and used to further compare RIP with CD59 antigen.

MATERIALS AND METHODS

Materials

Reagents for SDS/PAGE were from Bio-Rad (Hemel Hemstead, Herts., U.K.). Poly(vinylidene difluoride) (PVDF) Immobilon membrane was from Millipore (Watford, Herts,

Abbreviations used: MAC, membrane attack complex; RIP, rat inhibitory protein; PBS, phosphate-buffered saline; APB, alternative pathway buffer; MAb, monoclonal antibody; CVF, cobra venom factor; PI-PLC, phosphatidylinositol-specific phospholipase C; MIP, MAC-inhibiting protein; GPI, glycosyl-phosphatidylinositol; HRP, horseradish peroxidase; endo-F, mixture of endoglycosidase F and glycopeptidase; PVDF, poly(vinylidene difluoride); GPEs, guinea pig erythrocytes.

§ To whom correspondence should be addressed.

UK.). Prosep A was from Bioprocessing Ltd. (Consett, Co. Durham, U.K.). CNBr-activated Sepharose and phenyl-Sepharose were from Pharmacia (Milton Keynes, Bucks, U.K.). Fluorescein and horseradish peroxidase (HRP)-conjugated antibodies were from Jacksons Immunoresearch Laboratories Inc. (West Grove, PA, U.S.A.). Neuraminidase, O-glycosidase and endo-F (a mixture of endoglycosidase F and glycopeptidase F) were from Boehringer Mannheim (Lewes, E. Sussex, U.K.). Phosphatidylinositol-specific phospholipase C (PI-PLC) isolated from *Bacillus thuringiensis* was obtained from Peninsula Laboratories (St. Helens, Merseyside, U.K.). All other chemicals were obtained from BDH Chemicals (Poole, Dorset, U.K.) or from Sigma Chemical Co. (Poole, Dorset, U.K.) and were the best grade available. Phosphate-buffered saline (PBS) contained 0.137 M-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4 and 1.5 mM-NaH_2PO_4, pH 7.3.

Alternative pathway buffer (APB) contained 5 mM-sodium barbitone, 0.14 M-NaCl, 0.01 M-EGTA, 7 mM-$MgCl_2$ and 0.02% NaN_3. MAbs to human CD59 antigen were as follows: A35 was a gift from Dr. Alex Davies, Department of Medical Biochemistry, UWCM, and BRIC-229 was purchased from the Blood Group Reference Laboratory, Bristol, U.K.

Sera and proteins

Human, rat, rabbit, mouse, sheep and guinea pig blood were collected fresh and allowed to clot, and the serum was separated and stored at −70 °C prior to use. Serum from horse, goat, dog and swine were obtained from Serotech (Kidlington, Oxford, U.K.). Rat serum depleted of C8 (RS-8) and rat serum depleted of C9 (RS-9) were produced by passage of rat serum over columns of polyclonal anti-(rat C8) and polyclonal anti-(rat C9) respectively, as described previously (Scolding *et al.*, 1989). Cobra venom factor (CVF) was purified according to the method of Vogel & Muller-Eberhard (1984).

Purification of MAC-inhibitory proteins from rat erythrocyte membranes

Rats were exsanguinated by cardiac puncture under ether anaesthesia and blood (total volume of 500 ml for each preparation) was collected into heparin. Plasma was removed and the cells were washed three times with PBS. The buffy coat was discarded and the erythrocytes (200 ml of packed cells) were lysed by diluting 1:40 in lysis buffer (5 mM-sodium phosphate, 1 mM-benzamidine hydrochloride, 1 mM-phenylmethanesulphonyl fluoride, 2 mM-EDTA, pH 7.4) and stirring overnight at 4 °C. The erythrocyte ghosts were then washed and concentrated in a Pellicon Ultrafiltration System using a 300 kDa molecular mass cut-off membrane cartridge (Millipore). Final washing was carried out by centrifugation at 25000 *g* in the GSA rotor of a Sorvall centrifuge.

The pellet thus obtained consisted of a soft haemoglobin-free layer overlying a dense haemoglobin-rich pellet. The upper layer was resuspended in lysis buffer and 3 M-NaCl was added to give a final concentration of 0.15 M. To this suspension butan-1-ol was added to 20% (v/v) and the mixture was stirred at room temperature for 20 min. After centrifugation at 10400 *g* for 30 min in the Sorvall, the butanol-saturated aqueous phase was removed and dialysed exhaustively against start buffer for hydrophobic chromatography (0.3 M-NaCl/0.04 M-sodium phosphate buffer, pH 7.5, containing 0.05% CHAPS). The dialysed butanol extract was then concentrated to 50 ml in an Amicon ultrafiltration cell using a YM10 membrane (Amicon, Stonehouse, Gloucs., U.K.) before application to a 2 cm × 10 cm phenyl-Sepharose column previously equilibrated with the start buffer. The column was washed with 150 ml of the start buffer and eluted with a linear gradient consisting of 240 ml of start buffer and 240 ml of 0.04 M-sodium phosphate, pH 7.4, containing 1% CHAPS. Fractions (3 ml) were collected and tested for MAC-inhibitory activity and protein content as described below.

Active fractions from the phenyl-Sepharose column were pooled, dialysed against 0.02 M-sodium phosphate (pH 7.4)/0.05% CHAPS and applied to a Mono Q HR 5/5 f.p.l.c. column (Pharmacia) equilibrated with the above buffer. The column was washed with 20 ml of start buffer and then eluted with a linear salt gradient of 0–0.5 M-NaCl in start buffer. Fractions were assayed for MAC-inhibitory activity and protein content; the inhibitory fractions were pooled, dialysed against PBS containing 0.05% CHAPS and concentrated to 0.4 ml.

The concentrated inhibitory pool was applied in 0.2 ml aliquots to a Superose 6 gel-filtration f.p.l.c. column (Pharmacia) equilibrated with PBS/0.05% CHAPS, and 0.5 ml fractions were collected and assayed for inhibitory activity. The active fractions were pooled, dialysed against 0.02 M-sodium phosphate buffer, pH 8.1, containing 0.05% CHAPS and applied to the Mono Q column equilibrated in this buffer. The column was eluted using a linear salt gradient (0–0.5 M-NaCl) in the above buffer, active fractions were pooled and the purity of the eluted protein was assessed by SDS/PAGE.

Production of MAbs to RIP

MAbs to RIP were produced by standard methods (Galfre & Milstein, 1981). Briefly, Balb/C mice were immunized either with 25 μg of RIP, prepared as described above, in Freund's complete adjuvant, or with 0.1 ml of washed rat erythrocytes in pertussis toxin. Booster immunizations were given at 4 and 5 weeks of either RIP in incomplete adjuvant or rat erythrocytes in pertussis toxin. Individual animals were always immunized with the same immunogen each time. At 3 days after the final immunization, spleen cells were harvested and fused with P3/X63/AG8-653 myeloma cells (European Cell Culture Collection, Porton Down, Salisbury, Wilts., U.K.), and the resulting clones were screened by e.l.i.s.a. against purified RIP and for functional activity by measuring the enhancement of CVF-mediated lysis of rat erythrocytes (as detailed below). Wells positive in one or both of these screening assays were cloned either by limiting dilution or by single-cell sorting on a fluorescence-activated cell sorter (FACS 440; Becton–Dickinson). The MAb populations thus obtained were again screened in both assays and positive wells were expanded for production of ascites. Antibody was purified from ascitic fluid by affinity chromatography on Prosep A. Isotyping was carried out on tissue culture fluid using an MAb typing kit from Sigma.

Immunoaffinity purification of RIP

One of the anti-RIP antibodies obtained, coded 6D1, was coupled to CNBr-activated Sepharose (15 mg of antibody coupled to 10 ml of gel, according to the manufacturer's instructions). A column was poured and equilibrated in PBS/0.05% CHAPS, and then 40 ml of butanol extract of rat erythrocyte membranes, previously dialysed against equilibration buffer, was applied. After washing in the above buffer, bound protein was eluted with 0.02 M-diethylamine, pH 11.5, containing 0.05% CHAPS, with 1 ml fractions being collected into 0.1 ml of 1 M-Tris, pH 7.4, to effect immediate neutralization. The protein-containing fractions were pooled and dialysed against equilibration buffer.

SDS/PAGE and immunoblotting

SDS/PAGE was carried out by the method of Laemmli (1970) using 15% gels and a PROTEAN II mini gel system (Bio-Rad).

Gels were silver-stained (Merill *et al.*, 1981) or Western-blotted on to PVDF membranes by standard methods (Towbin *et al.*, 1979). Blots were probed with tissue culture supernatants or purified MAbs and developed using peroxidase-conjugated second antibody and peroxidase substrate.

N-Terminal sequence analysis

N-Terminal sequence analysis was performed using an Applied Biosystems 477 A protein sequencer equipped with a 120 A analyser and 610 A data analysis software. A sample of affinity-purified RIP (approx. 20 μg) was prepared for sequence analysis by SDS/PAGE under reducing conditions and electroblotting on to PVDF as described above. Protein bands were visualized by staining in 0.1% Coomassie Blue R in water, followed by extensive destaining in 40% methanol/10% acetic acid in water. The blot was dried and the RIP band (15 mm × 1 mm) was excised and placed in a Blott cartridge (Applied Biosystems) for sequencing.

Screening assay for MAC-inhibitory activity

MAC-inhibitory activity in column fractions was determined using a CVF-mediated reactive lysis system. Guinea pig erythrocytes (GPEs; 0.05 ml of a 1:10 suspension) were incubated for 30 min at 37 °C with 0.05 ml of a dilution of the fraction under test or an appropriate buffer blank, washed once and resuspended in 0.05 ml of APB. CVF was then added to a final concentration of 6 μg/ml, followed by 0.1 ml of a dilution of rat serum in APB previously titrated to give approx. 50% haemolysis of GPEs in the absence of inhibitor. Incubation was carried out for 30 min at 37 °C. Percentage haemolysis was calculated by comparing the A_{414} of the supernatants with a standard curve of haemolysis obtained using water-lysed GPEs.

Screening assay for antibodies against MAC-inhibitory proteins

Clones were screened for antibodies against MAC-inhibitory proteins in two ways. Firstly, clone supernatants were screened in an e.l.i.s.a. using RIP immobilized on the plate. The assay was developed with HRP-labelled anti-(mouse immunoglobulin) and HRP substrate. Secondly, clones were screened in a functional assay to identify antibodies which caused an enhancement of the MAC-mediated lysis of rat erythrocytes. In this assay, 0.05 ml of a 5% suspension of rat erythrocytes was incubated at room temperature for 30 min with 0.1 ml of tissue culture supernatant or purified IgG. After washing, the cells were resuspended in APB (0.05 ml), CVF (2 μg/ml final concentration) and rat serum (0.1 ml of a dilution previously titrated to give minimal haemolysis in the absence of antibody) were added and the mixture was incubated for 1 h at 37 °C. Percentage haemolysis was determined as before.

Stage of action of RIP in MAC inhibition

To determine the stage of action of RIP, rat C5b–7 and rat C5b–8 sites were formed on GPE (1%) by incubating with CVF (2 μg/ml) and rat serum depleted of C8 (1:50) or rat serum depleted of C9 (1:12) in APB for 1 h at 37 °C, followed by washing in APB. Purified RIP at two concentrations (1 μg/ml and 50 ng/ml) was incubated with the GPEC5b–7(rat) or GPEC5b–8 (rat) cells at 37 °C for 30 min, the cells were washed in APB and the MAC was completed by incubation with rat serum (1:800 in APB containing 0.1 M-EDTA) for 10 min at 37 °C. Haemolysis was calculated as described above.

Species-specificity of inhibition mediated by RIP

In order to determine whether RIP caused any inhibition of lysis by MAC from species other that rat, GPEC5b–7 (rat) cells, prepared as described above, were incubated with purified RIP at concentrations between 1 μg/ml and 1 ng/ml for 30 min at 37 °C. The cells was washed and MAC assembly was completed by incubation for 30 min at 37 °C with sera from various species (all in APB and containing 0.01 M-EDTA) previously titrated to give approx. 50% haemolysis of GPEC5b–7(rat) in the absence of RIP. Percentage haemolysis was calculated as described above.

Deglycosylation of RIP

Affinity-purified RIP was treated with three glycosidases of different specificities using the protocol recommended by the manufacturer. Briefly, 10 μg portions of RIP (in 0.01 ml of PBS containing 0.05% CHAPS) were denatured by boiling in 0.01 ml of 1% SDS for 2 min, followed by addition of 0.09 ml of buffer [0.02 M-sodium phosphate, pH 7.2, 0.01 M-NaN_3, 0.05 M-EDTA 0.5% (v/v) Nonidet P-40] and boiling for a further 2 min. The denatured protein was then treated with one of the following: (1) 0.4 units of endo-F to remove N-linked oligosaccharides; (2) 2.5 munits of O-glycosidase together with 2 munits of neuraminidase to remove O-linked oligosaccharides and terminal sialic acid residues; or (3) a mixture of the three enzymes. Controls included tubes containing only the enzymes and tubes containing only RIP. All samples were incubated for 18 h at 37 °C, after which they were subjected to SDS/PAGE and silver staining.

FACS analysis of RIP expression on rat blood cells

The expression of RIP on rat erythrocytes, lymphocytes and platelets was examined by flow cytometry. Blood was drawn under ether anaesthesia into heparinized tubes and the lymphocytes were separated by density centrifugation on Ficoll. Platelets were isolated by initial centrifugation at 650 ***g*** to pellet leucocytes and erythrocytes, followed by centrifugation of the plasma at 24000 ***g*** to pellet platelets. Erythrocytes, lymphocytes (each at approx. 10^5 cells/tube in 0.1 ml of PBS) and platelets (approx. 10^8 cells in 0.1 ml of PBS) were dispensed into tubes. Each cell type was then incubated with PI-PLC at several concentrations (1.9–380 munits/ml) or a buffer blank for 30 min at 37 °C. After washing three times in FACS buffer (PBS containing 1% BSA and 0.02% NaN_3), the cells were stained with anti-RIP MAb 6D1 or TH9 at 10 μg/ml on ice for 30 min. Control cells were incubated with mouse IgG at the same concentration. Following further washes, the cells were incubated for 30 min on ice with fluorescein isothiocyanate-labelled $F(ab')_2$ goat anti-(mouse IgG) (Serotech) diluted 1:40 in FACS buffer. All samples were then washed again and resuspended in 0.5 ml of FACS buffer before analysis. Parallel experiments were carried out with human erythrocytes using the anti-CD59 antibodies A35 and BRIC-229. In separate experiments, the two anti-RIP MAbs used above were examined for cross-reactivity with human, mouse and guinea pig erythrocytes.

RESULTS

Purification of RIP by classical chromatography and production of MAbs

The initial method used to isolate MAC-inhibitory proteins from rat erythrocytes was a modification of that described by Holguin *et al.* (1989) for the purification of human CD59 antigen. A typical preparation utilized 120 ml of the butanol extract of rat erythrocyte membranes (from 50 ml of packed washed cells) with a total protein content of 18 mg. The final yield was approx. 180 μg, with a specific inhibitory activity 500-fold that of the butanol extract (Table 1). The observed increase in specific activity was much greater than could be accounted for by the decrease in protein, suggesting that removal of lytic

Table 1. Purification of RIP

SA is the specific inhibitory activity in arbitrary units of activity per μg of protein. nd, not determined.

Purification stage	Elution point	Protein (mg)	SA*
Classical chromatography			
Butanol extract	–	18	1
Phenyl-Sepharose	0.3–0.5% CHAPS	2.6	nd
Mono Q (pH 7.4)	0.2–0.34 M-NaCl	0.92	nd
Superose 6	12.5 ml	0.40	nd
Mono Q (pH 8.1)	0.27–0.40 M-NaCl	0.18	700
Affinity chromatography			
Butanol extract	–	5.2	1
6D1 column	pH 11.5	0.23	3750

factors present in the butanol extract was partly responsible. SDS/PAGE analysis revealed that the preparation contained a major band at approx. 21 kDa, but in addition contained multiple higher molecular mass bands. Radiolabelling of this preparation and subsequent incubation with GPEs demonstrated that only the 21 kDa band became incorporated into cell membranes (Fig. 1*a*). On scanning densitometry, the protein was approx. 20% pure. This partially purified preparation was used to immunize mice and to screen clones for antibodies to the inhibitory component (RIP) as detailed above.

MAbs positive in the e.l.i.s.a. against the partially pure RIP and/or causing enhancement of CVF-mediated reactive lysis of rat erythrocytes by rat serum were produced from both fusions. All recognized an approx. 21 kDa band in Western blots of the RIP preparation under non-reducing conditions. Two MAbs, both of which caused enhancement of MAC lysis of rat erythrocytes in the functional assay, were chosen for further study and grown up in bulk as ascites. These antibodies, designated 6D1 and TH9, were both of the IgG_1 subclass. Western blots of a butanol extract of rat erythrocyte membranes probed with these antibodies are shown in Fig. 1(*b*). The antibodies did not blot reduced RIP. Neither antibody recognized any bands in solubilized human erythrocyte membranes, nor did either of the antibodies to human CD59 antigen (A35 and BRIC-229) recognize any bands in the rat membranes (results not shown).

Application of 40 ml of a butanol extract (5.2 mg of protein) of rat erythrocyte membranes to the affinity column yielded 230 μg of protein, which when analysed by SDS/PAGE gave a single band of molecular mass 21 kDa (Fig. 1*c*). This band was also identified following immunoblotting with the MAbs 6D1 and TH9 (Fig. 1*b*). The increase in specific inhibitory activity from butanol extract to pure protein was 3750-fold (Table 1). The possible reasons for this large increase in specific activity are discussed above.

Purified RIP inhibits, and anti-RIP MAbs enhance, lysis by rat MAC

Affinity-purified RIP was incorporated into GPEs and caused a dose-dependent inhibition of CVF-mediated lysis by whole rat serum (Fig. 2*a*). Binding of RIP to erythrocytes was assessed using radiolabelled protein. The proportion of protein binding to cells was dependent on the amount added, but was up to 50% when the dose added was below 100 ng/ml (results not shown). RIP was found to be active at concentrations as low as 20 ng/ml.

The anti-RIP MAbs 6D1 and TH9 both caused a dose-dependent enhancement of the CVF-mediated lysis of fresh rat erythrocytes. The two antibodies were equally effective at enhancing lysis (Fig. 2*b*). Non-immune mouse IgG caused no enhancement of lysis. Neither antibody caused any lysis when serum was added in the absence of CVF.

RIP acts late in MAC assembly to mediate inhibition

Incubation of GPEC5b-7(rat) or GPEC5b-8(rat) cells with RIP prior to addition of the missing components efficiently inhibited lysis of these intermediates, clearly demonstrating that RIP exerted its inhibitory effect even after the formation of C5b–8 sites (Table 2).

RIP inhibitory activity is not restricted to rat MAC

The species-specificity of RIP-mediated inhibition of lysis was examined to determine whether this activity was restricted solely to the rat system. GPEC5b-7(rat) cells, with or without

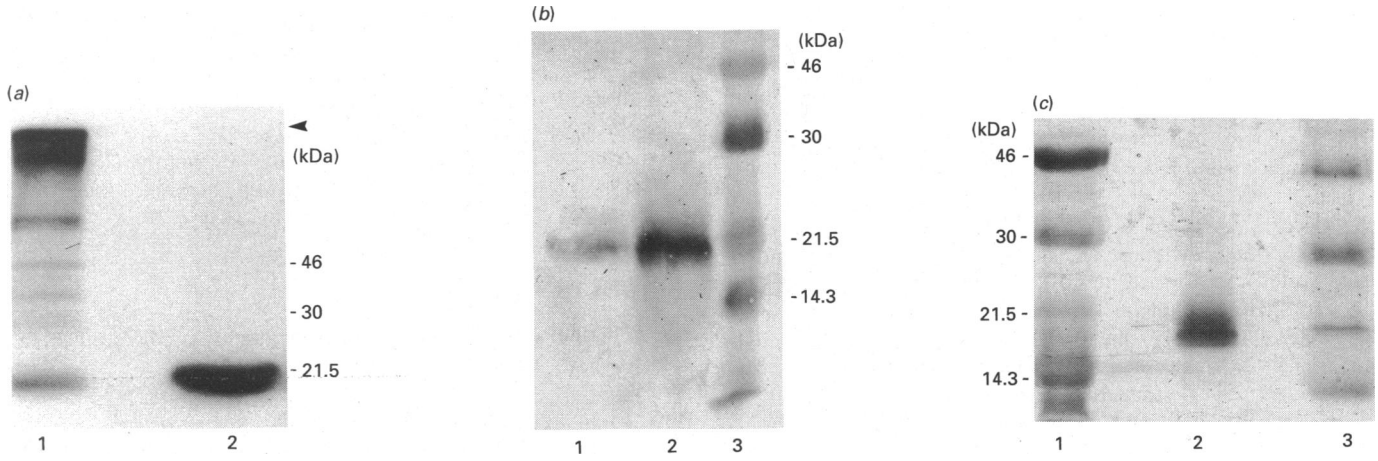

Fig. 1. SDS/PAGE and Western blotting of RIP

(*a*) Autoradiograph of partially purified RIP after radiolabelling and separation on SDS/PAGE. Lane 1, RIP preparation showing multiple protein bands; lane 2, radiolabelled RIP incorporated into erythrocyte membranes (only the 20 kDa band became incorporated). The same amount of radioactivity was loaded in each lane. The arrow indicates the top of the running gel; molecular masses are shown on the right. (*b*) Western blot of butanol extract of rat erythrocyte membranes (4 μg of total protein; lane 1) and affinity-purified RIP (2 μg; lane 2) separated on SDS/15%-PAGE under non-reducing conditions. The blot was probed with the anti-RIP MAb 6D1 and developed using a peroxidase-conjugated second antibody and HRP substrate. Lane 3 contains molecular mass markers. (*c*) Silver-stained gel (SDS/PAGE) of affinity-purified RIP (2 μg; lane 2) and butanol extract (4 μg of total protein; lane 3). Lane 1 contains molecular mass markers.

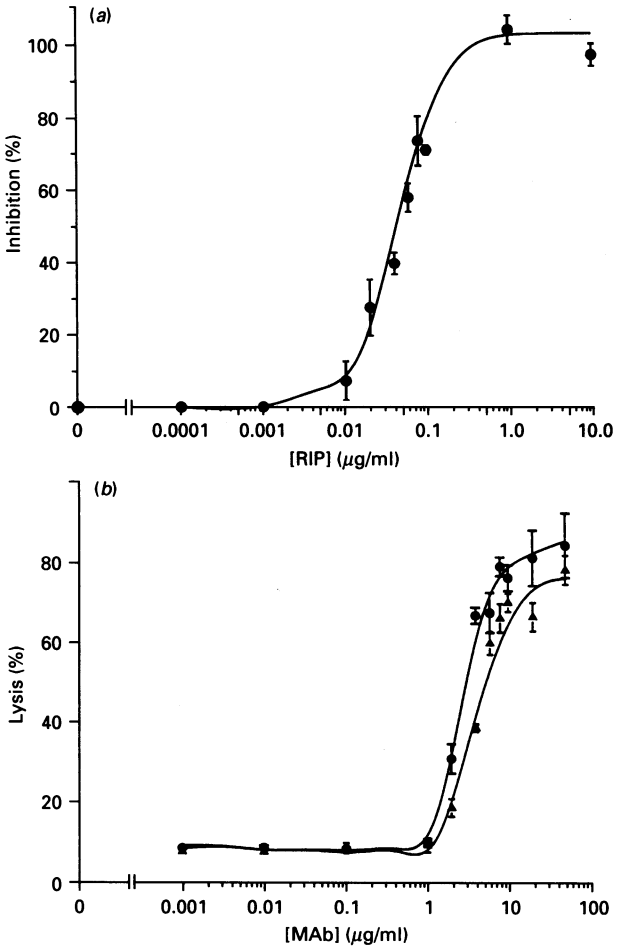

Fig. 2. Functional activity of RIP and anti-RIP

(*a*) Purified RIP (1–10000 ng/ml) was incubated with GPEs, and the cells were washed and exposed to CVF and rat serum. Haemolysis was calculated as described in the Materials and methods section. Controls consisted of non-RIP-treated cells incubated with CVF and rat serum (34% haemolysis), with rat serum alone (11% haemolysis) and with buffer alone (9% haemolysis). All points represent the means of triplicate determinations and the error bars show the S.D. of these values. (*b*) The anti-RIP MAbs 6D1 (●) and TH9 (▲) (1 ng–50 μg/ml) were incubated with rat erythrocytes. After washing, the cells were incubated with CVF and rat EDTA plasma at dilutions causing minimal haemolysis in the absence of antibody. Haemolysis was calculated as described in the Materials and methods section. Controls consisted of cells treated with non-immune mouse IgG (50 μg/ml) prior to addition of CVF and plasma, with plasma alone and with buffer alone (background lysis 7%). Controls gave no lysis above background. Each point is plotted with the background lysis subtracted and represents the means ± S.D. of triplicate determinations.

incorporated RIP, were incubated with EDTA sera from a number of species as a source of C8 and C9 at doses previously titrated to give approx. 50% lysis in the absence of RIP. RIP caused inhibition of lysis of GPEC5b–7(rat) cells exposed to rat, human, sheep, swine or rabbit serum (Table 3), but caused no inhibition of lysis by dog, mouse, guinea pig, goat or horse components.

Deglycosylation of RIP

Immunoaffinity-purified RIP was subjected to digestion with endo-F, neuraminidase and O-glycosidase, as described in the Materials and methods section. Samples were then analysed by SDS/PAGE on 15% gels, followed by silver staining to visualize the bands. As shown in Fig. 3, endo-F treatment, which removes

Table 2. Activity of membrane-incorporated RIP at the C5b–7 and C5b–8 stages of MAC assembly

GPC5b–7(rat) and GPC5b–8(rat) cells were incubated with or without RIP and then exposed to EDTA rat serum as a source of C8 and/or C9. The percentage of lysed cells was quantified as described in the Materials and method section. Means ± S.D. of triplicate determinations are given.

Stage of MAC assembly	RIP (1 μg/ml)	EDTA rat serum (1:800)	Haemolysis (%)
C5b–7	+	+	5.2 ± 0.5
	−	+	53.4 ± 1.8
	−	−	4.5 ± 0.9
C5b–8	+	+	5.4 ± 0.3
	−	+	63.7 ± 1.9
	−	−	5.2 ± 0.5

Table 3. Activity of RIP with MAC from other species

GPEC5b–7(rat) cells were incubated with RIP at a final concentration of 1 μg/ml (+) or with buffer alone (−), washed and incubated with dilutions of EDTA sera of various species as a source of C8 and C9, previously titrated to give about 50% haemolysis in the absence of RIP. Results are means ± S.D. of triplicate determinations.

Species	Serum dilution	Lysis (%) −	Lysis (%) +	Inhibition (%)
Rat	800	69.3 ± 3.2	22.1 ± 1.8	68.1
Human	800	53.9 ± 5.3	22.7 ± 5.0	57.9
Horse	10	60.8 ± 7.8	69.5 ± 6.6	(−14.0)
Sheep	100	46.6 ± 3.6	30.7 ± 2.2	34.1
Goat	100	48.6 ± 2.8	50.1 ± 4.1	(−3.1)
Swine	800	61.8 ± 1.3	25.9 ± 1.8	58.1
Dog	400	62.1 ± 7.8	56.6 ± 5.9	8.9
Rabbit	800	54.9 ± 1.7	18.8 ± 0.6	65.8
Guinea pig	100	69.7 ± 7.6	77.5 ± 5.5	(−11.2)
Mouse	10	61.6 ± 3.8	60.8 ± 3.0	1.3

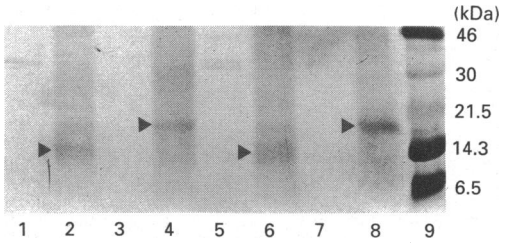

Fig. 3. Deglycosylation of RIP

RIP (10 μg) was treated with endo-F (lane 2), neuraminidase together with O-glycosidase (lane 4), or a mixture of the three enzymes (lane 6). Samples were analysed by SDS/PAGE on 15% polyacrylamide gels followed by silver staining. Lane 8 contained undigested RIP, and lane 9 contained molecular mass markers. Lanes 1, 3 and 5 contained only the enzymes used for digestion in the subsequent lanes. The RIP bands are arrowed.

N-linked oligosaccharides, reduced the apparent molecular mass of RIP by 3–4 kDa. Treatment with neuraminidase, which removes terminal sialic acid residues, together with O-glycosidase, which removes O-linked glycosyl groups, had no effect on the molecular mass of RIP. The activity of these latter enzymes was confirmed by successfully removing O-linked sugars

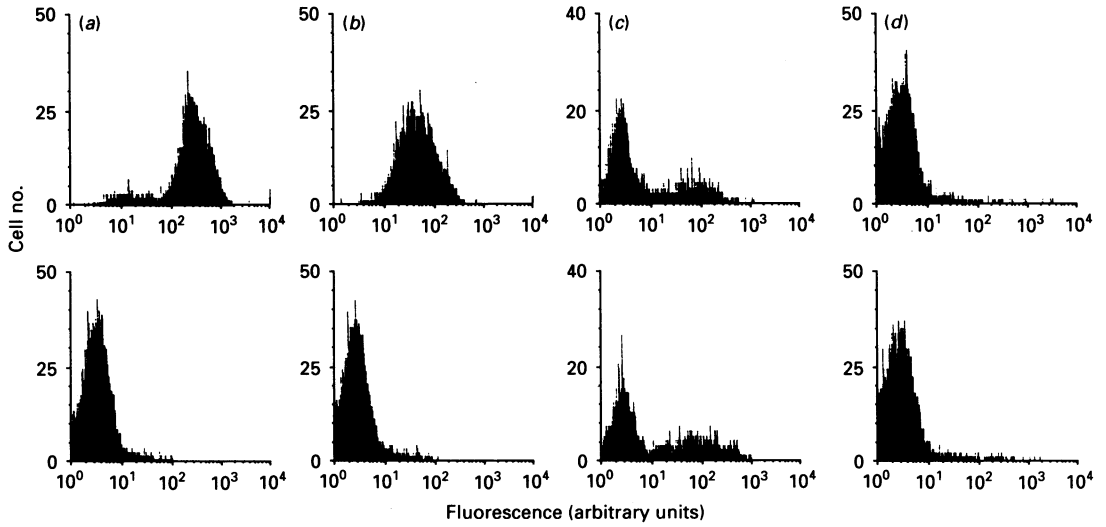

Fig. 4. Flow-cytometric analysis of RIP on rat blood cells

Rat erythrocytes, lymphocytes (10^5 cells/tube) and platelets (10^6 cells/tube) were stained for RIP with either of the anti-RIP MAbs 6D1 or TH9 as described in the Materials and methods section prior to analysis by flow cytometry. Identical results were obtained with both antibodies and those shown are for 6D1. The effects of pre-treatment of cells with PI-PLC on subsequent staining with anti-RIP antibodies was also assessed. (*a*) Rat erythrocytes stained with 6D1 (top) or with mouse IgG (bottom); (*b*) rat erythrocytes pre-treated with PI-PLC (19 munits/ml) and then stained with 6D1 (top) or with mouse IgG (bottom); (*c*) rat lymphocytes stained with 6D1 (top) or with mouse IgG (bottom); (*d*) rat platelets stained with 6D1 (top) or with mouse IgG (bottom).

Residue	1	5	10	15
CD59	LeuGlnCysTyrAsnCysProAsnPro		ThrAlaAspCysLysThr	
	: . : : . :		. : :	
RIP	LeuArg-X-TyrAsn-X-LeuAspPro		ValSerSer-X-LysThr	
	: . : .		: .	
LY6C	LeuGlnCysTyrGluCysTyrGlyValProIleGluThrSerCysProAla			
LY6A	LeuGluCysTyrGlnCysTyrGlyValProPheGluThrSerCysProSer			

Fig. 5. Aligned amino acid sequences of RIP, CD59 antigen and LY6A and LY6C antigens

N-terminal sequencing of purified RIP was performed as described in the Materials and methods section. Residue numbering is for RIP. A two-residue insertion in the LY6 antigen sequences is required for alignment of the third cysteine residues of LY6 with those of CD59 and RIP. Fully conserved residues are indicated by :, and presumptive cysteine alignments by ..

from complement component C9 under identical experimental conditions.

RIP is expressed on rat erythrocytes and is releasable by PI-PLC

RIP was identified by FACS analysis on rat erythrocytes using both 6D1 and TH9 anti-RIP antibodies. The two antibodies gave similar results: 90% of erythrocytes were positive when compared with cells stained with irrelevant antibodies (Fig. 4). Pre-treatment off cells with PI-PLC markedly decreased the staining of rat erythrocytes with anti-RIP antibodies (mean fluorescence 147 units before PI-PLC treatment and 50 units after). Lymphocytes and platelets did not stain with either of the anti-RIP antibodies (Fig. 4). No reactivity of the two MAbs was found with mouse, guinea pig or human erythrocytes. In addition, the anti-(human CD59) antigen MAbs A35 and BRIC-229 did not recognize rat erythrocytes on FACS analysis (results not shown).

Sequence analysis of RIP

An unambiguous sequence was followed for 15 cycles of Edman degradation. This, together with the *N*-terminal amino acid sequences of human CD59 antigen and the mouse LY6A and LY6C antigens, is shown in Fig. 5. The initial yield (Leu-1) was 16 pmol and the repetitive yield was 88%. No residues could be identified at cycles 3, 6 and 13. These positions are occupied by cysteine residues in human CD59; as cysteine residues had not been modified prior to sequence analysis and would therefore have been destroyed during sequencing, the data are consistent with RIP also having cysteine residues at these positions. Sequencing of reduced and alkylated RIP is needed to confirm this.

DISCUSSION

Although several human proteins which inhibit complement membrane attack have been characterized, little is known about regulation of the MAC in other species. Here we report the identification and isolation of a 21 kDa glycoprotein present on the surface of rat erythrocytes which has the capacity to insert into cell membranes and to exert a potent inhibitory effect on the MAC of rat complement.

Our first attempt to isolate a rat inhibitor of the MAC employed a modification of the method of Holguin *et al.* (1989) for the purification of CD59 antigen from human erythrocyte membranes. Using this method, we obtained a highly active MAC-inhibitory preparation which contained a major band at about 21 kDa, together with a number of higher-molecular-mass bands. After radiolabelling of this preparation and incubation with heterologous erythrocytes, only the 21 kDa component became incorporated into membranes. A series of MAbs was raised either against this partially purified preparation or against intact rat erythrocytes. Of these MAbs, several recognized the 21 kDa band on Western blots of the partly pure preparation and enhanced the CVF-mediated reactive lysis of rat erythrocytes, confirming that this component was the MAC-inhibitory factor in the mixture. Of the remaining antibodies, most recognized the 21 kDa band but did not enhance lysis, suggesting that they bound to a part of the inhibitor remote from the active site.

The two MAbs chosen for further study, 6D1 and TH9, both

enhanced the CVF-mediated lysis of rat erythrocytes by homologous complement in a dose-dependent manner. MAb 6D1 was immobilized on Sepharose and subsequently used to purify RIP to homogeneity in a single step. Purified RIP was efficiently inserted into heterologous cells and rendered them resistant to lysis by rat complement. RIP exerted its inhibitory effect even after the stage of C8 insertion into the MAC.

These properties closely resemble those reported for CD59 antigen. Antibodies against CD59 antigen enhance the reactive lysis of human erythrocytes (Davies *et al.*, 1989; Okada *et al.*, 1989*b*), and purified CD59 antigen is incorporated into heterologous cells, inhibiting MAC lysis at or after the C5b–8 stage (Meri *et al.*, 1990; Rollins & Sims, 1990). There are also structural similarities between the two inhibitors, in that they both have a molecular mass of about 20 kDa and are heavily glycosylated, each having between 3 and 6 kDa of N-linked oligosaccharides (Okada *et al.*, 1989a; Harada *et al.*, 1990). They each comprise a single polypeptide chain and cannot be detected by specific MAbs on Western blots after reduction (Okada *et al.*, 1989*b*). In addition to these similarities, we have found that RIP, like CD59 antigen (Rollins *et al.*, 1991), is not entirely homologously restricted, but is species-selective in its inhibitory action.

The capacity of purified CD59 antigen to insert into membranes is a consequence of its possession of a GPI anchor. Treatment of nucleated cells with PI-PLC cleaves the anchor and releases CD59 antigen (Stefanova *et al.*, 1989; Hideshima *et al.*, 1990; Whitlow *et al.*, 1990). However, erythrocyte CD59 antigen is resistant to release by PI-PLC (Taguchi *et al.*, 1990; Holguin *et al.*, 1990), as are other GPI-anchored proteins on human erythrocytes, including acetylcholinesterase and decay-accelerating factor (Roberts *et al.*, 1987; Walter *et al.*, 1990), and it has been shown that resistance to release is due to an extra-acyl-linked lipid moiety in the anchor which protects it from PI-PLC attack (Roberts *et al.*, 1988; Walter *et al.*, 1990).

The evidence presented in this paper demonstrates that RIP is also GPI-anchored: the purified protein was incorporated into membranes and PI-PLC treatment caused its release. The demonstration that erythrocyte RIP was not resistant to release by PI-PLC suggests that the anchor on rat erythrocytes is not protected. In support of this finding, rat erythrocyte acetylcholinesterase has previously been shown to be readily releasable by PI-PLC (Low & Finean, 1977; Futerman *et al.*, 1985). Human lymphocytes and platelets stain strongly for CD59 antigen (Whitlow *et al.*, 1990; Morgan, 1992). In contrast, rat lymphocytes and platelets did not stain with either of the anti-RIP antibodies used in this study. This finding may indicate either that the antibodies used recognize a determinant on RIP not present on these cells or that rat lymphocytes and platelets lack the inhibitor.

It is clear from our studies that RIP bears a close structural and functional resemblance to CD59 antigen. While complete sequence data are required to fully define the relationship between these proteins, *N*-terminal sequence comparison strongly supports the hypothesis that they are structural and functional homologues. Comparison of the CD59 and RIP sequences with those of LY6 is also instructive, as RIP appears more similar to CD59 than to LY6, with 9 of 15 versus 6 of 15 residues identical (assuming cysteines are correct) and the absence of a two residue insertion between residues 9 and 10 present in LY6. Given the phylogenetic proximity of rat and mouse, these data do not support suggestions that LY6 is the mouse homologue of CD59 (Bernard & Horejsi, 1989; Whitlow *et al.*, 1990; Korty *et al.*, 1991).

The isolation of a potent inhibitor of the membrane attack pathway in the rat, and the production of MAbs against this protein, provides useful tools which may help to elucidate the role of the MAC and of MAC-regulatory molecules in rat models of immune-mediated human diseases. These studies may provide clues to the possible relevance of CD59 antigen in human diseases, and enable the use of MAC-inhibitory molecules in therapy to be examined.

This work was supported by The Wellcome Trust, The U.K. Multiple Sclerosis Society and The Kidney Research Unit Foundation. B.P.M. is a Wellcome Senior Research Fellow. We thank Jane Jones for help with FACS analysis and Alex Davies for provision of antibodies against CD59 antigen.

REFERENCES

Bernard, A. & Horejsi, V. (1989) J. Immunol. **143**, 1073
Davies, A., Simmons, D. L., Hale, G., Harrison, R. A., Tighe, H., Lachmann, P. J. & Waldmann, H. (1989) J. Exp. Med. **170**, 637–653
Futerman, A. H., Low, M. G., Michaelson, D. M. & Silman, I. (1985) J. Neurochem. **45**, 1487–1494
Galfre, G. & Milstein, C. (1981) Methods Enzymol. **73**, 3–46
Groux, H., Huet, S., Aubrit, F., Tran, H. C., Boumsell, L. & Bernard, A. (1989) J. Immunol. **142**, 3013–3020
Harada, R., Okada, N., Fujita, T. & Okada, H. (1990) J. Immunol. **144**, 1823–1828
Hideshima, T., Okada, N. & Okada, H. (1990) Immunology **69**, 396–401
Holguin, M. H., Fredrick, L. R., Brenshaw, N. J., Wilcox, L. A. & Parker, C. J. (1989) J. Clin. Invest. **84**, 7–17
Holguin, M. H., Wilcox, L. A., Bernshaw, N. J., Rosse, W. F. & Parker, C. J. (1990) Blood **75**, 284–289
Korty, P. E., Brando, C. & Shevach, E. M. (1991) J. Immunol. **146**, 4092–4098
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Low, M. G. & Finean, J. B. (1977) FEBS Lett. **82**, 143–146
Meri, S., Morgan, B. P., Davies, A., Daniels, R. H., Olavesen, H., Waldmann, H. & Lachmann, P. J. (1990) Immunology **71**, 1–9
Merill, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) Science **211**, 1437
Morgan, B. P. (1992) Biochem. J. **282**, 409–413
Okada, N., Harada, R., Fujita, T. & Okada, H. (1989*a*) Int. Immunol. **1**, 205–208
Okada, N., Harada, R., Fujiat, T. & Okada, H. (1989*b*) J. Immunol. **143**, 2262–2266
Philbrick, W. M., Palfree, R. G. E., Maher, S. E., Bridgett, M. M., Sirlin, S. & Bothwell, A. L. M. (1990) Eur. J. Immunol. **20**, 87–92
Roberts, W. L., Kim, B. H. & Rosenberry, T. L. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7817–7821
Roberts, W. L., Myher, J. J., Kuksis, A., Low, M. G. & Rosenberry, T. L. (1988) J. Biol. Chem. **263**, 18755–18766
Rollins, R. A. & Sims, P. J. (1990) J. Immunol. **144**, 3478–3483
Rollins, R. A., Zhao, J., Ninomiya, H. & Sims, P. J. (1991) J. Immunol. **146**, 2345–2351
Salant, D. J., Quigg, R. J. & Cybulsky, A. V. (1989) Kidney Int. **35**, 976–984
Schonermark, S., Rauterberg, E. W., Shin, M. L., Loke, S., Roelcke, D. & Hansch, G. M. (1986) J. Immunol. **136**, 1772–1776
Schonermark, S., Filsinger, S., Berger, B. & Hansch, G. M. (1988) Immunology **63**, 585–590
Scolding, N. J., Morgan, B. P., Houston, A., Campbell, A. K., Linington, C. & Compston, D. A. S. (1989) Immunology **89**, 289–300
Shevach, E. M. & Korty, P. E. (1989) Immunol. Today **10**, 195–200
Stefanova, I., Hilgert, I., Kristofova, H., Brown, R., Low, M. & Horejsi, V. (1989) Mol. Immunol. **26**, 153–161
Stuart, J. M., Cremer, M. A., Townes, A. S. & Kang, A. H. (1982) J. Exp. Med. **155**, 1–16
Sugita, Y., Nakano, Y. & Tomita, M. (1988) J. Biochem. (Tokyo) **104**, 633–637
Sugita, Y., Tobe, T., Oda, E., Tomita, M., Yasukawa, K., Yamamji, N., Takemoto, T., Furuichi, K., Takayama, M. & Yano, S. (1989) J. Biochem. (Tokyo) **106**, 555–557
Taguchi, R., Funahashi, Y., Ikezawa, H. & Nakashima, I. (1990) FEBS Lett. **261**, 142–146
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Vogel, C. W. & Muller-Eberhard, H. J. (1984) J. Immunol. Methods **73**, 203–220

Walter, E. I., Roberts, W. L., Rosenberry, T. L., Ratnoff, W. D. & Medof, M.-E. (1990) J. Immunol. **144**, 1030–1036

Watts, M. J., Patel, A. K. & Morgan, B. P. (1987) Complement **4**, 236

Watts, M. J., Dankert, J. R. & Morgan, B. P. (1990) Biochem. J. **265**, 471–477

Whitlow, M. B., Iida, K., Stefanova, I., Bernard, A. & Nussezweig, V. (1990) Cell. Immunol. **126**, 176–184

Zalman, L. S., Wood, L. M. & Muller-Eberhard, H. J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 6975–6979

Zalman, L. S., Brothers, M. A. & Muller-Eberhard, H. J. (1989) J. Immunol. **143**, 1943–1947

Received 26 July 1991/28 November 1991; accepted 13 December 1991