Isolation and characterization of a membrane-attack-complex-inhibiting protein present in human serum and other biological fluids

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We have previously reported the isolation of a membrane-attack-complex-inhibiting protein (MIP) from human erythrocyte membranes [Watts, Patel & Morgan (1987) *Complement* 4, 236] and the production of polyclonal antibodies to this protein. Here we report the identification in plasma, urine, saliva and cerebrospinal fluid of a protein immunochemically identical with the membrane-derived MIP. The protein has been isolated from plasma by immunoaffinity chromatography on an anti-(erythrocyte MIP)–Sephacryl column and shown by SDS/polyacrylamide-gel electrophoresis to be of similar molecular mass to the erythrocyte protein (55 kDa non-reduced and 65 kDa under reducing conditions). Monoclonal antibodies have been raised against plasma MIP and used to establish a two-site enzyme-linked immunoadsorbent assay, enabling quantification of MIP in plasma, urine and cerebrospinal fluid. Plasma MIP, though not able to incorporate spontaneously into membranes, was deposited on heterologous and homologous erythrocyte membranes during complement activation in a C8-dependent manner. Depletion of MIP from plasma resulted in enhancement of the lytic capacity of the plasma on heterologous erythrocytes.

INTRODUCTION

Activation of complement leads to the production of biologically active molecules, including anaphylactic and chemotactic factors and the cytolytic membrane-attack complex (MAC). In order to limit damage to host tissues it is therefore essential that activation is strictly controlled. Numerous fluid-phase and membrane-associated proteins have been demonstrated to inhibit autologous complement at the level of the C3/C5 convertases, thereby preventing completion of the cascade and restricting lysis of homologous cells (Mollnes & Lachmann, 1988). The demonstration that reactive lysis, employing purified terminal components, is species-restricted provides evidence that assembly of the MAC is also under specific control (Yamamoto, 1977; Hansch *et al.*, 1981; Hu & Shin, 1984). Plasma S-protein and C8 both exert control at this stage by binding to the fluid-phase C5b-7 complex, hence preventing its insertion into membranes (Podack *et al.*, 1984; Nemerov *et al.*, 1979; Zeitz *et al.*, 1983). Recently, two groups have described membrane-associated proteins which also have MAC-inhibitory properties, C8 binding protein (C8bp), so-called because of its affinity for C8 (Schonermark *et al.*, 1986), and homologous restriction factor (HRF) (Zalman *et al.*, 1986). These proteins have been isolated from human erythrocyte membranes and identified on human peripheral blood cells. They exert their inhibitory effects at the stage of C8 and/or C9 binding and insertion into the MAC, although the exact mechanisms of action have yet to be elucidated.

We have also isolated a MAC-inhibiting protein (MIP) from human erythrocyte membranes (Watts *et al.*, 1987). Functional and molecular characterization of this protein demonstrates close similarities with C8bp and HRF, suggesting that all these proteins are identical.

In the present study we have employed antibodies produced to our cell-membrane-derived MIP to demonstrate the presence of fluid-phase MIP in normal plasma and other biological fluids. The protein has been isolated from plasma and demonstrated to be functionally active. Monoclonal antibodies raised against plasma-derived MIP detect molecules of identical size in membranes and in plasma, providing evidence for the identity of MIP derived from these two sources. This represents the first description of a fluid-phase inhibitor of the late stage of MAC assembly and may have important implications for the control of complement lysis.

MATERIALS AND METHODS

Materials

CNBr-activated Sepharose, acrylamide, *NN'*-methylene-bisacrylamide and ammonium persulphate were purchased from Pharmacia-LKB. Prosep A glass beads were obtained from Bioprocessing Ltd. (Consett, Co. Durham, U.K.). Nitrocellulose sheets were from Schleicher and Schuell and PVDF Immobilon membranes were obtained from Millipore. All other chemicals were purchased from BDH Chemicals or from Sigma Chemical Co. and were of the best grade available. Tris-buffered

Abbreviations used: MAC, membrane-attack complex; MIP, MAC-inhibiting protein; C8bp, C8-binding protein; HRF, homologous restriction factor; TBS, Tris-buffered saline (composition given in text); PBS, phosphate-buffered saline; NHS, normal human serum; DAF, decay-accelerating factor.

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saline (TBS) contained 10 mM-Tris and 200 mM-NaCl at pH 7.4.

Cells and sera

Outdated human erythrocytes were obtained from the blood bank at U.H.W., Heath Park, Cardiff. Fresh whole blood and plasma were donated by adult volunteers. Sheep blood was collected into acid citrate/dextrose anticoagulant and stored aseptically at 4 °C in Alsever's solution.

Normal human serum (NHS) was obtained from adult volunteers and stored in portions at -70 °C. Sera specifically depleted of C8 (NHS-C8) or C9 (NHS-C9) were produced by passage of NHS over monoclonal anti-C8- or anti-C9-Sepharose columns respectively (Abraha *et al.*, 1988; Morgan *et al.*, 1983).

Proteins

Human C9 and C8 were purified by immunoaffinity chromatography on immobilized monoclonal antibodies, as detailed previously (Morgan *et al.*, 1983; Abraha *et al.*, 1988).

MAC-inhibiting protein (MIP) was initially purified from human erythrocyte membranes utilizing methods essentially similar to those used by Zalman *et al.* (1986) for the isolation of HRF. Briefly, deoxycholate-solubilized, haemoglobin-free human erythrocyte membranes were subjected to affinity chromatography on a human C9-Sepharose column equilibrated in column buffer (20 mM-Tris/HCl, 50 mM-NaCl, 0.02 % deoxycholate, pH 8.4). After extensive washing with the same buffer the bound protein was eluted with the above buffer containing 2 M-NaCl. Later preparations utilized a solid-phase anti-MIP column, produced as described below, in place of the C9-Sepharose column, bound protein being eluted with 2 M-KBr in column buffer as described below for MIP purification from plasma.

Antibodies

Monoclonal anti-C9 and anti-C8 were produced as previously detailed (Morgan *et al.*, 1983; Abraha *et al.*, 1988). Amboceptor was purchased from Behring and used to produce sensitized sheep erythrocytes. Goat anti-rabbit peroxidase-conjugated antibody and HRP Colour Development Reagent were obtained from Bio-Rad. (Watford, Herts.). Polyclonal anti-MIP antiserum was produced by repeated immunization of albino high-antibody-titre half-lop rabbits (Ranch Rabbits, Crawley Down, Sussex, U.K.) with MIP isolated from human erythrocyte membranes as detailed above. The immunoglobulin fraction was isolated on Prosep A glass beads, and 40 mg of immunoglobulin was immobilized on CNBr-activated Sepharose to produce an anti-MIP-Sepharose immunoaffinity column used in subsequent purifications.

Monoclonal antibodies to plasma MIP were produced by standard methods (Galfre & Milstein, 1981) as described previously (Morgan *et al.*, 1983). BALB/C mice were immunized with 20 µg of plasma MIP in Freund's complete adjuvant. After 4 weeks the mice were boosted with 20 µg of MIP in incomplete adjuvant. The mice were test-bled 1 week later, and the mice with the highest titres of anti-MIP activity in an e.l.i.s.a. screening assay were boosted twice more with 10 µg of MIP in phosphate-buffered saline (PBS). On day 3 after the final boost the mice were killed, spleen cells fused with SP2 mouse

myeloma cells, and the products of fusion cultured in HAT medium in the presence of resident peritoneal macrophages. After 7 days in culture, viable hybrid cells were cloned using the fluorescence-activated cell sorter, one hybrid cell being delivered into 100 µl of macrophage-containing medium in each well of six 96-well plates. Culture supernatants were tested in an e.l.i.s.a. assay for anti-MIP antibody after about 14 days in culture and positive clones expanded before growth as ascites tumours in mice. Antibody was isolated from ascitic fluid by precipitation with Na₂SO₄ or by affinity purification on Prosep A beads. Antibodies were typed using a monoclonal antibody typing kit (ICN Immunobiologicals, High Wycombe, Bucks., U.K.).

Immunoblotting studies

SDS/PAGE was carried out by the method of Laemmli (1970) on 2.5 %–20 % gradient gels utilizing a Protean II mini-gel system (Bio-Rad). Proteins were electrotransferred onto nitrocellulose or PVDF membranes by the method of Towbin *et al.* (1979). MIP was then identified by incubation of the transfer membrane with polyclonal or monoclonal anti-MIP IgG at a final concentration of 10 µg/ml in TBS containing 0.2 % bovine serum albumin (BSA) for 3 h at room temperature. After extensive washing in the above buffer containing 0.1 % Tween 20, the blots were incubated with the appropriate peroxidase-conjugated second antibody diluted 1:1000 in TBS/0.2 % BSA for 1 h at room temperature, washed and developed using HRP substrate.

Identification of MIP on complement-attacked cell membranes

Optimally antibody-sensitized sheep erythrocytes were incubated with human serum at a final dilution of 1:10 for 30 min at 37 °C. Membranes and unlysed cells were pelleted, washed in water to complete lysis, and the haemoglobin-free membranes solubilized in SDS sample buffer. Controls included sensitized erythrocytes incubated with buffer alone and unsensitized erythrocytes incubated with buffer or NHS. Solubilized pellets from 5 × 10⁷ cells were then separated by SDS/PAGE, electroblotted and probed with polyclonal anti-MIP antibody as detailed above.

Identification of MIP in biological fluids

Serum (diluted 1:500 in TBS), saliva (undiluted), cerebrospinal fluid and urine (both concentrated 5-fold by ultrafiltration), were obtained fresh and subjected to SDS/PAGE and immunoblotting with polyclonal anti-MIP antibody as described above.

Isolation of MIP from plasma

Plasma was obtained fresh using heparin as anticoagulant, brought to 1 mM-phenylmethanesulphonyl fluoride, 1 mM-benzamidine and 1 mM-EDTA by addition of concentrated stock solutions, and specifically depleted of C8 and C9 by passage over monoclonal anti-C8- and anti-C9-Sepharose columns. The depleted plasma was then passed over a 30 ml column of anti-MIP-Sepharose to which 40 mg of polyclonal IgG was bound. After extensive washing with TBS the bound protein was eluted in 2 M-KBr in TBS and immediately dialysed into TBS containing 0.02 % sodium deoxycholate. Minor high-molecular-mass contaminants were

removed by gel filtration on an Ultrogel AcA44 column which had previously been equilibrated in TBS/0.02% sodium deoxycholate and the protein was eluted in the same buffer.

Depletion of MIP from plasma

Plasma depleted of C8 and C9, as described above, was passed over the anti-MIP column. This resulted in the removal of about 80% of the plasma MIP. Physiological amounts of C8 and C9 were added back to the MIP-depleted plasma and to control plasma which had been depleted of C8 and C9 but not of MIP. The haemolytic activity of control and MIP-depleted plasma was then assessed by measurement of haemolysis of antibody-sensitized sheep erythrocytes using standard methods (Whaley, 1985). Purified MIP was also added back to portions of the MIP-depleted plasma to assess the effects of repletion on haemolytic activity.

E.l.i.s.a. assay for MIP in biological fluids

In order to quantify the amount of MIP present in biological fluids a two-site e.l.i.s.a. assay was established utilizing polyclonal anti-MIP as first antibody and monoclonal anti-MIP as second antibody. Polyclonal anti-MIP IgG at a final concentration of 5 µg/ml in phosphate-buffered saline was incubated in the wells of microtitre plates (Flow Laboratories) overnight at 4 °C. The plates were then washed twice in PBS containing 0.1% Tween 20 and various dilutions of plasma, cerebrospinal fluid, urine or purified MIP in PBS added. After incubation at 37 °C for 1 h the wells were again washed and monoclonal anti-MIP antibody MIP-G3 was added at a final concentration of 2 µg/ml in PBS. The plates were again incubated at 37 °C for 1 h, washed and peroxidase-labelled goat anti-(mouse IgG) antibody at a final dilution of 1:1000 in PBS was added. After a further 1 h at 37 °C the plate was washed, peroxidase substrate (1,2-diphenylenediamine dihydrochloride; Pierce) was added and colour allowed to develop for 2 min. The reaction was then stopped with 10% H₂SO₄ and the A₄₉₂ in the wells read using a Titertek Multiscan microplate reader. Quantification of MIP in samples was achieved by comparison of results with standard curves produced using purified MIP.

Sample collection

Plasma samples for assay were collected from ten normal laboratory personnel into EDTA and were stored at -20 °C prior to assay. Urine and cerebrospinal fluid samples were a random selection of specimens arriving at the laboratory for routine analysis. Haemolysed samples were excluded but no selection on the basis of the presenting illness was made.

Immunogold ultrastructural studies

Group A human erythrocytes were antibody-coated using a human anti-A antibody. After washing, the coated erythrocytes were incubated for 30 min at 37 °C with NHS, NHS depleted of C8, NHS depleted of C9, or NHS depleted of both C8 and C9. Controls included unsensitized erythrocytes incubated with buffer alone or NHS and coated erythrocytes incubated with buffer alone. The cells were then washed once with buffer and three times in water. The washed membranes were placed on bacitracin-coated Formvar/nickel specimen grids and non-specific protein binding sites blocked by incubation

with a solution of 50% normal goat serum in Tris buffer supplemented with 0.1% bovine serum albumin and 0.1% ovalbumin for 20 min at room temperature. The grids were blotted dry and primary antibodies applied [rabbit anti-MIP and mouse anti-C9 (MC 47) at 10 µg/ml each] in 10% normal goat serum supplemented with 0.1% ovalbumin for 20 min at room temperature. The grids were washed three times in Tris buffer containing 0.1% BSA and then incubated with gold-labelled secondary antibodies, 5% each of 5 nm gold-labelled goat anti-(mouse IgG) and 10 nm gold-labelled anti-(rabbit IgG) (Janssen Life Sciences) in 0.1% BSA/Tris, for 20 min at room temperature. The grids were washed three times in 0.1% BSA/Tris, then in water and imaged at 50 kV in a Zeiss EM-10CR electron microscope.

RESULTS

Deposition of MIP-reactive material on heterologous and homologous erythrocytes

Immunoblotting of sheep erythrocyte membranes with polyclonal anti-MIP antibody showed that no anti-MIP-reactive material was present on these cells. However, after these erythrocytes had been antibody-sensitized and incubated with NHS, immunoblotting revealed a sharp MIP-reactive band of 65 kDa and a faint band at 85 kDa, identical with MIP detected on immunoblots of human erythrocyte membranes (Fig. 1, lane 4). Controls, including unsensitized or antibody-sensitized sheep erythrocytes incubated with buffer alone did not reveal any MIP-reactive material but unsensitized erythrocytes incubated with NHS showed faint bands on immunoblotting, perhaps reflecting the low level of complement activation occurring even without sensitization (Fig. 1, lane 2). Human erythrocytes that were antibody-coated

Fig. 1. Deposition of MIP-reactive material on heterologous erythrocytes

Sheep erythrocytes and optimally antibody-sensitized erythrocytes were incubated with either buffer or NHS. Membranes and unlysed cells were pelleted, washed in water to complete lysis and solubilized in SDS sample buffer. After separation by SDS/PAGE, the solubilized membranes were electroblotted and probed with polyclonal anti-MIP antibody. The blot was developed using peroxidase-conjugated anti-rabbit antibody and HRP substrate. Molecular masses are indicated on the left of the Figure. Lane 1, sheep erythrocytes and buffer; lane 2, erythrocytes and NHS; lane 3, sensitized erythrocytes and buffer; lane 4, sensitized erythrocytes and NHS.

Fig. 2. Deposition of MIP on homologous erythrocytes

Antibody-coated human erythrocytes were incubated with NHS, NHS depleted of C8, NHS depleted of C9 and NHS depleted of both C8 and C9 for 30 min at 37 °C. Controls included erythrocytes incubated with buffer and NHS and coated erythrocytes incubated with buffer alone. The erythrocytes were then placed on grids and MIP and C9 were localized using specific primary antibodies ($\square$, anti-MIP; $\blacksquare$, anti-C9) and gold-labelled secondary antibodies. The grids were imaged in the electron microscope and the average number of gold particles counted in randomly selected 10 μm^2 areas of membrane on each of 10 cells. Error bars represents the S.D. of these determinations. Conditions: A, erythrocytes and buffer; B, erythrocytes and NHS; C, coated erythrocytes and buffer; D, coated erythrocytes and NHS; E, coated erythrocytes and NHS depleted of C8; F, coated erythrocytes and NHS depleted of C9; G, coated erythrocytes and NHS depleted of C8 and C9.

and allowed to react with homologous human serum were also examined for deposition of MIP from serum. Immunoblotting, initially utilized to identify MIP on these cells, did not reveal significant deposition of more MIP on the cells during complement activation. Therefore, an alternative system was developed to determine if complement-dependent serum-derived deposition of MIP was also occurring on homologous target cells. This was achieved by electron-microscopic quantification of MIP and C9 on target cells following homologous complement attack. The number of gold particles associated with either human MIP (using 10 nm gold-labelled secondary antibodies) or human C9 (5 nm gold-labelled secondary antibodies) were counted on normal erythrocytes and on antibody-coated erythrocytes. Fig. 2 shows the number of gold particles (MIP and C9) deposited on these cells. Only small amounts of anti-MIP antibody bound prior to activation, suggesting that most of the membrane MIP was not accessible to antibody. Complement activation greatly increased the amounts of both MIP and C9 detectable on the cell surface when compared with controls, demonstrating deposition of MIP and C9 from serum. Removal of C9 from serum did not significantly decrease MIP deposition whereas removal of C8 markedly decreased MIP uptake on to cells (Fig. 2).

Fig. 3. Identification of MIP in biological fluids

The presence of MIP in biological fluids was investigated using SDS/PAGE followed by immunoblotting as described in the legend to Fig. 1. Lane 1, NHS (diluted 1:500); lane 2, urine (concentrated 5 $\times$); lane 3, saliva (unconcentrated); lane 4, cerebrospinal fluid (concentrated 5 $\times$).

MIP in serum and other biological fluids

The experiments described above suggested that MIP was present in NHS and was deposited on heterologous and homologous erythrocytes during complement activation. In order to investigate this possibility further serum was subjected to SDS/PAGE and after electroblotting onto nitrocellulose was probed with polyclonal anti-MIP antibody. The polyclonal anti-MIP antibody used in these studies showed weak cross-reaction with both C8 and C9 in e.l.i.s.a. assays (results not shown). Therefore sera specifically depleted either of C9 alone or of C8 and C9 were also examined. As shown in Fig. 3, lane 1, a single band of 65 kDa was detected by the polyclonal anti-MIP antibody in NHS. This band was also present and undiminished in sera depleted of C9 and of C8 and C9.

To investigate whether anti-MIP-reactive material was also present in other biological fluids, saliva, urine and cerebrospinal fluid were electrophoresed and immunoblotted as described above. Fig. 3 shows the presence in all of these fluids of a band of molecular mass identical with that seen in serum. The band from cerebrospinal fluid was very broad, perhaps reflecting overloading of the track, or heterogeneity arising from using pooled, concentrated cerebrospinal fluid.

Isolation of MIP from plasma

As noted above, the polyclonal anti-MIP antibody showed weak cross-reactivity with C8 and C9. To prevent saturation of the anti-MIP affinity column plasma was therefore specifically depleted of these components on monoclonal antibody affinity columns. The depleted plasma was then subjected to immunoaffinity chromatography on a solid-phase polyclonal anti-(erythrocyte membrane MIP) column.

On SDS/PAGE the purified protein had a molecular mass of 55 kDa under non-reducing conditions, and 65 kDa on reduction, identical with that of the membrane-derived protein isolated as described above (Fig. 4). The decreased electrophoretic mobility on

Fig. 4. SDS/PAGE of MIP isolated from human plasma

MIP was isolated from human plasma by immunoaffinity chromatography on an anti-MIP-Sepharose column. Non-reduced and reduced samples of the isolated protein were subjected to SDS/PAGE [10% (w/v) polyacrylamide separating gel]. The protein bands were visualized by Coomassie Blue staining. The top of the stacking gel is arrowed. Lane 1, molecular mass markers; lane 2, reduced MIP; lane 3, non-reduced MIP.

reduction implies further unfolding as a result of disulphide bond cleavage. All preparations also contained high-molecular-mass aggregates just entering the separating gel (molecular mass > 200 kDa) which on reduction co-migrated with the monomer or, in some preparations, produced two lower-molecular-mass bands of approx. 50 and 20 kDa. Aggregates were also present in our preparations of membrane MIP and the relative proportions of aggregate to monomer increased on storage of both proteins, even at -20°C . A minor band of molecular mass 85–90 kDa was often present in preparations of MIP from plasma and membrane and was detected by both polyclonal and monoclonal anti-MIP antibodies. The yield of MIP from plasma in several preparations was between 1.5 and 2.0 mg/l. The antibody column has now been used over 20 times with no appreciable loss of efficiency.

Production of monoclonal antibodies

Of the 576 wells screened following single-cell cloning of hybrid cells, five were strongly positive in an e.i.s.a. assay for anti-MIP reactivity. Of these, three were successfully grown up in bulk and injected intraperitoneally into mice to produce ascites fluid. Using class- and subclass-specific antisera two of these antibodies were IgG_{2a} (MIP-B5 and MIP-D8) and the other was IgG_{2b} (MIP-G3).

Effect of MIP depletion on the lytic activity of normal plasma

Plasma was depleted of C8 and C9 because of the cross-reactivity noted previously, and half was applied to the anti-MIP affinity column. Pure C8 and C9 were added to restore levels to 50 $\mu\text{g}/\text{ml}$ in both MIP-depleted and non-depleted sera and their lytic capacity compared in haemolytic assays utilizing antibody-sensitized sheep

Fig. 5. Effect of MIP depletion on the lytic activity of normal plasma

Plasma was immunochemically depleted of C8 and C9 and half was applied to the anti-MIP affinity column. Pure C8 and C9 were added back to restore levels to 50 $\mu\text{g}/\text{ml}$ in both MIP-depleted and non-depleted plasmas and their lytic capacity compared in haemolytic assays utilizing sensitized sheep erythrocytes as target cells. Lysis is expressed as 'Z units' calculated as described in Whaley (1985). Each point represents the mean value of triplicate determinations. O, Plasma depleted of MIP; ●, plasma containing MIP.

erythrocytes as target cells. Fig. 5 illustrates that plasma depleted of MIP was consistently more lytic towards the target erythrocytes than plasma containing MIP. Addition of pure MIP to a final concentration of about 50 $\mu\text{g}/\text{ml}$ diminished lytic activity in the depleted serum.

E.i.s.a. assay for MIP in biological fluids

A two-site e.i.s.a. assay utilizing polyclonal anti-MIP as first antibody and monoclonal anti-MIP G3 as second antibody was used to quantify MIP in plasma, urine and cerebrospinal fluid. The detection limit of the assay was 0.01 mg/l and the working range (< 10% CV) was 0.01–0.25 mg/l. All samples were diluted to bring the measured concentration within this working range. Dilution and recovery studies showed satisfactory linearity. The mean concentration of MIP in 10 normal plasma samples was 2.5 mg/l (range 1.2–2.9 mg/l, S.E.M. 0.18 mg/l), in urine the mean MIP concentration was 0.08 mg/l ($n = 10$, range 0.04–0.11 mg/l, S.E.M. 0.007 mg/l), and in cerebrospinal fluid the mean MIP concentration was 0.35 mg/l ($n = 10$, range 0.1–0.49 mg/l, S.E.M. 0.041 mg/l).

DISCUSSION

Since the description in the 1950s of a serum protein with C1 esterase inhibitory activity (Levy & Lepow, 1959) a large number of fluid-phase and membrane-associated proteins with complement controlling activity have been described (reviewed in Buscher & Opferkuch, 1988). The newest additions to this growing family of complement control proteins are the recently reported membrane-associated MAC-inhibiting proteins C8bp,

HRF and MIP which have been purified from human erythrocyte membranes (Schonermark *et al.*, 1986; Zalman *et al.*, 1986; Watts *et al.*, 1987). Functional and physicochemical similarities suggest that all three of these proteins are identical, a supposition supported by the demonstration of cross-reactivity of antiserum raised against C8bp (donated by G. M. Hansch) with MIP (M. J. Watts & B. P. Morgan, unpublished work). We have chosen to call our protein MAC-inhibitory protein (MIP) because neither of the other names is entirely appropriate. The protein does not solely bind C8, nor, at least in our hands, is its lytic inhibitory activity completely restricted to homologous systems. These molecules on incubation with cells re-incorporate into the membrane rendering the cell less susceptible to lysis by complement, particularly of the same species, and have also been reported to inhibit lysis by killer lymphocytes (Zalman *et al.*, 1987b, 1988). Hansch and co-workers have reported that C8bp interacts with the γ -subunit of C8 and diminishes lysis by inhibiting polymerization of C9 in the MAC (Hansch *et al.*, 1986, 1987; Schonermark *et al.*, 1988).

Several similarities exist between MIP and decay-accelerating factor (DAF). Both have been identified on circulating cells and have been implicated in the protection of homologous cells from complement lysis. More recently it has been shown that both of these proteins are deficient in blood cells from patients with paroxysmal nocturnal haemoglobinuria (Hansch *et al.*, 1987; Zalman *et al.*, 1987a; Halperin & Nicholson-Weller, 1989), and that C8bp (HRF/MIP), like DAF, is linked to the membrane by a phosphatidylinositol linkage (Hansch *et al.*, 1988). DAF has been demonstrated in body fluids, including plasma, tears, saliva and urine (Medof *et al.*, 1987). It therefore seemed possible that MIP would also be detectable in biological fluids.

Here we report the identification in plasma, urine, saliva and cerebrospinal fluid of a protein immunochemically identical with and functionally similar to that of the membrane-derived MAC-inhibiting proteins. Plasma, urine, saliva and cerebrospinal fluid MIP had similar molecular masses to and reacted with antibodies raised against the membrane form. The protein has been purified from plasma and monoclonal antibodies raised against plasma MIP all recognized membrane MIP. The concentration of the protein in normal plasma measured by e.l.i.s.a. was about 2.5 mg/l, and the yield from several purifications was between 1.5 and 2.0 mg/l, a recovery of 60–80%. The protein was immunochemically distinct from S protein and DAF as demonstrated by immunoblotting with monoclonal and polyclonal antibodies against these proteins (results not shown).

Unlike membrane-derived MAC-inhibitory proteins, purified plasma MIP did not spontaneously incorporate into cell membranes and inhibit lysis by the MAC, a further analogy with the properties of DAF, the urine-derived form of which cannot reinsert into membranes (Medof *et al.*, 1987). However, when sheep erythrocytes were lysed with human serum, or when human erythrocytes were attacked with homologous serum, MIP was deposited on the cell surface (Figs. 1 and 2). Thus, soluble MIP, though not able to spontaneously incorporate into membranes, is deposited on membranes during complement attack on heterologous or homologous target cells. Deposited MIP was detected on the cell surface by antibody much more efficiently than the

membrane protein, suggesting that it was present in a different conformation. The electron-microscopic studies also provided evidence of the stage of MAC assembly at which MIP binds. Deposition of MIP was not affected by the removal of C9 but was greatly inhibited in the absence of C8, suggesting that plasma MIP binding requires the C5b-8 complex. These data support the findings of Hansch and co-workers (Schonermark *et al.*, 1988), who have shown that C8bp (MIP/HRF) isolated from membranes binds to C8 in the MAC. Uptake of plasma MIP mediated by the MAC may render the cell less susceptible to subsequent attack, providing a mechanistic explanation for the intriguing findings of Houle *et al.* (1988), who showed that attack by homologous complement rendered cells resistant to lysis even by heterologous complement during a second attack.

In an attempt to demonstrate a functional role for fluid-phase MIP we depleted human serum of MIP. This resulted in enhancement of the lytic capacity of the serum on heterologous cells and restoration of MIP decreased lysis, providing evidence that fluid-phase MIP plays a role in the modulation of lysis by the MAC (Fig. 5). The concentration of MIP necessary to decrease the lytic activity of MIP-depleted serum back to the pre-depletion levels was much higher than the measured serum concentration, implying that functional activity was lost during the purification.

The source of soluble MIP is as yet uncertain, though the close similarities with the membrane protein suggest a common origin. Cells bearing MIP may constitutively secrete the protein as well as incorporating it into their membranes, or alternatively, soluble MIP may be derived from the membrane form, perhaps during the process of cell ageing. MIP, like DAF, has been shown to be linked to the membrane via a glycolipid membrane anchor (Hansch *et al.*, 1988), which may be slowly cleaved by endogenous phospholipases, releasing the protein. Although by gel electrophoresis the two proteins appeared to be of identical molecular masses, the possibility of cleavage close to or at the membrane-binding region remains.

The mechanism of action of soluble MIP remains uncertain. Our evidence suggests that it becomes associated with the membrane during MAC assembly, and may then function in a similar way to the membrane-associated form, interfering with C9 polymerization and thereby inhibiting MAC activity. Its biological importance also remains to be established. MIP is present in plasma and, intriguingly, in cerebrospinal fluid in relatively high concentrations. It may therefore play a part in the modulation of the effects of complement activation on cells exposed to these fluids.

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