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Erythrocytes of patients with paroxysmal nocturnal haemoglobinuria acquire resistance to complement attack by purified 20-kD homologous restriction factor

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SUMMARY

A 20-kD homologous restriction factor (HRF20) which is a membrane inhibitor of the terminal stage of human complement action can be detected by the monoclonal antibody 1F5, and is deficient on abnormal erythrocytes as well as leucocytes from patients with paroxysmal nocturnal haemoglobinuria (PNH). The erythrocytes of PNH patients significantly improved their resistance to homologous complement after adsorption of purified HRF20.

Keywords homologous restriction factor complement paroxysmal nocturnal haemoglobinuria haemolysis

INTRODUCTION

We have produced a monoclonal antibody (MoAb) 1F5 which sensitizes neuraminidase-treated human erythrocytes (HuE) to haemolysis by homologous complement via the alternative pathway (Okada *et al.*, 1987). The antigen (1F5 antigen) turned out to be a glycoprotein with M_r of 20 kD anchored to the membrane via phosphatidylinositol, which inhibits the terminal step of homologous complement attack (Okada *et al.*, 1989a, 1989b). The function of 1F5 antigen (1F5Ag) resembles that of homologous restriction factor (HRF) (Zalman, Wood & Müller-Eberhard, 1986) and C8 binding protein (C8bp) (Schonermark *et al.*, 1986). However, 1F5Ag is distinct from HRF and C8bp since the MoAb to 1F5Ag does not react in Western blots with any membrane proteins in the M_r range of 60–70 kD where HRF and C8bp migrate. Therefore, we designate 1F5Ag as HRF20 (20 kD HRF) (Okada *et al.*, 1989b). Its characteristics indicate that HRF20 is the same molecule as P18 reported by Sugita, Nakano & Tomita (1988). HRF and decay-accelerating factor (DAF) (Hoffman, 1969; Nicholson-Weller *et al.*, 1982; Medof, Kinoshita & Nussenzweig, 1984) have been shown to be deficient on abnormal erythrocytes of patients with paroxysmal nocturnal haemoglobinuria (PNH) causing the erythrocytes to be sensitive to homologous complement (Pangburn, Schreiber & Müller-Eberhard, 1983a; Pangburn *et al.*, 1983b; Nicholson-Weller *et al.*, 1983; Zalman *et al.*, 1987). We report here that not only is HRF20 deficient on cells of PNH patients but that adding purified HRF20 to these cells improves their resistance to homologous complement attack.

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MATERIALS AND METHODS

Diluents and buffers

Gelatin veronal-buffered saline (GVB) consisting of 0·1% gelatin (Difco laboratories, Detroit, MI), 10 mM veronal buffer (pH 7·4), and 148 mM NaCl was used as a standard solution for the complement reaction. Mg-EGTA-GVB (pH 7·4) was prepared by mixing 9 vol of GVB with 1 vol of a solution consisting of 20 mM $MgCl_2$ and 100 mM EGTA. EDTA-GVB (pH 7·4) consisted of a mixture of 6 vol of GVB and 4 vol of 100 mM EDTA. Glucose GVB (glGVB) was a mixture of GVB and an equal vol of 5% glucose. PBS consisted of 10 mM phosphate buffer (pH 7·4) and physiological saline. TPE-buffer was 10 mM Tris-HCl (pH 7·5) containing 1 mM PMSF and 1 mM EDTA.

Reagents

CHAPS (3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propanesulfonate) and SDS were purchased from Pierce Chemical Co. (Rockford, IL).

Cells

Blood was obtained from two PNH patients who exhibited a positive sugar-water lysis test (Hartmann & Jenkins, 1966). For controls, heparinized blood samples were obtained from healthy donors. Each fresh heparinized blood sample was separated by centrifugation on Ficol–Paque (Pharmacia Fine Chemicals, Upsala, Sweden) to obtain erythrocytes and mononuclear leucocytes (MNL) fractions. The cells were washed with PBS.

MoAbs

MoAb 1F5, which was obtained as described previously (Okada *et al.*, 1987, 1989a, 1989b), is directed to HRF20. A MoAb to

DAF, 1C6 (Fujita *et al.*, 1987), was kindly supplied by Dr T. Fujita (Tsukuba University School of Medicine, Tsukuba). MoAbs were purified from culture supernatants with protein A–Sepharose (Pharmacia Fine Chemicals) by the method described by Ey, Prowse & Jenkin (1978). Before use, MoAb was centrifuged at 10000 *g* for 10 min to remove any insoluble precipitate.

Western blot analysis

Washed HuE from PNH patients and from healthy individuals were ruptured in TPE-buffer and the stroma precipitated by centrifugation at 40000 *g*. These precipitated stroma were solubilized by boiling in 1% SDS and subjected to SDS–PAGE on a 12·5% gel followed by transfer to a Millipore membrane. Staining of HRF20 was with peroxidase-labelled anti-mouse immunoglobulin following treatment with 1F5.

Purification of HRF20

HRF20 was successfully purified from the butanol extract of HuE stroma using 1F5-coupled Sepharose as described previously (Okada *et al.*, 1989c). HRF20 was specifically bound to the immunosorbent column and was eluted with 0·1 M glycine-HCl (pH 3·0) containing 0·02% CHAPS. This purified HRF20 produced a single band around 20 kD on SDS–PAGE following staining with Coomasie blue.

FACS analysis of cell surface HRF20

Pellets containing 10^6 cells were resuspended on ice in 10 μl of normal sheep IgG (10 mg/ml) and allowed to settle for 30 min before addition of 20 μl of the MoAb (50 μg/ml) to be analysed. After incubation for 30 min on ice, the cells were subsequently washed with ice-cold PBS. The pellets were resuspended in 20 μl of 1/10 diluted FITC-labelled affinity-purified goat anti-mouse IgG (Cooper Biomedical, Malvern, PA) and further incubated for 30 min on ice. The cells were washed and resuspended in 1 ml of sheath solution for FACS analysis (Fujisawa Pharmaceutical Co., Osaka, Japan). Flow cytometric analysis was peformed with a FACS analyser (Becton Dickinson, Mountain View, CA). Data were stored in list mode and analysed using the Becton Dickinson Consort 30 FACS data analysis software package.

Acid serum (Ham) test and sucrose haemolysis test

The acid serum test (Dacie & Richardson, 1943; Logue, Rosse & Adams, 1973) and sucrose test (Hartmann & Jenkins, 1966) was performed as follows: for the Ham test, 50 μl of a 50% suspension of erythrocytes in PBS were incubated with 500 μl fresh human serum (HuS) containing 50 μl of 0·2 N HCl for 1 h at 37°C; for the sucrose test, 100 μl of a 50% suspension of erythrocytes were incubated with 850 μl 0·27 M sucrose solution containing 50 μl of HuS for 30 min at room temperature. After centrifugation, released haemoglobin in the supernatants was determined by absorbance at 414 nm to calculate percentage of lysis. Unlysed erythrocytes were pelleted and washed twice with PBS before determination of HRF20 on the cells by indirect immunofluorescence using the FACS analyser as described above.

PAGE and Western blotting

SDS–PAGE was performed according to the method of Laemmli (1970). Stacking gels of 2·5% and running gels of 12·5% were used. Apparent molecular weights were estimated by comparison with standards (Bethesda Research Lab., Rockville, MD). Western blotting was performed on an Immobilon-P membrane (Millipore, Bedford, MA) following electrophoretic transfer. HRF20 was enzymatically stained using 4-chloro-1-naphthol in 20 mM Tris-HCl (pH 7·4) following sequential treatment of the membrane with 1F5 (20 μg/ml) and peroxidase-conjugated goat anti-mouse IgG.

Adsorption of purified HRF20 to PNH-erythrocytes (PNH-E)

To 500 μl of a 50% suspension of PNH-E in PBS, 50 μl of purified HRF20 (100 μg/ml) were added and the mixtures incubated at 37°C for 1 h. After several washings, the amount of HRF20 adsorbed to PNH-E was determined by FACS analysis following indirect immunofluorescence staining with 1F5 as described above. After treatment with HRF20, the fluorescence intensity of the PNH-E increased by 19·5% that of normal erythrocytes. The number of HRF20 molecules on normal erythrocytes is about 3×10^4/cell as determined by Scatchard analysis using ^{125}I-labelled IF5 which is a MoAb to HRF20. Therefore, about 6×10^3 molecules/cell would have been adsorbed to the PNH-E. The HRF20 adsorbed PNH-E (PNH-E-HRF20) were compared with native PNH-E and normal erythrocytes for their sensitivity to haemolysis by complement in the sucrose test as follows: aliquots (25 μl) of a 50% suspension of erythrocytes in PBS were mixed with 400 μl of 0·27 M sucrose and 25 μl of human serum at 37°C, and the mixtures were incubated for 2, 10, 30, 60 and 120 min before separation of supernatants by centrifugation to determine the haemolytic extent spectrophotometrically.

RESULTS

Decrease in HRF20 on erythrocytes and MNL from PHN patients

By indirect immunofluorescence with FITC-conjugated anti-mouse immunoglobulin following treatment with 1F5, HRF20 was detected on essentially all erythrocytes from normal individuals as shown in Fig. 1a. An appreciable proportion of erythrocytes from PNH patients (PNH-E) were negative in their reactivity with 1F5 as shown in Fig. 1a. Peripheral MNL from PNH patients also contained significant amounts of HRF20 negative cells as shown in Fig. 1b. DAF was clearly lower on MNL of PNH patients; however, although diminished levels of DAF on PNH-E could be discerned, the sensitivity of the FACS analyser only allowed us to observe a slight tendency for this to be so (Fig. 1a).

Western blot analysis of HRF20 from PNH-E

Western blot analysis of HuE from a PNH patient and a healthy individual are shown in Fig. 2. The extent of HRF20 staining observed with PNH-E stroma was appreciably weaker than that of normal erythrocytes (Fig. 2, lane 2). Pretreatment with 1F5-Sepharose removed the HRF20 band (lane 3). On duplicate gels not used for Western blotting, the intensity of staining with Coomasie blue was essentially the same in all lanes (data not shown).

Haemolysis by acidified serum and sucrose serum

Abnormal erythrocytes of PNH patients can be haemolysed by acidified serum (Dacie & Richardson, 1943; Logue *et al.*, 1973)

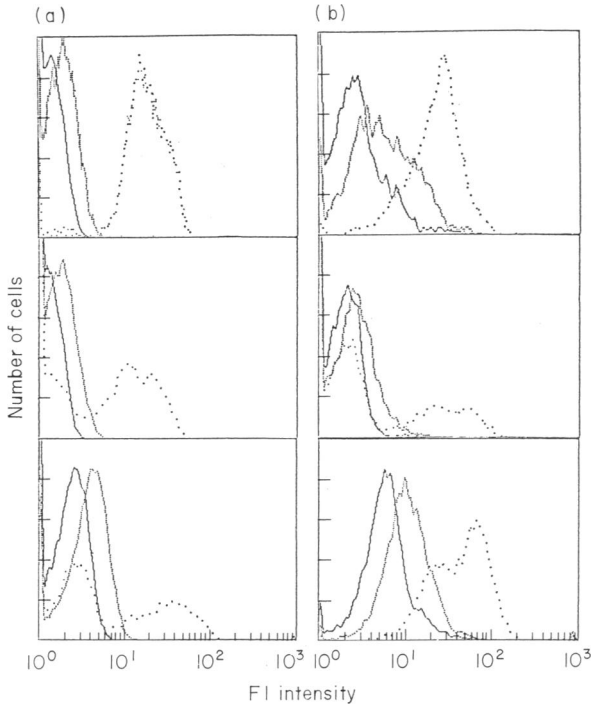

Fig. 1. Flow cytometric analysis of cell surface HRF20 detected by MoAb 1F5 (. . . .) and DAF detected by MoAb 1C6 (.....). (——) indicates negative control performed with IgG1 MoAb directed to a guinea pig membrane protein. (a) Analysis on erythrocytes; (b) on peripheral mononuclear leucocytes. Erythrocytes and MNL from a healthy individual (top) and two PNH patients (middle and bottom) were analysed.

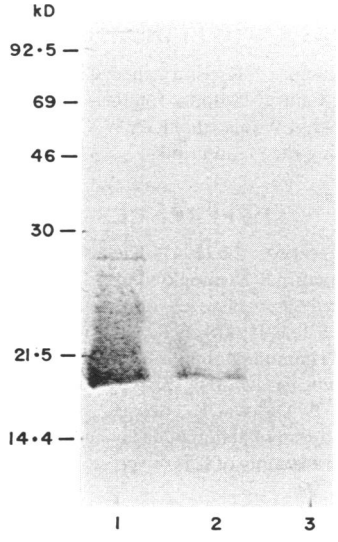

Fig. 2. Western blot analysis of membrane proteins from normal erythrocytes (lane 1), PNH-E from two PNH patients (lane 2), and that from PNH-E after passing through a 1F5-coupled Sepharose column to remove HRF20 (lane 3); 10 μg of solubilized membrane proteins were applied to each lane.

as well as sucrose serum (Hartmann & Jenkins, 1966). After haemolysis of PNH-E by acidified serum and sucrose serum, the residual erythrocytes were stained with FITC-conjugated anti-mouse immunoglobulin following treatment with 1F5. As shown in Fig. 3, after haemolysis by acidified serum as well as sucrose serum, HRF20-negative cells were eliminated.

Development of PNH-E haemolysis resistance by purified HRF20

PNH-E adsorbed with HRF20 (PNH-E-HRF20) prepared as described in Materials and Methods were incubated with HuS in the presence of sucrose at 37°C for 2, 10, 30, 60 and 120 min. As shown in Fig. 4, HRF20 conferred resistance to haemolysis by homologous serum at the initial stage of incubation although prolonged incubation overcame this resistance.

DISCUSSION

In the absence of specific antibodies, an individual has to provide some means of eliminating invading organisms in order to survive. As the alternative complement pathway is continuously active (Pangburn, Schreiber & Müller-Eberhard, 1981), it readily reacts with invaders even in the absence of specific antibody (Schreiber *et al.*, 1979). However, initially the complement system must distinguish the invaders as non-self. For this purpose, several membrane molecules act as passwords communicating with complement molecules so as to prevent any reaction on homologous cell membranes (Okada, Tanaka & Okada, 1983). Since password molecules are absent on non-self invaders, the complement system can react on these without restriction.

Recently, we were successful in producing a MoAb, 1F5, which reacts with a novel membrane inhibitor, 1F5Ag (Okada *et al.*, 1987, 1989a, 1989b); 1F5Ag, also referred to as HRF20 kD HRF, inhibits the terminal steps (C8 and C9) of complement attack as does HRF (Zalman *et al.*, 1986) or C8bp (Schonermark *et al.*, 1986); however, it is distinct in having a M_r of 20 kD while those of C8bp and HRF are about 65 kD. Since 1F5 does not produce any band around 60–70 kD by Western blotting analysis of freshly solubilized HuE membranes, HRF20 is not a fragment of HRF or C8bp. Therefore, HRF20 is a novel membrane molecule which can be regarded as a password molecule for prevention of complement attack on homologous cells.

If such password molecules happen to be absent or impaired on cell membranes, the complement system is capable of reacting on homologous cells. This is the case with erythrocytes of PNH patients. PNH-E have been demonstrated to be deficient in DAF (Nicholson-Weller *et al.*, 1983) as well as HRF (Zalman *et al.*, 1987).

In a preliminary experiment, 1F5Ag (HRF20) was shown to be deficient on PNH-E as briefly reported previously (Okada *et al.*, 1989a). We undertook to determine whether HRF20 is also deficient on cell membranes of PNH patients. As expected, HRF20 has been found to be deficient on about 50% of erythrocytes as well as peripheral blood mononuclear cells from PNH patients tested (Fig. 1). On Western blot analysis with 1F5, the HRF20 band at a M_r of 20 kD was clearly less intense in PNH-E compared with normal erythrocytes (Fig. 2). To establish that the absence of HRF20 on PNH-E actually contributes to the susceptibility to hemolysis by homologous

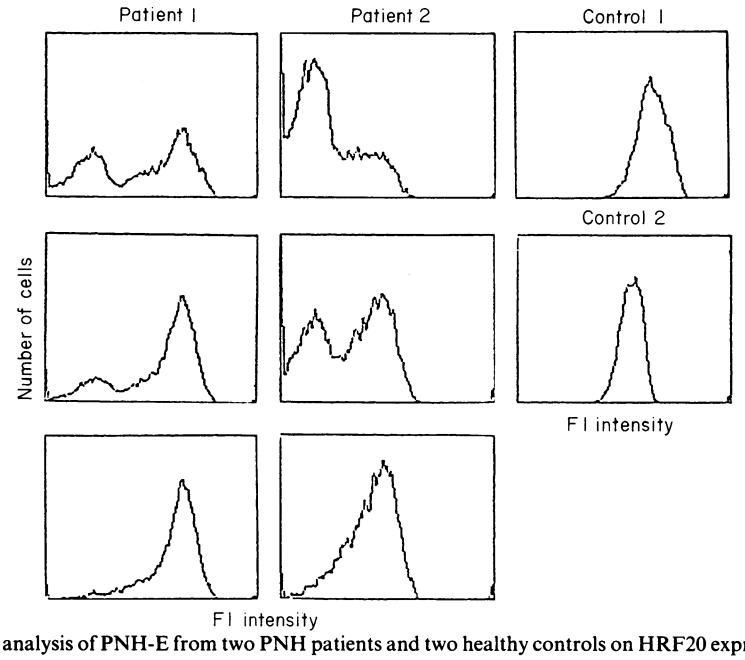

Fig. 3. Flow cytometric analysis of PNH-E from two PNH patients and two healthy controls on HRF20 expression detected by MoAb, IF5. Original erythrocyte population (top), erythrocytes remaining unlysed after the acid test (middle) and after the sucrose test (bottom) were analysed on the PNH patients.

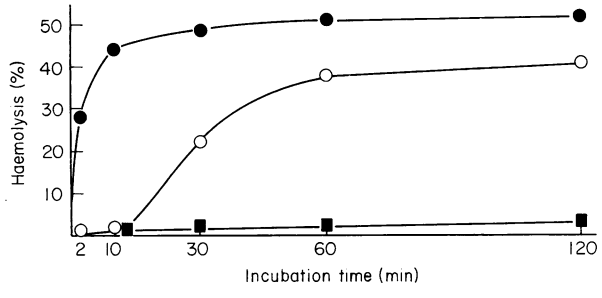

Fig. 4. Acquisition of resistance to homologous complement in the sucrose test by adsorption of HRF20 to PNH-E. Native PNH-E (●), PNH-E treated with HRF20 (○) as well as normal erythrocytes (■) were incubated at 37°C with human serum in the presence of sucrose solution. Among the PNH-E, about 50% were sensitive to the sucrose test.

complement, the amount of HRF20 on PNH-E was determined before and after haemolysis by homologous serum using the standard Ham test (Dacie & Richardson, 1943) and sucrose test (Hartmann & Jenkins, 1966). Most erythrocytes surviving haemolysis by homologous serum were HRF20 positive, as shown in Fig. 3. This indicates that erythrocytes haemolysed in the Ham test and the sucrose test were HRF20 negative and that HRF20-positive erythrocytes were resistant.

To confirm that deficiency of HRF20 on PNH-E contributes to the abnormal sensitivity to haemolysis by homologous complement, PNH-E were incubated with purified HRF20. After this treatment, the abnormal erythrocytes which were HRF20 negative acquired about 20% of the HRF20 found on normal erythrocytes on flow cytometric analysis using MoAb 1F5. Since HRF20-treated PNH-E acquired appreciable resistance to haemolysis by sucrose tests (Fig. 4), deficiency of HRF20 on a portion of PNH-E would account for the increased sensitivity to homologous complement although the cells were still rather sensitive to complement after prolonged incubation. This could result from significant complement activation which might have overcome the inhibitory capacity of added HRF20 due to their deficiency in DAF. Therefore, haemolysis may be suppressed completely by increasing the amount of HRF20 for adsorption to the cells. Recently, Holguin *et al.*, (1989) independently demonstrated inhibition of haemolysis of PNH-E by an 18-kD membrane inhibitor which seems to be identical to HRF20. These phenomena suggest that PNH patients may be treated by administration of purified HRF20 as well as DAF to provide resistance to homologous complement. Since we have cloned the cDNA for HRF20 (Okada *et al.*, 1989c), the possibility of using recombinant HRF20 may also be envisioned.

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