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COMPLETE DEFICIENCY OF 20 KDa HOMOLOGOUS RESTRICTION FACTOR (HRF20) AND RESTORATION WITH PURIFIED HRF20

Noriko Okada*, Ryoko Harada*, Ryo Taguchi$ and Hidechika Okada#

** Department of Microbiology, Fukuoka University school of Medicine, Nanakuma, Fukuoka 814-01, Japan*

$ Department of Microbial Chemistry, Nagoya City University School of Pharmacy, Tanabedori, Nagoya 467, Japan

 # Department of Molecular Biology, Nagoya City University School of Medicine, Mizuho-cho, Nagoya 467, Japan

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SUMMARY: **20 KDa homologous restriction factor (HRF20) is a membrane glycoprotein which inhibits formation of membrane attack complexes of homologous complement. Erythrocytes from a patient who is completely deficient in HRF20 were readily hemolyzed by homologous complement activated by sucrose or by acidification as in paroxysmal nocturnal hemoglobinuria (PNH). After incubating PNH erythrocytes (PNH-E) with purified HRF20, the cells were analyzed by flow cytometry using a monoclonal antibody to HRF20 and shown to have the antigen absorbed. These PNH-E acquired resistance to hemolysis by homologous complement suggesting that HRF20 may be successfully used for treatment of these patients.**

For complement to distinguish autologous cells from invading organisms, membrane molecules serve to identify host cell membranes as "self" so as to prevent any adverse reaction on host cells (1,2). Therefore, complement can act without restriction only on non-self particles that lack self-identifying molecules.

We produced a monoclonal antibody (mAb), 1F5, which reacts with a novel membrane inhibitor of complement, 1F5 antigen (3). 1F5 antigen proved to be a 20 KDa membrane glycoprotein bound to cell membranes via a phosphatidylinositol anchor and to be an inhibitor of the terminal step of homologous complement attack (4,5). We now call this antigen HRF20 which stands for 20 KDa homologous restriction factor (5) because its inhibitory capacity

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is similar to that of homologous restriction factor (HRF) reported by others (6) although its molecular size is much smaller. HRF20 is now considered to be the same membrane molecule reported independently by Sugita et al as P18 (7). Furthermore, the base sequence of cDNA coding HRF20 (8) coincides with that of CD59 to be reported by Davies et al (9).

HRF20 as well as decay accelerating factor (DAF) (10-15) can be regarded as regulatory molecules that communicate with complement so as to prevent any adverse effects on host cell membranes. If such complement regulatory molecules are absent or impaired, complement action is unhindered on such deficient membranes. This is the case with erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH) which have been demonstrated to be deficient in DAF (13) and HRF (14) as well as HRF20 (3-5). Recently we obtained erythrocytes from a PNH patient which are completely deficient in HRF20 but not in DAF (15). We established that addition of purified HRF20 to these erythrocytes can restore resistance to homologous complement.

MATERIALS AND METHODS

Diluents and reagents: Phosphate buffered saline (PBS) consisted of 10 mM phosphate buffer (pH 7.4) and physiological saline. Sodium dodesylsulfate (SDS) and 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propanesul-fonate (CHAPS) were purchased from Pierce Chemical Co. (Rockford, Illinois).

Cells: Blood was obtained from a PNH patient (K.N.; male, 21 y.o.) who exhibited a positive sugar-water lysis test (16). 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 without lymphocytes. The erythrocytes were washed with PBS.

Monoclonal antibodies (mAbs): 1F5 has been produced as a mAb which sensitizes human erythrocytes to hemolysis by homologous human complement and has been demonstrated to neutralize the regulatory function of HRF20 on formation of membrane attack complexes of homologous complement (3-5).

Purified HRF20: HRF20 was purified from the butanol extract of erythrocyte stroma using 1F5 coupled Sepharose as described previously (4). 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 KDa on SDS-PAGE following staining with Coomasie blue. The N-terminal partial amino acid sequence was Leu-Gln-Cys-Tyr-Asn-Cys-Pro-Asn-Pro-Thr-Ala-Asp-Cys-Lys-Thr-Ala-Val-X-X-Ser-Ser-Asp-Phe-Asp-Ala-X-Leu-X-Thr-Lys-Ala-Gly-X-Gln-Val-Tyr-Asn-Lys- as reported previously (4).

Flow cytometric analysis of cell surface HRF20: About 10^6 cells were resuspended on ice in 10 µl of normal sheep IgG (10 mg/ml) before addition of 20 µl of the mAb (50 µg/ml) to be analyzed. 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-labeled affinity purified goat anti-mouse IgG (Cooper Biomedical Inc. Malvern, PA) and incubated for 30 additional min on ice. The cells were washed and resuspended in 1 ml of sheath solution for FACS analysis (Fujisawa Pharm. Co., Osaka). Flow cytometric analysis was performed with a FACS analyzer (Becton Dickinson, Mountain View, CA).

Acid serum (Ham) test and sucrose hemolysis test: The sucrose test (16) and acid serum test (17,18) were performed as follows. For the acid serum test (Ham test), 50 µl of a 50% suspension of erythrocytes in PBS were incubated with 500 µl fresh human serum 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 fresh human serum for 30 min at room temperature. After centrifugation, released hemoglobin in the supernatants was determined by absorbance at 414 nm to calculate % lysis. Unlysed erythrocytes were pelleted and washed twice with PBS before determination of HRF20 on the cells by indirect immunofluorescence using the FACS analyzer as described above.

Treatment of PNH-E with purified HRF20: 10 µl of purified HRF20 at varying concentrations (100, 10, 1 and 0 µg/ml) was added to 500 µl of 50% suspension of PNH-E in PBS and the mixtures were incubated at 37°C for 1 h. After incubation, the treated erythrocytes were washed 3 times with PBS. The amount of HRF20 adsorbed to PNH-E was determined by flow cytometric analysis with 1F5 which is a mAb to HRF20. The HRF20 adsorbed PNH-E (PNH-E-HRF20) were compared with native PNH-E and normal erythrocytes for their sensitivity to hemolysis 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 10, 30, and 60 min before separation of supernatants by centrifugation to determine the hemolytic extent spectrophotometrically.

RESULTS

Adsorption of HRF20 to PNH-E: 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 at 2 µg/ml (final concentration), the

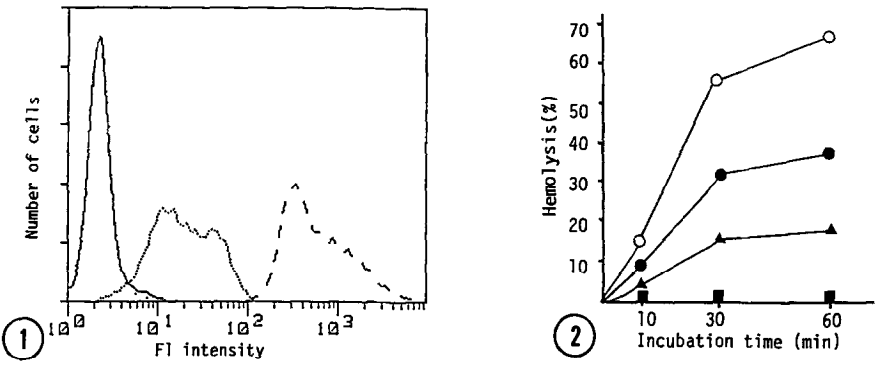

Fig. 1. Flow cytometric analysis of cell surface HRF20 detected by mAb 1F5 followed by staining with FITC-anti mouse IgG. The solid line (————) indicates PNH-E, the dotted line (·····) indicates PNH-E treated with 2 ug/ml of HRF20, and the broken line (— — —) indicates normal erythrocytes. Negative control of PNH-E stained without the first antibody is indicated by the sporadic dots (· · · · ·) which almost coincide with the solid line.

Fig. 2. Acquisition of resistance to homologous complement by adsorption of HRF20 to PNH-E. Native PNH-E (○), PNH-E treated with HRF20 at 2 μg/ml(■), 0.2 μg/ml (▲) and 0.02 μg/ml (●) were incubated at 37°C with human serum in the presence of sucrose solution.

fluorescence intensity of the PNH-E, which had been negative, rose to 4% of that of normal erythrocytes (Fig. 1).

PNH-E acquisition of resistance to homologous complement by adsorption of purified HRF20: PNH-E treated with HRF20 (PNH-E-HRF20) as described were incubated with human serum in the presence of sucrose at 37°C for 10, 30 and 60 min. As shown in Fig. 2, HRF20 conferred resistance to hemolysis by homologous serum in a dose dependent manner.

DISCUSSION

The alternative complement pathway is able to react with microorganisms in the absence of specific antibodies (19). However, the complement system must be able to recognize the invaders as non-self. To discriminate between self and non-self, host membrane molecules serve to prevent complement from acting on homologous cell membranes (2). If these regulatory molecules are absent on non-self invaders, the complement system can act on these without restriction.

Erythrocytes of PNH patients (PNH-E) have been demonstrated to be deficient in complement regulatory membrane proteins such as DAF (13), HRF (14) and HRF20 (3-5).

Recently, we encountered a PNH patient whose erythrocytes are completely deficient in HRF20 but not DAF (15). In spite of the presence of DAF, these cells showed abnormal sensitivity to complement attack. When these PNH-E were incubated with purified HRF20, HRF20 was efficiently adsorbed. By incubation of a 50% suspension of PNH-E in 2 ug/ml of HRF20, the cells acquired 4% of the amount of HRF20 found on normal erythrocytes as determined by flow cytometry (Fig. 1). Incubation of PNH-E in 0.2 μg/ml HRF20 gave about 1% of the HRF20 level found on normal erythrocytes.

The PNH-E treated with HRF20 acquired resistance to homologous complement as determined by the sucrose test (Fig. 2). To induce significant resistance to homologous complement, 2 μg/ml was required (Fig. 2). Holguin et al (20) also reported that an 18 KDa membrane protein from normal erythrocytes had the capacity to protect PNH-E from complement attack activated by cobra venom factor-Bb complex. This 18 KDa protein should be identical to HRF20.

Assuming that the total blood volume of an adult person is 6,000 ml, 12 mg HRF20 would theoretically be required to render whole blood cells of a HRF20 deficient patient resistant to homologous complement attack.

Recently we have cloned the cDNA for HRF20 (8), and the production of recombinant HRF20 for possible therapeutic use may be considered.

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