DEFICIENCY OF THE HOMOLOGOUS RESTRICTION FACTOR IN PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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The abnormal erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH) are deficient in decay-accelerating factor (DAF) (1, 2). DAF is a membrane-associated complement regulatory protein that accelerates the spontaneous decay of the classical (3) and the alternative C3 convertase (1) and inhibits assembly of these enzymes (4). The protein is a membrane constituent of normal human E (3), platelets, polymorphonuclear leukocytes, monocytes, lymphocytes (5, 6), and endothelial cells (7). DAF deficiency explains the markedly extended half-life of C3 convertase on the surface of PNH-E (1, 8) compared with normal cells and therefore, at least in part, the susceptibility of PNH-E to lysis in serum acidified to pH 6.4 (9). However, DAF deficiency does not account for the enhanced sensitivity of PNH-E to reactive lysis by C5b-9 (10, 11). Both C9 binding and C9 polymerization have been reported to be abnormally high on PNH-E (12).

Recently, we isolated (13) from human E a membrane protein called the homologous restriction factor (HRF) that can inhibit complement-mediated transmembrane channel expression, including the C5b-8, C5b-9, and tubular poly C9 channels (13). Antibody produced to isolated HRF caused a marked enhancement of reactive lysis of normal human E by C5b-9 and an increase in C9 uptake. Because anti-HRF treated normal human E resembled PNH-E in susceptibility to reactive lysis, the question arose as to whether PNH-E are deficient in HRF. Therefore, the E of two patients with PNH were examined and in both cases the affected E lacked the HRF protein.

Materials and Methods

Isolation of HRF. Hemoglobin-free E membranes (~200 mg protein) were dissolved in 2% deoxycholate (DOC), 50 mM NaCl, 2 mM PMSF, 1 mM benzamidine, 2 mM EDTA, 2 mM EGTA, 20 mM Tris-HCl, pH 8.4, at 4°C overnight in a total volume of 100 ml. The mixture was centrifuged at 12,000 g for 30 min to remove particulate matter and the supernatant was dialyzed against 0.02% DOC, 50 mM NaCl, 20 mM Tris-HCl, pH 8.4 (starting buffer). The material was applied to a Sepharose column containing 300
mg of bound anti-HRF Ig. The column was washed with five column volumes of starting buffer and HRF was eluted with 2 M NaCl in starting buffer and then with 2 M KBr. The final yield was ~850 μg of protein. To maintain the protein in solution it was necessary to have 0.02% of DOC in the buffer.

Separation of PNH-E from Normal E. The PNH-E population of patients TF and KM was enriched by treating the E with anti-DAF and guinea pig serum. 2 × 10⁹ E were incubated with 500 μl of rabbit anti-DAF for 30 min at 37°C, washed twice, and further incubated with 1 ml of fresh guinea pig serum for 10 min at 37°C. ~60% of the E of TF and 42% of KM were lysed under these conditions and the remaining cells were washed and used as PNH-E.

Functional Assay for Cell-Bound HRF. 10⁷ normal or 10⁷ abnormal PNH-E in 10 μl were incubated with various amounts of anti-HRF for 30 min at 4°C. The cells were washed twice with 0.01 M EDTA containing veronal buffer, pH 7.4, and were subjected to reactive lysis by isolated C5b6, C7, C8, and C9 as described (13).

Insertion of ¹²⁵I-HRF into PNH-E. Purified HRF was radiolabeled with ¹²⁵I to 2.8 × 10⁶ cpm/μg using the iodogen method (Pierce Chemical Co., Rockford, IL). 5 × 10⁷ PNH-E were incubated with varying amounts of HRF in 20 μl of 0.02% DOC or 20 μl of 0.02% DOC alone (control) in a total volume of 1.04 ml for 2 h at 37°C. The cells were washed twice and the amount of HRF incorporated was determined.

Results

Lack of HRF in Membranes of the Abnormal PNH-E. The blood of patient KM contained 58% and that of TF 40% abnormal E as determined by the acidified (pH 6.4) serum lysis test. The unlysed cells were considered normal. To enrich the abnormal cells, E of both individuals were treated with anti-DAF and guinea pig serum. This treatment lysed the DAF containing normal cells (42 and 60% for KM and TF, respectively) and left the DAF-deficient abnormal cells intact. The membranes of the enriched abnormal cells were dissolved and subjected to SDS-PAGE and immunoblotting (Fig. 1). Compared with normal human E, the abnormal cells clearly lacked the 65-kD protein detected by the anti-HRF.

Lack of Enhancement by Anti-HRF of Reactive Lysis of PNH-E. Anti-HRF inhib-
Anti-HRF activity on normal human E, allowing reactive lysis by C5b-9 to increase from 1.0 to 20%. No such effect was observed with the abnormal PNH-E (Fig. 2). Lysis in absence of anti-HRF was 14% and did not increase with antibody addition. It was not explored why the antiserum caused a reduction in lysis of PNH-E. The normal E population of KM obtained after lysis of the PNH cells in acidified serum was also tested and behaved like the normal E control shown in Fig. 2.

**Effect of Insertion of Exogenous HRF into the Membrane of PNH-E.** $^{125}$I-HRF isolated from normal E membranes in buffer containing 0.02% DOC was incubated with the enriched abnormal PNH-E of TF for 1 h at 37°C and 1 h at 30°C in the presence of DOC (final concentration, 0.0004%). The concentration of HRF ranged from 150 to 1,540 ng/ml and the cell concentration was $4.8 \times 10^7$/ml. The cells were washed and the cell-associated radioactivity determined. Uptake of $^{125}$I-HRF was 2-3% of input and the number of HRF molecules bound per cell ranged from 144 to 2,700. With increasing HRF incorporation, reactive lysis by C5b-9 decreased from 230% of the normal control to 110% (Fig. 3). Increasing the number of HRF molecules per cell from 1,080 to 2,700 did not further decrease the cell sensitivity to reactive lysis.

**Effect of Anti-HRF on the Lysis of Normal Human E by Acidified Human Serum.** Anti-HRF by itself did not render normal E susceptible to lysis by acidified serum. However, it did enhance the induction of lysis by anti-DAF in 10 mM EGTA containing acidified serum (not shown).

**Discussion**

DAF deficiency of the affected E in PNH explains in part their abnormal sensitivity to complement. Incorporation of exogenous DAF into the membranes of the affected PNH cells decreased their susceptibility to lysis in acidified serum (14). Treatment of normal human E with anti-DAF rendered these cells susceptible to lysis in acidified serum (14, 15). DAF was found normally expressed on
FIGURE 3. Inhibition of reactive lysis by isolated HRF inserted into abnormal PNH-E.

erythroid progenitors of PNH bone marrow and this expression was lost during differentiation in vitro (16). DAF was shown to be anchored in the cell membrane by a glycolipid involving phosphatidylinositol (17) and it is possible that DAF deficiency is due to an acquired defect in the attachment mechanism.

It has been established that PNH-E are also abnormally sensitive to reactive lysis by C5b-9 (10) and that this sensitivity is seen only when the C9 used is of human origin, but not when the C9 is from another species (18). This work suggested the existence of a regulatory mechanism on normal human E designed to inhibit the function of the membrane attack complex and related the postulated regulation to the phenomenon of homologous species restriction (19). The latter term refers to the observation that lysis by complement is inefficient when C8 and C9 are of the same species as the target cells. Since DAF lacks the ability to interfere with C8 or C9 action (11), human E membranes were explored for the presence of a protein that could interact with C8 or C9. A C8 binding protein (C8bp) was described (20) that functioned as an inhibitor in the lysis of chicken E bearing C5b-7 by human C8 and C9.

The HRF isolated previously (13) exhibits affinity for human C8 and C9 and is probably closely related to C8bp. Anti-HRF rendered normal human E PNH-like with respect to susceptibility to reactive lysis. The enhancing effect of anti-HRF in this system was seen only when C8 and C9 were of human origin. HRF has also been detected on the surface of polymorphonuclear leukocytes, Raji lymphoblastoid cells (13), as well as monocytes and lymphocytes.

The data presented above indicate that in the two cases studied, the abnormal PNH-E membranes lack the HRF protein and activity. This finding suggests that HRF deficiency constitutes an additional molecular defect in PNH. Since both of the other missing proteins, DAF (17) and acetylcholinesterase (21) are linked to the membrane of normal E by phosphatidylinositol, it has been suggested (17) that it is the biosynthetic pathway generating the anchoring mechanism that is defective in this disease. It is conceivable that HRF is also anchored through phosphatidylinositol.
Whereas up to 2,700 molecules of isolated $^{125}$I-HRF were inserted into the abnormal PNH cells, 1,000 molecules were sufficient to reduce the sensitivity of the cells to reactive lysis close to the normal level. Anti-HRF did not render normal human E susceptible to lysis by acidified serum. However, anti-HRF enhanced lysis induced by anti-DAF, further demonstrating the two distinct regulatory mechanisms of normal E, DAF functioning at the C3/C5 convertase stage and HRF at the stage of C8/C9 action.

Summary

The affected E of two patients with paroxysmal nocturnal hemoglobinuria (PNH) were enriched by lysing the unaffected, normal E with anti–human decay-accelerating factor (DAF) and guinea pig serum. The membranes of the unlysed, DAF-deficient cells (PNH-E) were dissolved and examined by SDS-PAGE and immunoblotting using an antiserum to homologous restriction factor (HRF). Whereas the 65 kD complement regulatory protein was readily detectable in the normal controls, it was completely lacking in both samples of PNH-E membranes. Functional studies likewise indicated the absence of HRF activity from PNH-E. When radiolabeled, isolated HRF protein was offered to PNH-E, it became firmly attached to the cells. 1,000 molecules of HRF per cell reduced the characteristic susceptibility of these cells to reactive lysis by C5b-9 to nearly normal levels. The results suggest that HRF, which is known to control the action of C8 and C9 on normal human E membranes, is deficient in PNH, as well as acetylcholinesterase and DAF.

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