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Immunology

Isolation of a human erythrocyte membrane protein capable of inhibiting expression of homologous complement transmembrane channels

[membrane attack complex/poly(C9)/homologous restriction factor/liposomes]

LEORA S. ZALMAN, LORRAINE M. WOOD, AND HANS J. MÜLLER-EBERHARD

Division of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037

Contributed by Hans J. Müller-Eberhard, May 27, 1986

ABSTRACT **Erythrocytes are poorly lysed by homologous complement, whereas they are readily lysed by heterologous complement. This phenomenon had been attributed to an interference by the cell surface with the action of complement components C8 and C9. To isolate the responsible membrane constituent, detergent-solubilized human erythrocyte (E_H) membranes were subjected to affinity chromatography by using human C9-Sepharose. The isolated protein had a mass of 38 kDa and, incorporated into liposomes, was highly effective in inhibiting complement-mediated channel expression, including the C5b-8, membrane attack complex, and tubular polymer of C9 channels. Antibody produced to the 38-kDa protein caused a 20-fold increase in reactive lysis of E_H by isolated C5b6, C7, C8, and C9. The antibody did not enhance C5b-7 uptake, but it affected C9 binding to the target cell membrane. Antibody to human decay-accelerating factor, used as a control, had no effect on reactive lysis of E_H. Anti-38-kDa protein did not enhance the action on E_H of C8 and C9 from other species, indicating that the action of this regulatory protein is species specific. It was therefore termed homologous restriction factor (HRF). Blood cells other than erythrocytes, such as polymorphonuclear leukocytes, also exhibited cell-surface HRF activity. In immunoblots of freshly isolated E_H membranes, anti-38-kDa HRF detected primarily a 65-kDa protein, suggesting that the 38-kDa protein constitutes an active fragment of membrane HRF. Because of the specific binding reaction observed between HRF and C8 or C9, HRF was tested with anti-human C8 and anti-human C9. A limited immunochemical relationship of HRF to C8 and C9 could be established and solid-phase anti-C9 proved an efficient tool for the isolation of HRF from solubilized E_H membranes.**

It is known that complement is much more efficient in lysing heterologous erythrocytes than it is in lysing homologous or autologous erythrocytes. The phenomenon has been referred to as homologous species restriction (1) of complement-mediated hemolysis and has been attributed variously to interference of an unknown erythrocyte membrane constituent with the action of complement component C9 (2), C8 and C9 (1), or C8 (3). Complement regulatory membrane proteins have been described and appear to fulfill the function of protecting host cells, particularly those of the blood, from accidental attack by autologous complement. For example, decay-accelerating factor (DAF) (4, 5) is a powerful regulator of cell-bound C3/C5 convertase (6, 7), which, however, does not control transmembrane channel formation by C8 and C9 (8). The homologous restriction factor (HRF) of human erythrocytes (E_H) thus constitutes a distinct membrane-associated regulator that appears to act directly on C8 and C9. A C8-binding protein of 65 kDa was recently purified from E_H and shown to inhibit the action of homologous C8 and C9 in complement-mediated hemolysis (3).

In this communication, work is described that led to the isolation of a protein from membranes of E_H that exhibits marked affinity for C9, readily incorporates into liposomes, and strongly inhibits the expression of homologous complement channels on erythrocytes and liposomes. It also occurs on peripheral blood leukocytes. Immunochemical analysis of the protein showed it to be antigenically related to C8 and C9. Part of this study has been presented in abstract form (9, 10).

MATERIALS AND METHODS

Materials. C5b6 (11), C7 (12), C8 (13), and C9 (14) were purified as described. C9 was polymerized by incubation with 1 mM Ca^{2+} or 50 μM Zn^{2+} for 1 hr at 37°C (15). DAF was isolated as described (6). Sera from various animals such as monkeys, sheep, rats, etc., were obtained from the Division of Animal Resources, Research Institute of Scripps Clinic. Polyacrylamide gel electrophoresis was performed according to Laemmli (16) using an acrylamide gradient of 1.7% to 17%.

Antisera. Anti-human DAF (6) and anti-human C9 (14) were raised in rabbits with purified proteins. Anti-human HRF was also produced in rabbits, but with gel pieces containing 38-kDa HRF that were excised from Coomassie blue-stained $NaDodSO_4$/polyacrylamide gels. A potent antiserum was obtained after injection of 3 × 5 μg of protein.

Purification of the 38-kDa Protein from E_H. Hemoglobin-free human erythrocyte membranes (≈86 mg of protein) were dissolved in 2% deoxycholate/20 mM Tris·HCl, pH 8.4, at 4°C overnight in a total vol of 100 ml. The mixture was centrifuged to remove particulate matter and the supernatant was dialyzed against 0.2% deoxycholate/20 mM Tris·HCl, pH 8.4 (starting buffer). The material was applied to a Sepharose column with 6 mg of isolated human C9 bound to it. The column was washed with starting buffer and then with 0.4 M NaCl in starting buffer. The active material was eluted with 1 M NaCl in starting buffer. The total yield was 90 μg of protein.

Liposome-Swelling Assay. The liposome-swelling assay was carried out as described (17, 18). HRF was incorporated into liposomes made of egg phosphatidylcholine and dicetyl phosphate by sonicating 2 μg of protein with 3 μmol of phospholipid and drying the mixture onto the bottom of a test tube. The liposomes were formed by resuspending the dried material in 0.6 ml of 13% dextran (average size, 71.2 kDa) solution. After 2 hr in a water bath at 37°C, C5b-9 was assembled onto the surface of the liposomes by sequential addition of 3 μg of C5b6, 2 μg of C7, 2 μg of C8, and 15 μg

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Abbreviations: E_H, human erythrocyte(s); HRF, homologous restriction factor; DAF, decay-accelerating factor; MAC, membrane attack complex; poly(C9), tubular polymer of C9; PMN, polymorphonuclear leukocytes.

of C9 and incubation at 37°C for another hour. To assemble poly(C9) (tubular polymer of C9) onto the liposomes, C9 and then 50 μM $ZnCl_2$ were added to the liposomes and the mixture was incubated for 1 hr at 37°C.

Functional Assay of Cell-Bound HRF. E_H (10^7) were incubated with either anti-HRF (25–100 μl), anti-DAF, or normal rabbit serum (all sera were heat-inactivated) for 30 min at 4°C. The cells were washed twice with 10 mM EDTA containing Veronal buffer (pH 7.4) and subjected to reactive lysis by adding human isolated C5b6 (3 μg), C7 (2 μg), C8 (2 μg), and C9 (15 μg) sequentially. The total reaction volume was 50 μl. After 1 hr at 37°C, the cells were suspended in 1 ml of buffer, centrifuged, and the amount of hemolysis was determined spectrophotometrically at 412 nm. Nucleated cells were treated similarly. The cells were first allowed to incorporate ^{51}Cr and the amount of ^{51}Cr release was determined as a measure of cell lysis.

DAF Assay. The DAF assay was performed according to a published method (6).

C7 and C9 Uptake Studies. E_H (10^7) were pretreated with either anti-HRF or anti-DAF as described above. To determine the amount of C7 binding, C5b6 and ^{125}I-labeled C7 were added to the cells. After 15 min at 37°C, the cells were layered onto 20% sucrose and centrifuged in a microfuge for 1 min. The bottom of the tube was cut off and the radioactivity was measured in both the cell pellet and the supernatant. For the C9 binding experiments, the pretreated cells were incubated with C5b6, C7, C8, and ^{125}I-labeled C9 for 15 min at 37°C and layered onto 20% sucrose as described above.

RESULTS

Isolation and Properties of the 38-kDa HRF from E_H Membranes. To date, three preparations have been obtained from different batches of pooled E_H. C9 rather than C8 affinity chromatography was used because exploratory experiments indicated that the former produced a much higher yield than the latter. For one of the preparations, the starting material consisted of 86 mg of protein of hemoglobin-free E_H membranes, which had been stored at −70°C for more than a year. The membranes were dissolved in 2% deoxycholate/20 mM Tris·HCl, pH 8.4, at 4°C overnight. The soluble material was dialyzed against 0.2% deoxycholate/20 mM Tris·HCl, pH 8.4 (starting buffer) and applied to a human C9-Sepharose column, which was then washed with starting buffer and 0.4 M NaCl. The C9-binding protein was eluted with 1 M NaCl in starting buffer. The total yield was 90 μg of protein. On $NaDodSO_4$/polyacrylamide gel electrophoresis, the protein appeared ≈95% homogeneous and to have a molecular mass of 38 kDa. By immunoblot analysis using a rabbit antiserum to the 38-kDa HRF, the 38-kDa band was strongly stained, but, in addition, a lightly stained band (≈65 kDa) was visualized that was not detected by protein stain (Fig. 1). The third HRF preparation, which was obtained from freshly prepared E_H membranes, consisted primarily of a 65-kDa component (Fig. 1). Furthermore, immunoblots of dissolved E_H membranes using the anti-38-kDa HRF detected a distinct band in the 65-kDa region (Fig. 1).

Incorporation of the 38-kDa HRF into Liposomes and Inhibition of Channel Formation by the Membrane Attack Complex (MAC). Several batches of liposomes were prepared from egg phosphatidylcholine containing different amounts of the first preparation of HRF. The liposomes were exposed to C5b6, C7, C8, and C9 to allow formation and insertion of MAC into the liposomes. The expression of MAC channel function was measured by the liposome-swelling assay. A direct correlation was found between the amount of HRF incorporated and the degree of inhibition of MAC channel formation (Fig. 2). The channel formation by C5b-8 or by polymerizing C9 was similarly inhibited. However, when preformed poly(C9) was incorporated together with HRF during the preparation of the liposomes, the poly(C9) channel was unimpaired. By using material of the second preparation

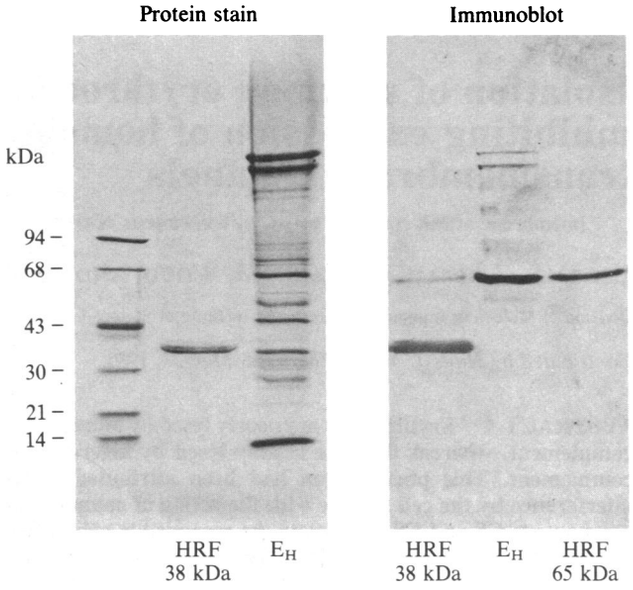

FIG. 1. Analysis of isolated and solubilized E_H membranes by $NaDodSO_4$/polyacrylamide gel electrophoresis and immunoblot using rabbit antiserum to the 38-kDa protein. The amount of protein applied was as follows: 38-kDa HRF, 6 μg; 65-kDa HRF, 4 μg; E_H membranes, 40–50 μg. The gel concentration gradient was 1.7% to 17%.

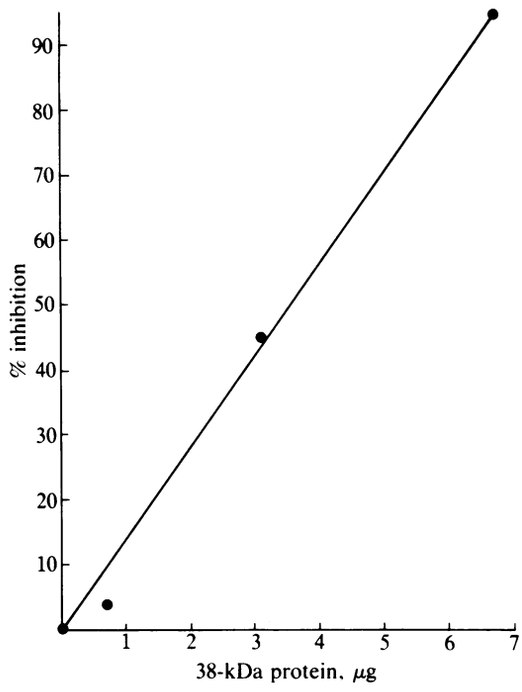

FIG. 2. Inhibition of MAC channel formation by the 38-kDa protein of E_H incorporated into liposomes. Various amounts of purified HRF were added to 3 μmol of dried lipid in a test tube and sonicated. The protein/lipid mixture was dried, 14% dextran was added, and the liposomes were allowed to form at 37°C for 2 hr. The liposomes reacted with 3 μg of C5b6, 2 μg of C7, 2 μg of C8, and 15 μg of C9 for 1 hr at 37°C. Portions of the liposomes were added to isotonic sugar solutions, and the initial rate of change in light scattering was measured. The rates of swelling with raffinose were compared with those of liposomes that contained no HRF.

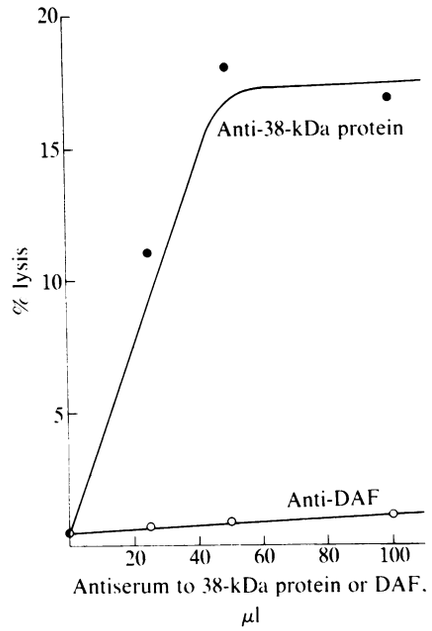

FIG. 3. Effect of anti-38-kDa protein on reactive lysis by C5b-9 of human erythrocytes. Number of cells was 10^7, time of incubation was 1 hr, and the amounts of protein were as follows: C5b6, 3 μg; C7, 2 μg; C8, 2 μg; C9, 15 μg. Control, E_H treated with anti-DAF.

of HRF, 90% inhibition of MAC channel function was observed with 2 μg of protein. Two different preparations of isolated human DAF had no HRF activity in the same assay system, and isolated HRF contained no DAF activity.

Inhibition of HRF Activity on E_H by Anti-HRF. E_H were pretreated with anti-38-kDa HRF in presence of 10 mM EDTA for 30 min at 4°C and then washed twice. Controls were pretreated with anti-DAF. The cells were then subjected to reactive lysis using C5b6, C7, C8, and C9. Anti-38-kDa HRF effected a dose-dependent increase in lysis, which at saturating amounts of antibody was 20-fold higher than the lysis observed in the controls (Fig. 3). These results show that the anti-HRF was capable of inhibiting HRF activity and that the HRF on E_H markedly suppressed the lytic efficiency of the MAC.

Effect of Anti-HRF on Uptake of Radiolabeled C7 and C9 by E_H Undergoing Reactive Lysis. That the enhancing effect of anti-HRF on reactive lysis of E_H was not due to an increase in C5b-7 uptake is shown in Fig. 4. The E_H cells were pretreated with 100 μl of anti-HRF or anti-DAF (Fig. 3) and

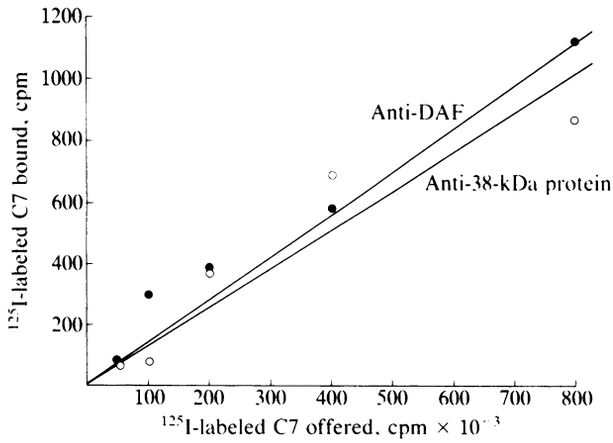

FIG. 4. Effect of anti-38-kDa protein on uptake of C5b-7 by E_H. Conditions are described in *Materials and Methods*. Control, E_H treated with anti-DAF.

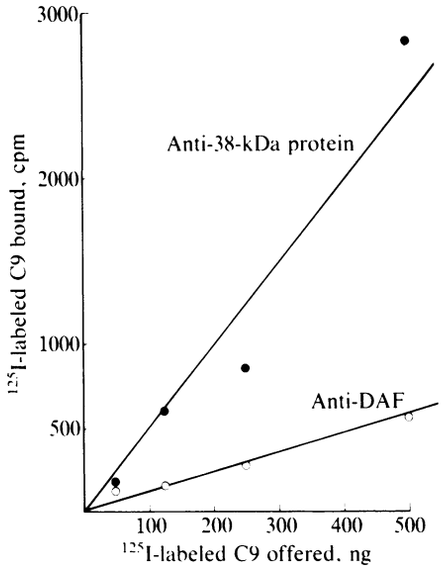

FIG. 5. Effect of anti-38-kDa protein on uptake of human ^{125}I-labeled C9 by E_H during reactive lysis by C5b-9. Conditions are described in *Materials and Methods*. Control, E_H treated with anti-DAF.

then subjected to treatment with C5b6 and ^{125}I-labeled C7. Uptake of C7 was comparable in both cases. However, the uptake of ^{125}I-labeled C9 was greatly enhanced during reactive lysis by pretreatment of the E_H with anti-HRF, but not with anti-DAF (Fig. 5). In this particular experiment, the enhancement was 5-fold.

The 38-kDa HRF Restricts Homologous but not Heterologous C8 and C9 Action. E_H were coated with anti-HRF or anti-DAF, and C5b-7 was deposited on the cells. As a source of C8 and C9, EDTA serum from different species was added and incubated for 1 hr at 37°C. As shown in Table 1, the hemolytic efficiency of human C8 and C9 was increased 20-fold by anti-HRF, whereas the C8 and C9 of none of the eight nonhuman species tested showed any increase.

Evidence for the Occurrence of HRF on Human Leukocytes. Polymorphonuclear leukocytes (PMN) were isolated from blood and tested for the possible presence of HRF in an activity assay analogous to that used for HRF on E_H. The PMN were labeled with ^{51}Cr, pretreated with anti-HRF or anti-DAF, and subjected to reactive lysis with C5b6, C7, C8, and C9. The anti-HRF caused a 9-fold increment in ^{51}Cr release compared to the control in which only 8% cell death occurred (Fig. 6). These results suggest that HRF is not only present on erythrocytes, but is a regular membrane constituent of other blood cells. The Raji lymphoblastoid cell line also exhibited HRF activity.

Immunochemical Relationship of HRF to C8 and C9. Dot blots revealed that isolated HRF reacts positively with

Table 1. Effect of anti-HRF on lysis of E_H C5b-7 by C8 and C9 from different species

Species of C8 and C9	% lysis with anti-HRF/ % lysis with anti-DAF
Human	20
Monkey	0.95
Sheep	1.1
Mouse	1.1
Donkey	0.85
Emu	0.80
Rabbit	1.0
Goat	1.0
Rat	1.0

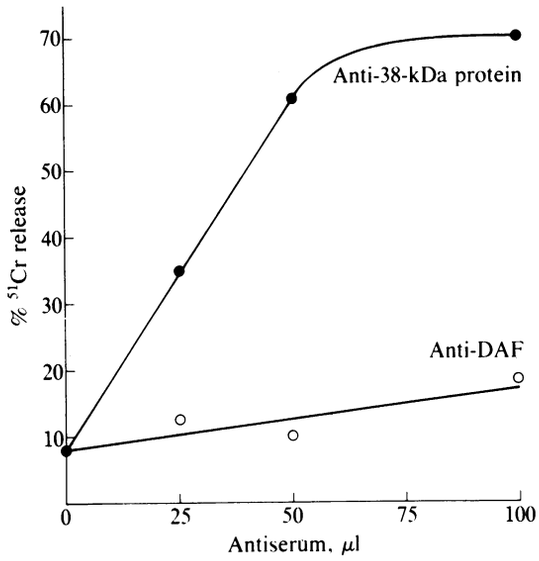

FIG. 6. Enhancement of reactive lysis by human C5b-9 of human PMN by anti-38-kDa protein. Human peripheral blood was separated on a Ficoll-Hypaque gradient. The PMN layer was removed and washed in phosphate-buffered saline (pH 7.4). ^{51}Cr-labeled PMN (1×10^5) were incubated with either anti-HRF or anti-DAF in presence of 2 mM EDTA for 30 min at 4°C. The cells were washed twice and treated with purified human C5b6 and C7 and then with human EDTA serum as a source of C8 and C9. Percentage ^{51}Cr release was measured after 1 hr at 37°C.

anti-C9 and anti-C8. Immunoblots of E_H membranes developed with anti-C9 showed weak staining of the band that is detected by anti-HRF. Ouchterlony tests in agarose gels containing 0.1% Triton X-100 gave a positive reaction of anti-HRF with C8. Anti-HRF (38 kDa) did detect C9 in Ouchterlony test but not by immunoblots. But Sepharose-anti-C9 selectively retained from solubilized E_H membranes the 65-kDa HRF as well as a 38-kDa and a 28-kDa component. These data suggest that HRF bears a limited structural relationship to C8 and to C9.

DISCUSSION

The 38-kDa protein was isolated from deoxycholate-dissolved membranes of E_H by using its affinity for C9. Although the protein may have a greater affinity for C8 than for C9, Sepharose-C9 was more efficient in isolating the protein than Sepharose-C8. Two preparations obtained from purified E_H membranes stored at −70°C for prolonged periods of time yielded highly active bifunctional 38-kDa protein. It was capable of incorporating into liposomes and as such it inhibited C5b-8, MAC and poly(C9) channel formation. Whereas the protein stain after $NaDodSO_4$/polyacrylamide gel electrophoresis of the isolated protein only revealed the 38-kDa protein, an immunoblot of the same gel using anti-38-kDa protein also showed the presence of a 65-kDa component (Fig. 1). A third preparation made from freshly obtained E_H membranes in the presence of protease inhibitors consisted primarily of a 65-kDa protein, which was also detected by the anti-38-kDa protein. In an immunoblot analysis of freshly prepared dissolved E_H membranes, this antibody recognized only a 65-kDa protein band, suggesting that HRF is present in E_H membranes primarily in this form of larger molecular mass and that the 38-kDa protein is a fully active cleavage product thereof.

HRF described here may therefore be related to the C8-binding protein (3), which was shown to have a molecular mass of 65 kDa on $NaDodSO_4$/polyacrylamide gel electrophoresis of E_H membranes. After isolation from papain-treated E_H membranes by phenol/water extraction and isoelectric focusing, the protein was shown to bind C8 and to inhibit lysis of C5b-7-bearing chicken erythrocytes by human C8 and C9. Lysis by rabbit C8 and C9 was not affected by the human C8-binding protein.

In the present study, it was demonstrated that anti-38-kDa protein inhibits HRF on normal E_H and that HRF exercises a powerful regulatory function on E_H with respect to the action of homologous C8 and C9, but not C8 and C9 of eight other species tested (Table 1). Enhancement of lysis of E_H by the antibody in the reactive lysis system using human C5b-9 was up to 20-fold (Fig. 3). Enhancement of lysis was due to increased C9 uptake and probably to greater functional efficiency of C8 and C9. The occurrence of HRF is not restricted to erythrocytes. HRF appears to be present also on PMN (Fig. 6) and Raji cells and may well be a normal surface constituent of mononuclear leukocytes, platelets, and endothelial cells.

HRF binds to both C8 and C9 and it is likely that it interferes with their function through this binding reaction. It may be relevant to the mechanism of action of HRF that C9 is capable of binding to C8α (19) and that both share antigenic determinants (20). Our preliminary data suggest that HRF shows an immunochemical cross-reaction with C8 and C9. Future studies might reveal that HRF also binds to and inhibits the cytotoxic C9-related protein of killing lymphocytes, which immunochemically cross-reacts with C9 (21, 22).

Although the deficiency of DAF on the abnormal E_H in paroxysmal nocturnal hemoglobinuria (6, 23) has explained their increased susceptibility to alternative pathway-mediated lysis in acidified serum (6, 24), it does not account for their enhanced sensitivity to reactive lysis of C5b-9 (25–27). In this system, the DAF-controlled C3/C5 convertase is not involved. Further studies have shown that C5b6, C7, and C8 binding to paroxysmal nocturnal hemoglobinuria cells is not higher than to normal E_H (26, 28). Rather, C9 binding and poly(C9) formation have been reported to be abnormally high on paroxysmal nocturnal hemoglobinuria cells (28). Since DAF exhibits no HRF activity, the question of whether HRF is also deficient in paroxysmal nocturnal hemoglobinuria needs pursuing.

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