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DIFFERENTIAL SUSCEPTIBILITY OF TYPE III ERYTHROCYTES OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA TO LYSIS MEDIATED BY COMPLEMENT AND PERFORIN

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Summary Previous reports have suggested that a 65 kDa membrane protein, termed homologous restriction factor (HRF), in addition to protecting erythrocytes (E) against lysis by homologous complement (C), may also be involved in protecting cytolytic lymphocytes against lysis mediated by a pore-forming protein (PFP/perforin), one of their own lytic mediators. Here, we used HRF-deficient type III E of patients with paroxysmal nocturnal hemoglobinuria (PNH) to study their susceptibility to lysis mediated by homologous C and perforin, and compared it with lysis of HRF-bearing control or PNH type I E. We show that type III E of PNH patients are indeed more susceptible to lysis mediated by homologous C than control or type I E, but they are as susceptible to perforin-mediated lysis as type I E. In addition, all human E (type I or III) tested here are equally susceptible to lysis mediated by either human (homologous) or murine (heterologous) perforin. By immunoblot analysis, we confirm that type III E, in contrast to type I E, were deficient in the 65 kDa HRF.
These results support the notion that homologous species restriction is seen in the C- but not in the lymphocyte perforin-system and argue against an active participation of HRF in protecting cells from perforin-mediated lysis.

Paroxysmal nocturnal hemoglobinuria (PNH) is a disease characterized by an abnormal sensitivity of the patient's erythrocytes (E) to lysis mediated by complement (C) (1,2). The E in PNH can be divided into types I, II and III, based on differences in their susceptibility to C, especially that of homologous species (3,4). Thus, type III E are most susceptible to C-mediated lysis while type I (normal) E are least sensitive; type II E have an intermediate sensitivity. It is now known that type III E lack a number of phosphatidylinositol-anchored surface proteins (5), such as acetylcholinesterase (AchE) (6), decay-accelerating factor (DAF) (7,8) and homologous restriction factor (HRF) or C8-binding protein (C8BP) (4,9). The latter, HRF/C8BP, is a 65 kDa protein species that has been shown to protect cells from reactive lysis by homologous C components (10-12), a phenomenon

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kown as homologous restriction (13,14). HRF/C8BP is thought to bind to C8 in the target bilayer, thereby interrupting the assembly of C8 and C9 into functional C lesions. This would explain the exquisite sensitivity of type III E to reactive lysis by C.

The two effector lymphocyte populations, cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, have previously been shown to produce a potent cytolytic pore-forming protein (PFP, perforin or cytolysin) stored in the cytoplasmic granules of these cell types (15-18). Like the terminal C components, purified PFP/perforin lyses cells non-specifically by forming ion channels in the target membranes. While PFP/perforin lyses a variety of target cells, it does not lyse CTL or NK cells (19-23), suggesting that a mechanism of self-protection spares cytolytic lymphocytes. Recently, it has been reported that the perforin pathway could be inhibited by both membrane-bound and soluble HRF/C8BP (24-26). In particular, PNH-E and sheep E were shown to be susceptible to large granular lymphocyte (LGL)- and perforin-mediated lysis but were made more resistant upon incorporation of HRF into E membrane (26). This is an exciting finding that converges the C- and perforin-protective pathways into a single shared mechanism. This protection model would be consistent with the structural and functional homologies shared by perforin and the terminal C components (27-32) and if correct, would predict that the HRF-deficient type III E of PNH patients ought to be more susceptible to perforin-mediated lysis than type I E. Here, we have tested this hypothesis using PNH E. Our results show that HRF-deficient type III E are indeed highly susceptible to homologous C-mediated lysis, yet they are as susceptible to perforin-mediated lysis as HRF-bearing type I E of the same patients or E of normal individuals. Thus, our results do not support a role for HRF in protecting cells against perforin-mediated lysis.

Materials and Methods

Complements and antisera. Rapidly processed and pooled guinea pig sera used as source of guinea pig C were purchased from Diamedix Co. (Miami, FL). Fresh human sera collected from healthy individuals were absorbed with normal human E at 4 °C for 2 h. The absorbed sera were kept at -20 °C until use as a source of human C.

The C components C5b-6, C7, C8 and C9 were obtained from outdated human plasma, as described (27,33). Gel profiles of these purified C components were similar to those presented elsewhere (33,34). Rabbit antiserum specific for C8 (boiled in 1% SDS) was prepared by multiple subcutaneous injections of 50 μg of purified C8.

Separation of human erythrocytes. Human blood was collected from 4 PNH patients and 4 healthy donors. E were processed as detailed elsewhere (6,35). Briefly, type I E of PNH patients were obtained as cells that resisted lysis in response to cobra venom factor (CVF) (6). E from healthy individuals were similarly treated with CVF as control. Type III E from PNH patients were separated using monoclonal antibodies specific for AChE (35). All E were stored under sterile conditions in Alsever's solution and used within 2 days of collection and separation.

Preparation of human perforin-containing extract. Human perforin was extracted from interleukin (IL-2)-stimulated human NK cells as previously

described (23). Briefly, human peripheral blood mononuclear cells (PBMC) from healthy donors were cultured with 50 Gy-irradiated RPMI 8866 cells and harvested on day 9. A homogeneous cell population of CD16(B73.1)$^+$/CD56(NKH1)$^+$/CD3(OKT3)$^-$/CD5(B36.1)$^-$ NK cells was isolated from these PBMC by an indirect rosetting technique with a mixture of anti-CD3 (OKT3), anti-CD5 (B36.1), anti-CD14 (B52.1) monoclonal antibodies (mAbs) and $CrCl_3$-treated goat anti-mouse Ig-coated E, followed by density gradient centrifugation (36,37). The NK cells purified by this negative selection procedure generally exceeded 95% purity as tested by indirect immunofluorescence with a panel of anti-NK and anti-T mAbs (36). After stimulation with rIL-2 (100 U per 2 x 10^6 cells/ml) for 4 days, the NK cells were collected, washed with PBS for 3 times, and resuspended (2 x 10^7/ml) in relaxation buffer (130 mM KCl, 5 mM NaCl, 1 mM disodium ATP, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM phenylmethylsufonyl fluoride, 10 mM Hepes, pH 6.8). Cells were disrupted by nitrogen cavitation at 400 p.s.i. for 25 min and centrifuged at 800 xg for 10 min. The fluffy pellet on top of the nuclei, containing most of the hemolytic activity, was carefully resuspended in PBS (2 x 10^8 cell equivalents/ml), followed by three cycles of freezing and thawing. The supernatant after a 5-min centrifugation in a microfuge was collected and kept at -20 °C until use.

Purification of murine perforin. Murine perforin was purified from CTLL-R8 as previously described (23,38). Briefly, R8 cells were cavitated under nitrogen in relaxation buffer. The nuclei and intact cells were removed by centrifugation at 200 xg for 10 min. After another centrifugation at 39,000 xg for 20 min, the organelle-enriched pellet was resuspended in extraction buffer (2 M $NaKHPO_4$, pH 7.4, 1 mM EGTA and 0.2 mM diisopropyl fluorophosphate). The perforin-containing supernatant was then collected and diluted in buffer A (20 mM Tris-HCl, pH 7.2, 1 mM EGTA). Ion-exchange and molecular sieving chromatography steps were performed by applying samples sequentially on DEAE-Sepharose, Q sepharose, polyanion SI and Superose 12 columns (all from Pharmacia, Uppsala, Sweden) connected to a Fast Protein Liquid Chromatography (FPLC, Pharmacia) system. The perforin activity in the various column fractions, quantitated in hemolytic units (HU) using murine E as targets, was assessed with a hemolytic microassay system (23).

Complement-mediated hemolysis. The sensitivity of the various types of E to human (homologous) and guinea pig (heterologous) C-mediated hemolysis was assessed following modification made on a published procedure (23). In brief, E were washed, resuspended (5 x 10^8/ml) in GVB^{2+} buffer and incubated with rabbit anti-human E antiserum (purchased from Organon Teknika corp, West Chester, PA.) at 1:400 dilution for 1 h at 4 °C. After another wash, the antibody-coated E were resuspended in GVB^{2+} buffer (2.5 x 10^8/ml). A 10-μl aliquot of this suspension was mixed, in triplicates, with 90 μl of GVB^{2+}-diluted human or guinea pig sera (1:160 to 1:5) in a microtiter plate and incubated at 37 °C for 1 h, after which 100 μl of cold GVB^{2+} buffer was added to each well. The plate was centrifuged at 200 xg for 10 min. A 100-μl aliquot of the supernatant from each well was transferred to another plate and the hemoglobin in the supernatant was measured spectrophometrically at 410 nm using an automated microplate reader (model MR 700, Dynatech Laboratories Inc., Alexandria, VA). The percent of hemolysis was calculated according to the following formula: % hemolysis = (A_{410} experimental release - A_{410} spontaneous release)/(A_{410} total release - A_{410} spontaneous release) x 100. Spontaneous or total release of hemoglobin was determined using E incubated with C-free GVB^{2+} buffer or water, respectively. Experiments were performed in triplicates.

Reactive lysis using purified C5b-6, C7, C8, and C9 were performed as described (26).

Perforin-mediated hemolysis. Perforin-mediated hemolysis was performed as described (23), with minor modifications. Briefly, 10 μl of E suspension (2.5 x 10^8/ml in PBS) were incubated with 140 μl of Ca^{2+}-free PBS, containing human or murine perforin at 0.125 to 4 HU, and 10 μl of 20 mM $CaCl_2$ at 37 °C for 30 min. After centrifugation of the plate and transfer of the supernatant to a new plate, the hemoglobin release was determined and the percent of hemolysis was calculated as described before for C-mediated hemolysis. All experiments were done in triplicates.

Assay for antibody-dependent cellular cytotoxicity (ADCC). E from PNH patients or normal donors were sensitized with rabbit anti-human E antiserum as

described above. These antibody-coated E (10^7 E/well, final volume of 200 μl/well) were incubated in triplicates with purified human NK cells at the indicated effector-to-target ratios at 37 °C for 4 h as described (26). After centrifugation at 200 xg for 10 min, 100 μl of supernatant was collected. The percent of ADCC was measured on the basis of hemoglobin release as above.

Immunoblot analysis of HRF/C8BP. HRF/C8BP in E membrane was detected following a published procedure (10). Briefly, E ghost membranes, washed extensively in PBS, were extracted with a high molarity phosphate buffer (1.5 M $NaKHPO_4$, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsufonyl fluoride) followed by a final wash in 50 mM $NaKHPO_4$, pH 7.4. Washes were performed by centrifugation at 11,000 xg for 10 min using a Sorvall SS-34 rotor. The membrane pellets were then extracted twice with acetone (1 vol membrane:20 vol acetone) at -20 °C for 60 min, followed by centrifugation. The protein pellet was then resuspended in Neville's buffer and reduce with dithiothreitol (DTT) and alkylated with iooacetamide, as described (38). Fifty μg of proteins were applied per lane on a 10% SDS-polyacrylamide gel. Gel electrophoresis and transfer to a nitrocellulose membrane were performed as described (34,38). After blocking with gelatin-containing buffer (10 mM Tris:HCl, pH 7.3, 150 mM NaCl, 2% glycine, 0.05% sodium azide, 1% gelatin), the membrane was then incubated with Tris-buffered saline, pH 7.4, containing 0.1% gelatin and 50 μg/ml of purified C8, for 4 h at room temperature. After 3 washes in detergent-containing buffers (10 mM Tris-HCl, pH 7.4, 400 mM NaCl, 2 mM EDTA, 0.25% NP-40, 0.25% Tween-20), the membrane was further incubated with rabbit anti-C8 antiserum. The blots were developed with ^{125}I-labeled goat F(ab)'$_2$ anti-rabbit IgG (New England Nuclear, Boston, MA). Autoradiography was performed with intensifying screens.

Results

Detection of HRF/C8BP in membranes of type III E. We first determined whether the type III PNH E studied here lacked surface HRF/C8BP. E membrane proteins extracted with high salt buffers were subjected to immunoblot analysis, using the C8-anti-C8 sandwich binding assay described elsewhere (10). As shown in Fig. 1, a band of 65 kDa reacting with C8 was detectable only in membranes of type I PNH E and control E, while no such band was detected in type III PNH E (data shown here for 2 patients). In some experiments, a band of 32 kDa in type I E was also

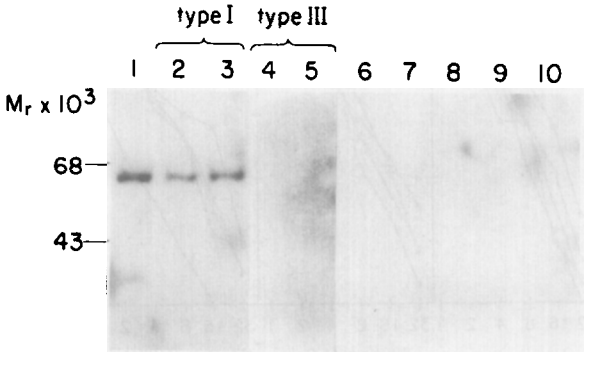

Figure 1. Immunoblot analysis of E membranes from type I and III PNH E and control E. Membranes of untreated E obtained from a control healthy donor (lane 1), of type I (lanes 2 and 3) and type III (lanes 4 and 5) E from 2 different PNH patients were subjected to SDS-PAGE (10%). C8 and anti-C8 antibodies were used, as described in Methods, to detect C8BP. Lanes 6-10 are identical replica of lanes 1-5 reacted with preimmune serum. Autoradiography was performed for 48 h.

detected that reacted with C8 and anti-C8 antibodies but this was not a consistent finding. These results are in agreement with previously published observations showing that type III E membranes lack HRF/C8BP (4,9).

Differential sensitivity of PNH and control E to homologous and heterologous C-mediated hemolysis. The lysis, by homologous C, of E from PNH patients and healthy individuals was compared. Both type I E, obtained after CVF treatment of E from PNH patients, and E from healthy individuals were markedly resistant to lysis mediated by human C (the upper panel of Fig. 2 shows data for 4 patients, assessed individually). In contrast, type III E obtained from all 4 patients were sensitive to human C-mediated lysis (Fig. 2, upper panel). As expected, unseparated E from these PNH patients (containing a mixture of types I, II and III E) showed a susceptibility to human C-mediated lysis that was intermediate between that of purified types I and III E (Fig. 2, upper panel, compare the various hemolysis curves).

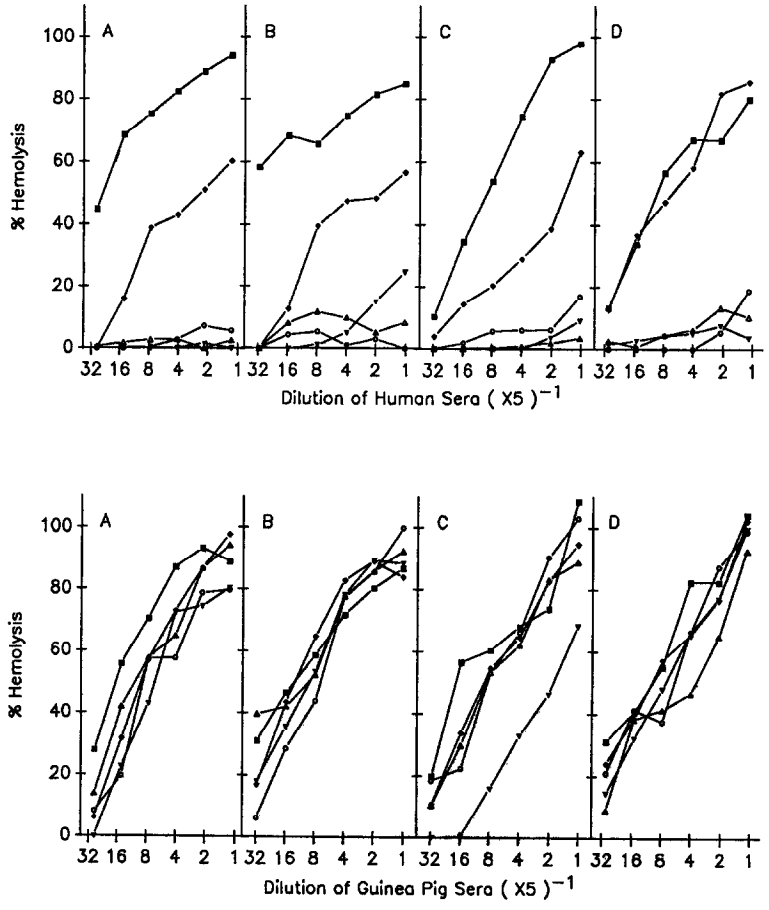

Figure 2. Lysis of E from healthy individuals and PNH patients mediated by human (upper panels) and guinea pig (lower panels) C. Data in panels A-D are from 4 individual PNH patients and 4 healthy controls. ▼, type I; ■, type III; and ◆, unseparated E from PNH patients; ○, CVF-treated; and △, CVF-untreated E from healthy individuals. Dilutions of human and guinea pig sera are reported on the X axis. Data points represent means of triplicates.

As a control, lysis of the same E populations was carried out in parallel using heterologous guinea pig C (Fig. 2, lower panel). At low concentrations of guinea pig C, type III E were more susceptible to lysis than other types of E, including type I and control E. Higher concentrations of guinea pig C, however, lysed equally well types I, III and unseparated E from PNH patients and control E, a result consistent with the notion that types I and III E differ mainly in their sensitivity to lysis by homologous C.

Our initial studies were performed with human sera as a source of C (Fig. 2, upper panel), but similar results were obtained when reactive lysis was performed using purified C5b-9 (data not shown). That is, type III E showed high susceptibility to reactive lysis, while type I E remaining marked refractory to lysis.

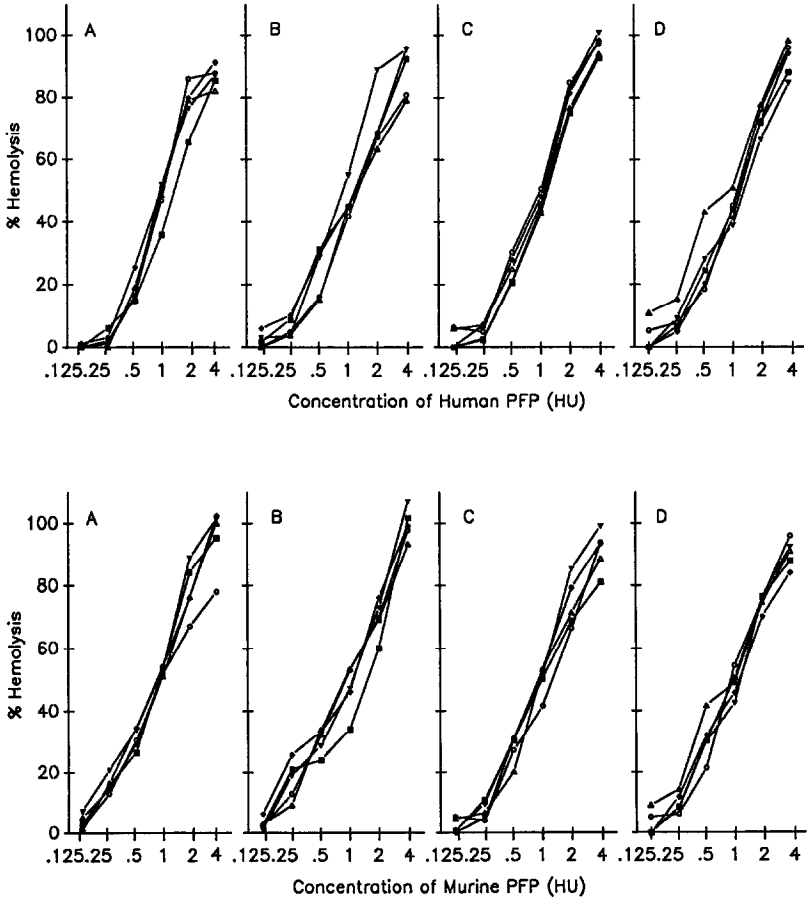

Figure 3. Lysis of E from healthy individuals and PNH patients mediated by human (upper panels) and murine (lower panels) perforin. Human and murine perforin-mediated lysis of type I (▼), type III (■), unseparated (◆) E from 4 PNH individual patients, CVF-treated (○) and untreated (△) E from four healthy individuals are reported in panels A-D. Concentrations of human and murine perforin are indicated. Data points represent means of triplicates.

Susceptibility of type I and III PNH E to lysis mediated by human and murine perforin. When tested against partially enriched human (Fig. 3, upper panel) and purified murine (Fig. 3, lower panel) perforin, all E populations studied, including types I, III and unseparated E from the 4 PNH patients, and E from 4 healthy donors, showed similar sensitivity to lysis and no differential sensitivity to perforin from the two species (compare upper and lower panels of Fig. 3), indicating that the phenomenon of homologous species restriction described before for the C system (10-14) is not applicable to the perforin system.

Susceptibility of types I and III PNH E to NK cell-mediated ADCC. It has been reported (26) that purified HRF incorporated into E membrane could reconstitute the resistance of PNH E against lysis by large granular lymphocyte (LGL) in an ADCC reaction. We performed a similar cytotoxicity experiment using types I, III and unseparated E from two PNH patients and E from two healthy individuals as target cells. Purified NK cells were used as effectors (Fig. 4). All E populations tested were equally sensitive to this type of NK cell-mediated ADCC. In parallel experiments using monocyte-depleted total lymphocyte populations as effectors, similar results were obtained (data not shown).

Discussion

HRF/C8BP has been shown to confer protection against cytotoxicity mediated by both C (10-12) and lymphocyte perforin (24-26,39). In the C-system, HRF/C8BP is supposedly responsible for homologous species restriction, that is, resistance of E of a given species to lysis by C of homologous, but not heterologous, species

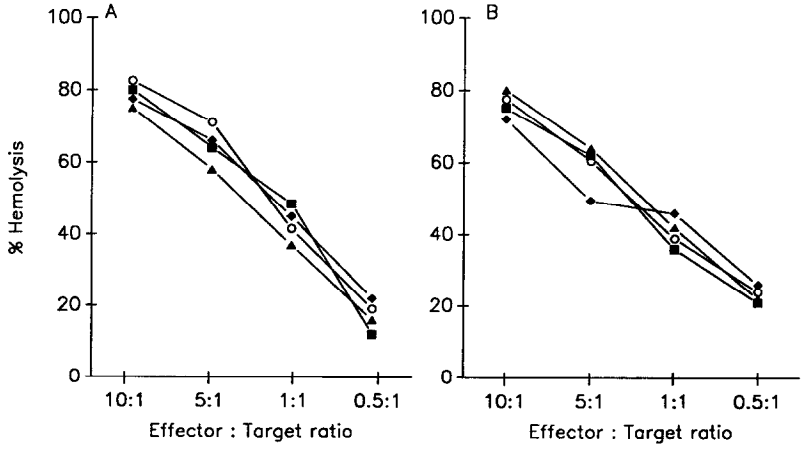

Figure 4. NK cell-mediated ADCC of antibody-coated E. (A,B) show data from 2 different PNH patients and healthy individuals. NK cells from 1 healthy individual were tested for their ability to lyse antibody-sensitized type I (▼), type III (■), unseparated (◆) PNH E and CVF-untreated control E (○). 10^7 E/ml were used for each data point while the density of NK cells varied as indicated.

(13,40). In addition to this function, purified HRF that had been reconstituted into the membranes of type III E of PNH patients was shown to confer to these cells protection against both perforin- and LGL-mediated lysis (26). Furthermore, stimulation of resting peripheral blood lymphocytes with anti-CD3 antibodies was shown to induce expression of surface HRF as well as cell resistance against lysis mediated by perforin, and this enhanced resistance was abrogated by $F(ab')_2$ anti-HRF (39). Together, these results are provocative in suggesting that HRF represents the common denominator preventing lysis of cells by homologous C and perforin (24-26).

We have shown recently that the phenomenon of species restriction, while applying well to the C-system, does not hold for the lymphocyte perforin-system, as indicated by the observation that some target cells are sensitive to lysis by homologous but not heterologous C yet are equally sensitive to lysis by perforin of both the same and different species (23). The hypothesis that the HRF molecule plays a role in the resistance of cells to perforin had not been addressed directly in that study. Here, we have used the E of PNH patients to address this question. By immunoblot analysis, type III PNH E were shown to lack the 65 kDa C8-binding species or HRF, in accord with published observations (4,9). We have shown that these type III E were highly susceptible to homologous C-mediated lysis, when compared to type I and control E, while all E populations tested were lysed equally well by both homologous and heterologous perforin. Moreover, we found that type III PNH E, like type I and normal untreated E, were equally susceptible to lysis mediated by NK cells in an ADCC reaction which has previously been shown to require perforin (41,42). These results suggest that HRF does not play a direct role in the resistance of cells to perforin-mediated injury. Thus, its previously postulated role in this reaction must now be carefully reexamined.

Several laboratories have independently reported evidence for the existence of a self-protective mechanism by which perforin-producing cytolytic lymphocytes protect themselves from lysis by this protein (19-23,43). However, the molecular basis of this phenomenon is still unknown. HRF, the only putative protective factor studied to date, does not seem to explain this type of reaction. Other protective molecules (protectins?) need to be sought to explain resistance of cytolytic lymphocytes to perforin-mediated lysis.

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