

INHIBITION OF ANTIBODY-DEPENDENT LYMPHOCYTE  
CYTOTOXICITY BY HOMOLOGOUS RESTRICTION  
FACTOR INCORPORATED INTO  
TARGET CELL MEMBRANES

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The purpose of this study was to determine whether cell lysis by cytotoxic lymphocytes may be affected by the presence of homologous restriction factor (HRF)<sup>1</sup> on the surface of the target cells. HRF is a membrane protein that inhibits the transmembrane channel-forming function of C8 and C9 of complement (1, 2). It inhibits homologous C8 and C9, but not their analogs of other species. The cytolytic protein of human large granular lymphocytes (LGL) (3-5) and human cytotoxic T lymphocytes (6) was shown to be immunochemically related to C9. This C9-related protein (C9RP) was found also to crossreact with C6, C7 (7), and C8 (4, 7). Because of certain structural and functional similarities between C9RP and C9 (4, 8), the possibility arose that HRF might affect the function of killer lymphocytes using C9RP. To explore this question, the antibody-dependent cellular cytotoxicity (ADCC) reaction was chosen using human LGL as effectors and erythrocytes (E) as targets. E were selected as targets because these cells are known to be lysed in the ADCC reaction by simple channel insertion into their membrane (9), whereas killing of nucleated cells by lymphocytes is thought to involve additional factors (10, 11). HRF isolated from human E (E<sub>H</sub>) was found to bind readily to sheep E (E<sub>S</sub>) and to retain its activity. Also, the abnormal E of paroxysmal nocturnal hemoglobinuria (PNH) were used as target cells since these are deficient in HRF and capable of being reconstituted with isolated HRF (12). In the following, evidence will be reported suggesting that HRF can inhibit target cell lysis by LGL.

### Materials and Methods

*Isolation of HRF.* HRF derived from E<sub>H</sub> membranes was purified using a rabbit anti-HRF Sepharose column (12). ~100 mg of E<sub>H</sub> membrane protein was dissolved in 2% deoxycholate (DOC), 50 mM NaCl, 2 mM PMSF, 1 mM benzamidine, 2 mM EDTA, 2

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<sup>1</sup> *Abbreviations used in this paper:* ADCC, antibody-dependent, cell-mediated cytotoxicity; C9RP, C9-related protein; DAF, decay-accelerating factor; DOC, deoxycholate; HRF, homologous restriction factor; LGL, large granular lymphocyte; PNH, paroxysmal nocturnal hemoglobinuria.

mM EGTA, 20 mM Tris-HCl, pH 8.4, at 4°C overnight in a total volume of 100 ml. The material was centrifuged at 12,000 *g* to remove undissolved matter, and the supernatant was dialyzed against 0.02% DOC, 50 mM NaCl, 20 mM Tris-HCl, pH 8.4 (starting buffer). The dissolved protein was passed first over a column containing rabbit IgG bound to Sepharose to remove IgG-binding proteins and then over a Sepharose column containing 300 mg of bound anti-HRF Ig. The column was washed with starting buffer and then with 0.1 M NaCl and 0.2 M NaCl in starting buffer. HRF was eluted with 2 M NaCl and then 2 M KBr in starting buffer. The yield was about 100 µg of purified protein. HRF in 0.02% DOC was radiolabeled with <sup>125</sup>I using the iodogen method (Pierce Chemical Co., Rockford, IL).

*Isolation of C9RP.* C9RP was isolated from human LGL by affinity chromatography using a rabbit anti-C9RP-Sepharose immunoabsorbent column (4, 6).

*Preparation of Abnormal PNH-E.* The abnormal E of a PNH patient were enriched by lysing the normal E with anti-DAF (decay accelerating factor) and guinea pig serum.  $2 \times 10^9$  E were incubated with 500 µl of rabbit anti-DAF for 30 min at 37°C, washed twice, and then incubated with 1 ml of fresh guinea pig serum for 10 min at 37°C. The cells that remained after this treatment were washed thoroughly and used as PNH-E.

*LGL.* Peripheral blood mononuclear cells were isolated from heparinized normal human blood by Ficoll-Isopaque gradient centrifugation using lymphocyte separation medium (Litton Bionetics, Irving, TX). Monocytes were removed by adherence to plastic. The cell culture was stimulated with 0.2% (vol/vol) phytohemagglutinin, M form (Gibco, Grand Island, NY) and grown in the presence of 5 U/ml of human recombinant IL-2 (Amgen, Thousand Oaks, CA) for several weeks (4).

*Incorporation of <sup>125</sup>I-HRF.*  $E_s$  or  $E_H$  ( $3-5 \times 10^7$ ) were incubated with varying amounts of <sup>125</sup>I-HRF in a total volume of 1 ml for 2 h at 37°C in a final concentration of 0.004% DOC. The cells were washed twice and the amount of HRF bound was determined by quantitation of radioactivity.  $E_s$  or  $E_H$  not receiving HRF (negative controls) were also treated with 0.004% DOC.

*Incorporation of <sup>125</sup>I-DAF.*  $5 \times 10^7$   $E_sA$  (antibody-coated  $E_s$ ) were incubated with varying amounts of <sup>125</sup>I-labeled, isolated DAF (13, 14) in a total volume of 3 ml for 2 h at 37°C in a final concentration of 0.0013% DOC and 0.0013% NP-40 (Sigma Chemical Co., St. Louis, MO). The cells were washed twice and the amount of DAF incorporated was determined by quantitation of cell-associated radioactivity. Controls not receiving DAF were treated with the same amount of detergent for the same length of time. The preparation of DAF used was active, as indicated by accelerated dissociation of <sup>125</sup>I-Bb from zymosan-C3b,Bb complexes (14).

*Reactive Lysis.*  $10^7$   $E_s$  with or without bound HRF were incubated at 37°C with ~3 µg C5b6, 2 µg C7, 2 µg C8, and 15 µg C9 in a total volume of ~50 µl. After 2 h, hemoglobin release was measured at 412 nm.

*Sensitization of E with IgG Antibody.*  $E_s$  were coated with antibody (A) using the IgG fraction of rabbit anti- $E_s$  (Cooper Biomedical, Malvern, PA) according to the package instructions.  $E_HA$  were made using the IgG fraction of anti- $E_H$  (Cooper Biomedical).  $E_s$  or  $E_H$  at  $5 \times 10^8$  cells/ml were incubated with a 1:8,000 dilution of antibody in the presence of 10 mM EDTA for 30 min at 37°C and washed three times.

*ADCC.*  $10^7$   $E_sA$  or PNH-EA with or without bound HRF were incubated with  $1-2 \times 10^7$  human LGL for 4 h at 37°C. Lysis was determined spectrophotometrically at 412 nm.

## Results

*65 kD HRF, Incorporation into  $E_s$ , and Inhibition of Reactive Lysis.* HRF was isolated from DOC-solubilized  $E_H$  membranes by affinity chromatography using rabbit anti-human HRF coupled to Sepharose 4 B. The column was washed successively with 0.1 M and then 0.2 M NaCl in 0.02% DOC and eluted with 2 M NaCl in 0.02% DOC. Throughout the isolation procedure the pH was

maintained at 8.4. ~100  $\mu\text{g}$  of HRF was isolated from 100 mg of solubilized  $E_H$  membrane protein. A representative SDS-PAGE pattern of HRF is shown in Fig. 1. The  $M_r$  was 65,000.

$^{125}\text{I}$ -labeled HRF in 0.02% DOC was added to a suspension of  $E_S$  ( $4 \times 10^7$  cells/ml), which reduced the final DOC concentration to 0.004%. The cells were kept at  $37^\circ\text{C}$  for 2 h and washed thoroughly. In several such experiments, uptake of HRF by the cells varied between 1 and 4%. Fig. 2 shows the binding curve of one of the studies in which 1,500–15,900 molecules per cell were bound in direct proportion to input. The results of all uptake experiments indicate that the 65 kD HRF isolated from  $E_H$  retained its membrane anchoring function.

HRF incorporated in the membrane of  $E_S$  ( $E_S$ -HRF) was tested for inhibition of channel formation by C5b-9 using reactive lysis. The number of HRF molecules on  $E_S$ -HRF ranged from 530 to 8,500 per cell. Lysis of unmodified  $E_S$ , which had also been treated with 0.004% DOC, was 58%, and declined to 3%

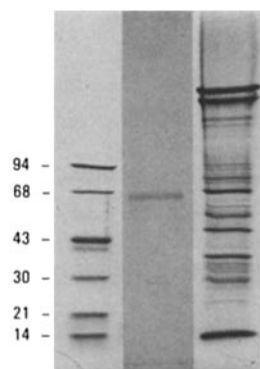


FIGURE 1. Demonstration of HRF, isolated from human erythrocyte membranes, by SDS-PAGE. The  $M_r$  of the protein is 65 kD, and comparison with the pattern of whole  $E_H$  membranes reveals no major protein band corresponding to HRF. Amounts applied were 3  $\mu\text{g}$  of HRF (*center*) and 40  $\mu\text{g}$  of membrane protein (*right*). The gel concentration was 1.7–17%. The gel was stained with Coomassie blue. Molecular mass standards are in the left lane.

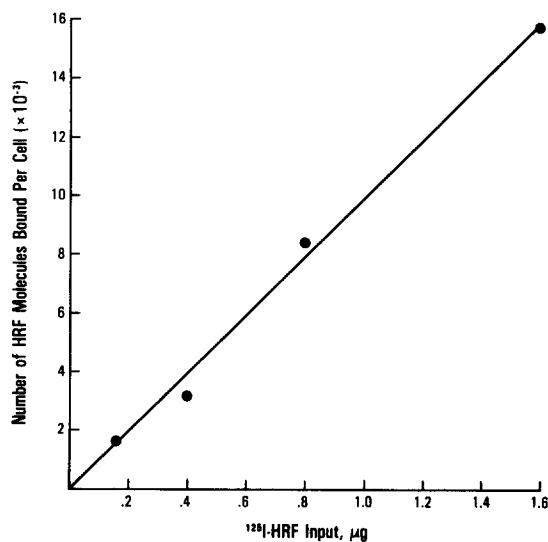


FIGURE 2. Uptake of  $^{125}\text{I}$ -HRF of human erythrocyte membranes by  $E_S$ .  $4 \times 10^7$   $E_S$  were treated with varying amounts of  $^{125}\text{I}$ -HRF (0.16–1.58  $\mu\text{g}$ ), which was contained in 0.02% DOC for 2 h at  $37^\circ\text{C}$ . The total volume was 1 ml and the final DOC concentration 0.004%.

at 8,500 HRF molecules per  $E_s$  (Fig. 3). The results indicate that HRF isolated from  $E_H$  also retained its function in inhibiting hemolysis by C5b-9.

*Inhibition of ADCC by HRF Bound to  $E_sA$  or PNH-EA.*  $E_s$ -HRF bearing up to 3,200 molecules of HRF per cell were sensitized with rabbit IgG anti- $E_s$  antibody and subjected to ADCC using human LGL. The reaction mixtures were incubated at 37°C for 4 h. In two experiments (Fig. 4) lysis of  $E_sA$  devoid of human HRF was 35 and 32%, respectively. In both cases, the incorporated HRF reduced

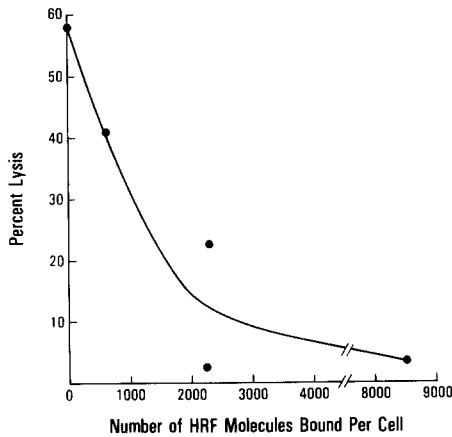


FIGURE 3. Inhibition of reactive lysis with C5b-9 by isolated HRF bound to  $E_s$ .  $^{125}I$ -HRF was incorporated into the membrane of  $E_s$ , the number of molecules per cell ranging from 530 to 8,500.  $10^7$   $E_s$ -HRF were incubated for 2 h at 37°C with ~3  $\mu$ g C5b6, 2  $\mu$ g C7, 2  $\mu$ g C8, and 15  $\mu$ g C9 in a volume of 50  $\mu$ l.

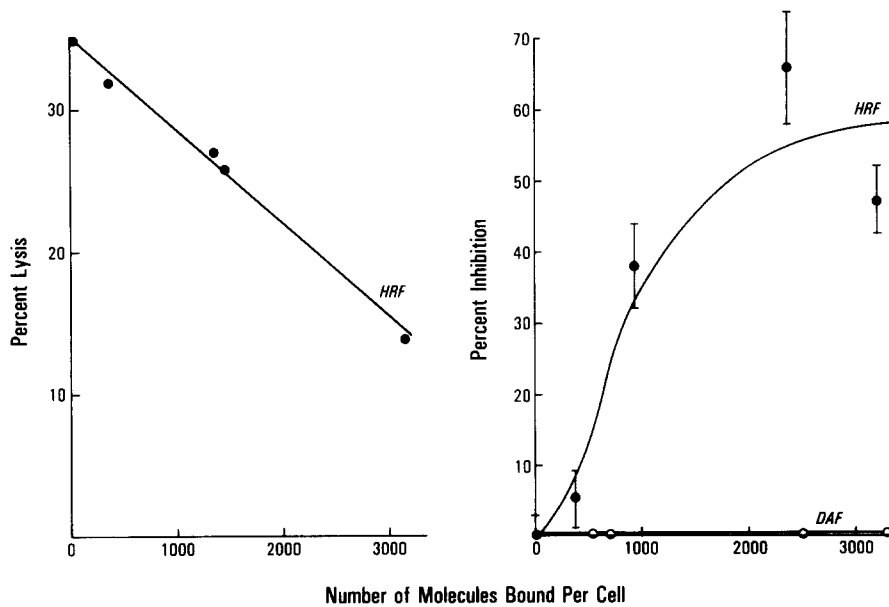


FIGURE 4. Inhibition of ADCC by HRF bound to  $E_s$  sensitized with IgG antibody. Samples of  $10^7$   $E_sA$  bearing varying amounts of HRF were incubated for 4 h at 37°C with  $10^7$  human LGL in a total volume of 30  $\mu$ l. Lysis was measured by determining hemoglobin in the supernatant at  $OD_{412}$ . The variation of lysis is indicated by the standard error bars (right), each point representing three separate determinations. Also shown (right) is the lack of effect of incorporated DAF on ADCC. Each point represents the average of two determinations.

lysis by ~60%. Different preparations of HRF and different batches of  $E_s$  were used in these experiments. Detergent-treated  $E_sA$  were used as HRF<sup>-</sup> controls in all experiments. In a separate experiment, the effect of reinserted DAF on ADCC was determined. Up to 3,300 molecules of <sup>125</sup>I-DAF were bound per  $E_sA$ , and no effect on cellular cytotoxicity was observed (Fig. 4).

Abnormal PNH-E were enriched from the  $E_H$  population of a patient with PNH by lysis of the normal  $E_H$  with rabbit anti-DAF and guinea pig complement. After thorough washing, the unlysed DAF- (and HRF-) deficient cells were sensitized with IgG rabbit anti- $E_H$  antibody and incubated for 4 h with a twofold excess of human LGL. PNH-E without HRF underwent 20% lysis, and this degree of lysis was completely abolished by 17,700 molecules of HRF per cell (Fig. 5).

*HRF Bound to  $E_s$  Inhibits Hemolysis by Isolated C9RP.* <sup>125</sup>I-HRF was incorporated into  $E_s$  as described above, and  $10^7$   $E_s$ -HRF bearing 330 to 7,600 molecules of HRF per cell were exposed to 5  $\mu$ g of isolated C9RP in 20  $\mu$ l containing 5 mM  $Ca^{2+}$ .  $E_s$  treated with 0.004% DOC in the absence of HRF were used as control. After 5 h at 37°C, the controls had undergone 55 and 70% lysis, whereas  $E_s$ -HRF, with 4,250 or 7,600 molecules per cell, showed only 20 or 18% lysis (Fig. 6). Thus, HRF incorporation into  $E_s$  resulted in 64–75% reduction of  $E_s$  lysis by C9RP.

### Discussion

When HRF was first isolated in this laboratory from -70°C-stored  $E_H$  membranes, it was found to be a 38 kD protein (1). This protein was capable of incorporating into the lipid bilayer of liposomes, and in this form was active in inhibiting channel formation by C5b-8, C5b-9, and polymerizing C9. Rabbit

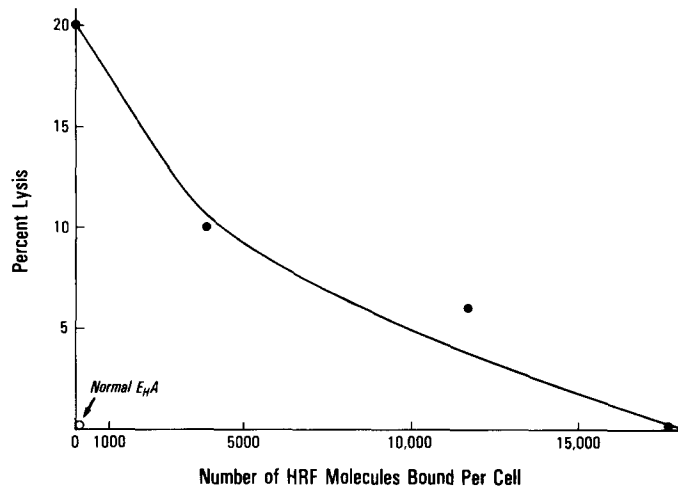


FIGURE 5. Inhibition of ADCC by HRF bound to the abnormal PNH-E. The abnormal cells were selectively enriched by removing the normal cells from the total E population. The cells were sensitized with IgG antibody and were then incubated with varying amounts of <sup>125</sup>I-HRF for 2 h at 37°C. After washing, the uptake of HRF was determined and  $10^7$  treated cells were then incubated with  $2 \times 10^7$  LGL for 4 h at 37°C in a total volume of 30  $\mu$ l.

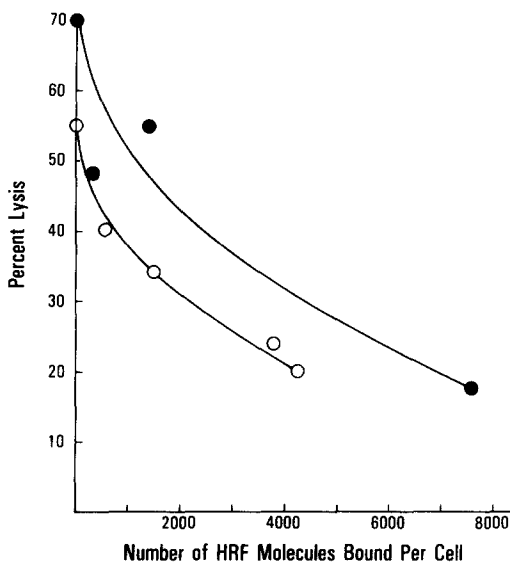


FIGURE 6. Inhibition of C9RP-induced lysis of  $E_s$  by cell bound HRF. In two separate experiments,  $E_s$  were treated with various amounts of  $^{125}\text{I}$ -HRF, washed, and then  $10^7$   $E_s$ -HRF were exposed to  $5\ \mu\text{g}$  C9RP in  $20\ \mu\text{l}$  containing  $5\ \text{mM}$   $\text{Ca}^{2+}$  for 5 h at  $37^\circ\text{C}$ .

antibody to the 38 kD protein inhibited HRF activity on  $E_H$ , human polymorphonuclear leukocytes, and Raji cells. The same antibody detected, in immunoblots of freshly isolated  $E_H$  membrane, primarily a 65 kD protein. This observation suggested that the 38 kD protein was an active fragment of the 65 kD membrane HRF. It also related HRF to the C8 binding protein described by others (2).

Initially HRF was isolated from DOC-solubilized  $E_H$  membranes by affinity chromatography using human C9-Sepharose (1). Since then, more than 20 HRF preparations have been obtained using rabbit anti-HRF immunoadsorbent columns. In each instance, 65 kD HRF was isolated, including one preparation derived from membranes of human LGL (our unpublished observation). Because abnormal PNH-E exhibit enhanced sensitivity to reactive lysis by C5b-9 (15-18), the membranes of such cells were investigated for HRF content and found completely deficient in HRF (12). In that study, radioactive HRF was inserted into PNH-E, and  $\sim 1,000$  molecules per cell were sufficient to confer normal resistance to reactive lysis.

In this paper the present method of isolation of HRF is described, the 65 kD HRF is demonstrated by SDS-PAGE, a binding curve describing the binding of  $^{125}\text{I}$ -HRF to  $E_s$  is presented, and the acquisition of resistance to lysis by C5b-9 is shown as a function of HRF uptake by  $E_s$ .

Evidence has accumulated showing that killer lymphocytes use a discrete protein of  $M_r \sim 70,000$  to produce aqueous pores in target membranes (3-6, 19, 20), and that this protein is immunochemically related to C9 (3-6, 21) and other proteins of the membrane attack complex of complement (7, 22). In view of the similarities between the cytolytic C9RP of cytotoxic lymphocytes and C9, the possibility was considered that HRF may inhibit channel formation, not only by complement, but also by cytotoxic lymphocytes. Erythrocytes have been used extensively as target cells in antibody-dependent cytolysis by lymphocytes (9, 23,

24). Since isolated human HRF may be readily incorporated into the membrane of  $E_S$  and  $E_H$ , these cells were chosen as targets in the ADCC reaction with human LGL. The results suggest that HRF is capable of inhibiting this reaction in a dose-dependent manner, which is in contrast to DAF, insertion of which into  $E_S$ A had no effect on ADCC. They also imply that HRF can interact with C9RP in a manner similar to its known interaction with C8 and C9. HRF binds to both C8 (1, 2) and C9 (1) and it reduces uptake of C9 by EC5b-8 (1 and our unpublished data). In the case of cytolysis by lymphocytes, HRF may interfere with oligomerization and polymerization of C9RP. More work is clearly needed to elucidate the molecular mechanism of action of HRF in the inhibition of complement and lymphocyte cytotoxicity. To test the postulated interaction of HRF with C9RP more directly, the effect of membrane-bound HRF on the lysis of  $E_S$  by isolated C9RP was examined. It should be emphasized that, unlike murine perforin (20), human C9RP is a poor hemolytic agent. It is not clear why erythrocytes are relatively resistant to lysis by C9RP whereas nucleated cells such as K562 or M21 melanoma cells are readily killed (4, 6). Nevertheless, under the conditions described, C9RP did cause lysis of  $E_S$ , and the fact that this reaction could be inhibited by  $E_S$ -bound HRF suggests strongly that HRF and C9RP can interact directly. The question arises, therefore, as to whether HRF may be operative in self-protection of cytotoxic lymphocytes.

### Summary

The 65 kD homologous restriction factor (HRF) was isolated from normal human erythrocytes (E) by immunoadsorption using rabbit anti-human HRF. The protein was radiolabeled and incorporated into the membrane of sheep erythrocytes ( $E_S$ ).  $E_S$  bearing HRF exhibited a markedly reduced susceptibility to reactive lysis by C5b-9.  $E_S$ -HRF with 1,000–3,000 HRF molecules per cell and sensitized with rabbit IgG anti- $E_S$  also were less susceptible to lysis by human large granular lymphocytes (LGL) than untreated  $E_S$  sensitized with IgG antibody. Similarly, human E of a patient with paroxysmal nocturnal hemoglobinuria (PNH), lacking HRF and sensitized with IgG antibody underwent lysis by human LGL. Lysis was abrogated by incorporation of isolated human HRF. Incorporation of human decay-accelerating factor (DAF) into sensitized  $E_S$  had no effect on antibody-dependent, cell-mediated cytotoxicity. Furthermore, lysis of  $E_S$  by the isolated cytolytic C9-related protein (C9RP) of human cytotoxic lymphocytes could be inhibited by cell bound human HRF. These results suggest that HRF inhibits channel formation not only by C5b-9, but also by cytotoxic lymphocytes.

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