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Immunology

Induction of expression of cell-surface homologous restriction factor upon anti-CD3 stimulation of human peripheral lymphocytes

(cytotoxic T cells/natural killer cells/complement component 9-related protein/self-protection of cytotoxic lymphocytes)

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ABSTRACT **Homologous restriction factor (HRF) is a 65-kDa membrane protein that inhibits transmembrane channel formation by the membrane-attack complex of complement and by the complement component C9-related cytolytic lymphocyte protein. Stimulation of resting peripheral human lymphocytes with the anti-CD3 monoclonal antibody OKT3 has been shown to induce cytotoxicity in the $CD8^+$ subpopulation. As demonstrated here, OKT3 stimulation also induces expression of cell-surface HRF by $CD4^+$ and $CD8^+$ T lymphocytes. The small proportion of Leu 19^+ natural killer lymphocytes present in peripheral blood mononuclear cells was found to express HRF prior to stimulation. Whereas unstimulated peripheral blood mononuclear cells were susceptible to lysis by the membrane-attack complex or by the C9-related protein, OKT3-stimulated peripheral blood mononuclear cells were relatively resistant to both the membrane-attack complex and C9-related protein. This acquired resistance was abrogated by blocking surface HRF with $F(ab')_2$ anti-HRF, suggesting that resistance was due to lymphocyte-membrane HRF. By using solid-phase anti-HRF, a 65-kDa protein was isolated from the activated peripheral blood mononuclear cells and shown to be capable of conferring upon sheep erythrocytes the characteristic activity of human HRF.**

Homologous restriction factor (HRF) is a cell membrane-associated 65-kDa protein that was originally isolated from human erythrocyte membranes (1). Isolated HRF could be incorporated into the lipid bilayer of liposomes, and, in this form, it inhibited transmembrane channel formation by the membrane-attack complex of human complement (1). Anti-HRF inhibits the activity of HRF on human erythrocytes and thereby enhances lysis of these cells by the membrane-attack complex. Present evidence indicates that HRF acts by interfering with the channel-forming function of complement components C8 and C9 (1, 2).

Because of the immunochemical and functional relationship of C8 and C9 to the cytolytic C9-related protein (C9RP) of human natural killer (NK) cells (3, 4) and human cytotoxic T lymphocytes (CTL) (5), the question was explored of whether the channel-forming function of this protein, too, is controllable by HRF. Highly purified C9RP efficiently kills metabolically active cells, such as K562 erythroleukemia cells, human M21 melanoma cells (4, 5), and many other human tumor cell lines (unpublished observations). Whereas peripheral human NK cells have a high content of C9RP, peripheral human $CD8^+$ lymphocytes contain little or no C9RP. However, upon activation of peripheral blood mononuclear cells (PBMC) with the anti-CD3 monoclonal antibody OKT3 (6–8), $CD8^+$ cells acquire a high content of C9RP (9) concomitant with cytotoxic activity (9–11). An excellent correlation has been found to exist between cellular C9RP content and cytotoxic activity of human peripheral killer lymphocytes stimulated with either OKT3 or interleukin 2 (9).

To test whether cellular cytotoxicity is inhibitable by target-cell-bound HRF, the antibody-dependent cellular cytotoxicity reaction was chosen using human large granular lymphocytes as effector cells and antibody-sensitized sheep erythrocytes as targets. Human HRF inserted into the membranes of the erythrocytes inhibited antibody-dependent cellular cytotoxicity in a dose-dependent manner. Cell-bound HRF also inhibited the lysis of these erythrocytes by isolated C9RP (12). Thus, it was demonstrated that HRF not only inhibited complement cytotoxicity but also potentially inhibited cell lysis by lymphocytes. The possibility arose, therefore, that HRF is involved in the self-protection of cytotoxic lymphocytes.

Killing lymphocytes avoid killing themselves while destroying target cells (13). Apparently they are resistant to the cytolytic proteins they release upon contact with the recognized target cells (14). The molecular mechanism of self-protection is unknown. It will be shown below that the anti-CD3 activation of peripheral T lymphocytes was accompanied by cell-surface expression of HRF. Activated, HRF-expressing cells were relatively resistant to lysis by C9RP compared to resting lymphocytes. This acquired resistance to C9RP lysis was abrogated by antibody to HRF.

MATERIALS AND METHODS

Human PBMC. PBMC were isolated from heparin-treated blood from healthy donors by Ficoll/Hypaque (Pharmacia) density gradient centrifugation and were cultured in RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 2 mM L-glutamine, 50 μg of gentamicin per ml, and 10% (vol/vol) heat-inactivated fetal bovine serum. Cultures were stimulated with OKT3 at 100 ng/ml for various lengths of time prior to harvesting. OKT3 was purified from supernatants of hybridomas obtained from the American Type Culture Collection.

Phenotypic Analysis. $F(ab')_2$ fragments of rabbit anti-HRF IgG (1, 12) were produced, and conjugated with fluorescein isothiocyanate (FITC, Sigma) at pH 9 with subsequent separation of bound and free FITC on a Sephadex G-25 column, PD-10 (Pharmacia). Nonimmune $F(ab')_2$ fragments were conjugated in an identical manner for use as a control. Phycoerythrin-conjugated monoclonal antibodies OKT4 (anti-CD4), OKT8 (anti-CD8) (Ortho Diagnostic Systems), and anti-Leu 19 (anti-NKH-1) (Becton Dickinson) were used as recommended. Analyses were performed on a Becton Dickinson FACS IV flow cytometer. For single-color anal-

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Abbreviations: HRF, homologous restriction factor; C5b6, C7, C8, and C9, complement components 5b6, 7, 8, and 9, respectively; C5b–9, complex of these complement components; CTL, cytotoxic T lymphocytes; PBMC, peripheral blood mononuclear cells; NK, natural killer; C9RP, C9-related protein; FITC, fluorescein isothiocyanate; DOC, deoxycholate.

yses of HRF expression, propidium iodide (Sigma) was added at 5 μg/ml to exclude dead cells from analysis.

Purification of Complement Components and of C9RP. Complement components C5b6 (15), C7 (16), C8 (17), and C9 (18) were purified as described. C9RP was isolated from interleukin 2- (Amgen Biologicals, Thousand Oaks, CA) stimulated human PBMC by immunoaffinity chromatography on a Sepharose anti-human C9RP column (4, 5).

Purification of HRF from OKT3-Activated PBMC. PBMC (1.1×10^9 cells) stimulated for 3 days with OKT3 were solubilized in 2% (wt/vol) deoxycholate (DOC), 50 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 mM EDTA, 2 mM EGTA, and 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 material, and the supernatant was dialyzed against starting buffer (0.02% DOC/50 mM NaCl/20 mM Tris·HCl, pH 8.4). The dissolved protein was passed first over a column of rabbit IgG-Sepharose to remove IgG-binding proteins and then over a Sepharose column containing 300 mg of bound anti-HRF immunoglobulin (12). The column was washed with starting buffer and then with 0.2 M NaCl in starting buffer. HRF was eluted with 2 M NaCl in starting buffer and then 2 M KBr in starting buffer.

Cytotoxic Assays. PBMC (5×10^6 cells) were labeled with ^{51}Cr (300 μCi/ml; 1 Ci = 37 GBq) for 60 min at 37°C and washed three times. Aliquots of 10^6 cells were incubated with various concentrations of $F(ab')_2$ anti-HRF for 30 min at 4°C and washed, and 10^5 cells were added per well to 96-well microtiter plates. Cells were subjected to reactive lysis by the combination of isolated C5b6 (10 μg/ml), C7 (4 μg/ml), C8 (5 μg/ml) and C9 (30 μg/ml) (1) or to lysis by isolated C9RP (20–30 μg/ml) in parallel wells that were incubated for 4 hr at 37°C. After centrifugation, supernatants were removed for measurement of radioactivity, and cytotoxicity was calculated using a standard formula (10). In some experiments, cells were treated with rabbit anti-decay-accelerating factor antiserum at a concentration known to inhibit decay-accelerating factor activity on human erythrocytes (19) and peripheral lymphocytes (unpublished data).

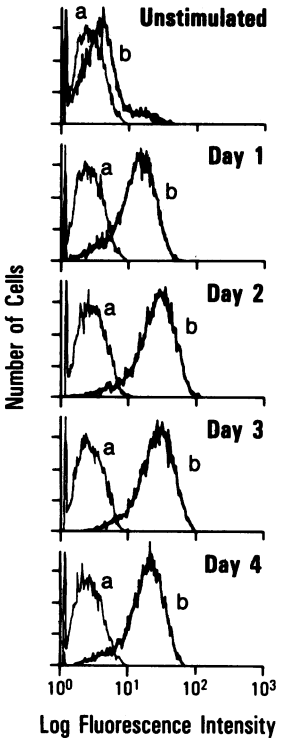

FIG. 1. Induction of expression of HRF on the surface of PBMC upon stimulation with OKT3. Serial histograms were obtained by fluorescence-activated cell sorter analysis of unstimulated and OKT3-stimulated PBMC stained with FITC-conjugated $F(ab')_2$ of rabbit anti-HRF IgG (traces b) or FITC-conjugated $F(ab')_2$ of rabbit IgG from an unimmunized animal (traces a).

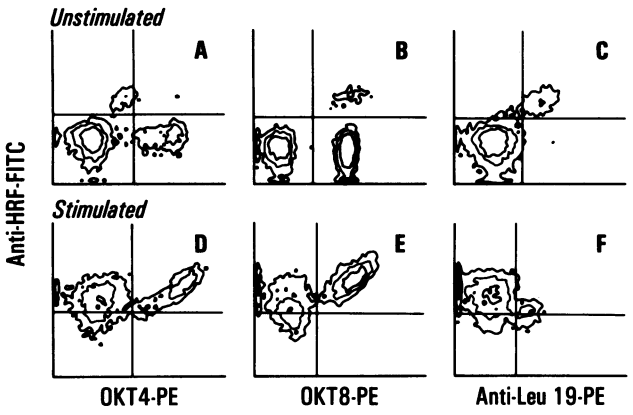

FIG. 2. Phenotype of PBMC expressing surface HRF before and after OKT3 activation. Two-color FACS analyses were performed. (*A–C*) Unstimulated PBMC. (*D–F*) Cells from the same donor stimulated for 3 days with OKT3 (100 ng/ml). PE, phycoerythrin.

Insertion of HRF Isolated from PBMC into the Membrane of Sheep Erythrocytes. Various amounts of lymphocyte HRF (20–112 ng) were added to separate aliquots of sheep erythrocytes (5×10^7 cells). The HRF was allowed to insert into the cells for 2 hr at 37°C in a final DOC concentration of 0.0016% and a total volume of 1 ml. Control cells not receiving HRF were treated with 0.0016% DOC alone for the same length of time (12).

Reactive Lysis of Erythrocytes. Sheep erythrocytes (10^7 cells) with or without bound HRF were incubated at 37°C with the combination of ≈3 μg of C5b6, 2 μg of C7, 2 μg of C8, and 15 μg of C9 in a total volume of ≈50 μl. After 2 hr, hemoglobin release was measured at 412 nm.

RESULTS

Induction of Expression of HRF on the Surface of Anti-CD3-Stimulated PBMC. PBMC stimulated for various lengths of time with the anti-CD3 monoclonal antibody OKT3 were harvested and stained with FITC-conjugated $F(ab')_2$ of rabbit anti-HRF IgG. The serial histograms depicted in Fig. 1, which were obtained by fluorescence-activated cell sorter analysis, demonstrate induction of surface expression of HRF. The majority of unstimulated cells were virtually HRF negative, with a mean fluorescence of approximately twice background. A small population (5–10%) was positive for HRF. At day 2 and 3 of OKT3 stimulation, HRF surface expression of the PBMC reached a maximum of ≈10 times background mean fluorescence, and >90% of the cells were stained.

Phenotype of PBMC Expressing Surface HRF Before and After OKT3 Activation. Phenotypic characterization of OKT3-activated PBMC was performed to correlate known proliferative and cytotoxic activities of subpopulations with sur-

Table 1. Phenotypic distribution of HRF expression in unstimulated and OKT3-stimulated PBMC

PBMC	HRF^+ PBMC, %	HRF-positive cells, %		
		$CD4^+$	$CD8^+$	Leu 19^+
Unstimulated	≈10	<1	≈50	≈80
Day-3 OKT3-stimulated	≈90	≈40	≈30	≈10

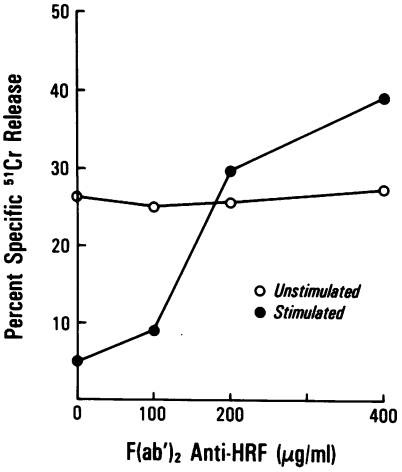

FIG. 3. Reactive lysis by C5b–9 of OKT3-stimulated PBMC: Enhancement by treatment of cells with anti-HRF. ^{51}Cr-labeled unstimulated or day-3 OKT3-stimulated PBMC were incubated with F(ab')$_2$ anti-HRF and subsequently subjected to reactive lysis with isolated C5b6, C7, C8, and C9. Anti-HRF had virtually no effect on unstimulated PBMC. Controls with rabbit anti-decay-accelerating factor at 1:10 final dilution (data not shown) gave 25% specific lysis for unstimulated PBMC and 6% for stimulated PBMC, which was comparable to the zero-antibody controls. Data represent the average of duplicate determinations.

face HRF expression. Two-color analyses performed by fluorescence-activated cell sorter are shown in Fig. 2 for OKT4 (CD4), OKT8 (CD8), and anti-Leu 19 (NKH-1) positive subpopulations. In unstimulated PBMC (Fig. 2 *A–C*) a small percentage (5.3%) of CD8$^+$ cells expressed HRF. Most HRF-positive cells were also Leu 19$^+$ (8.5%) (Fig. 2*C*). In PBMC stimulated for 3 days with OKT3 (Fig. 2 *D–F*), both CD4$^+$ and CD8$^+$ populations similarly stained positive for HRF. The Leu 19$^+$ phenotype consistently (four donors) demonstrated a small population of slightly weaker HRF staining. Table 1 summarizes the phenotypic distribution of HRF expression in unstimulated and stimulated PBMC.

Resistance of OKT3-Stimulated PBMC to Lysis by C5b–9. ^{51}Cr-labeled, unstimulated or day-3 OKT3-stimulated PBMC were incubated with F(ab')$_2$ anti-HRF at 4°C for 30 min. After washing, the cells were incubated with the combination of isolated C5b6, C7, C8, and C9 at 37°C for 4 hr. ^{51}Cr release as a function of anti-HRF dose is shown in Fig. 3. Lysis of unstimulated cells was independent of anti-HRF dose. Stimulated cells demonstrated a marked resistance to reactive lysis that was overcome by treatment of the cells with anti-HRF.

Resistance of OKT3-Stimulated PBMC to Lysis by C9RP. In analogous experiments, anti-HRF-treated PBMC were incubated with purified C9RP in the presence of 10 mM Ca^{2+} for 4 hr at 37°C. As shown in Fig. 4, two representative experiments revealed lytic patterns very similar to those observed with reactive lysis by C5b–9. Upon activation, PBMC acquired resistance to attack by C9RP that, however, was abrogated by blocking of surface HRF with F(ab')$_2$ anti-HRF.

Isolation of HRF from OKT3-Activated PBMC. HRF was isolated from two batches of OKT3-stimulated PBMC. The material shown in Fig. 5 was obtained from 1.1 × 10^9 activated PBMC after solubilization of the cells with DOC, removal of particulate material, and anti-HRF immunoadsorption. The yield of the protein was ≈70 μg, and its molecular weight was ≈65,000, which is comparable to that of HRF isolated from human erythrocyte membranes. Inserted into the membrane of sheep erythrocytes, the activated PBMC-derived HRF caused marked inhibition of hemolysis by C5b–9 (Fig. 6).

DISCUSSION

The killing process executed by CTL and the measures taken by these cells to avoid killing themselves must be closely correlated and interrelated events. Kranz and Eisen (14) have reported that nine tumor cell lines and four noncytolytic T cell lines were effectively lysed by a given murine CTL clone,

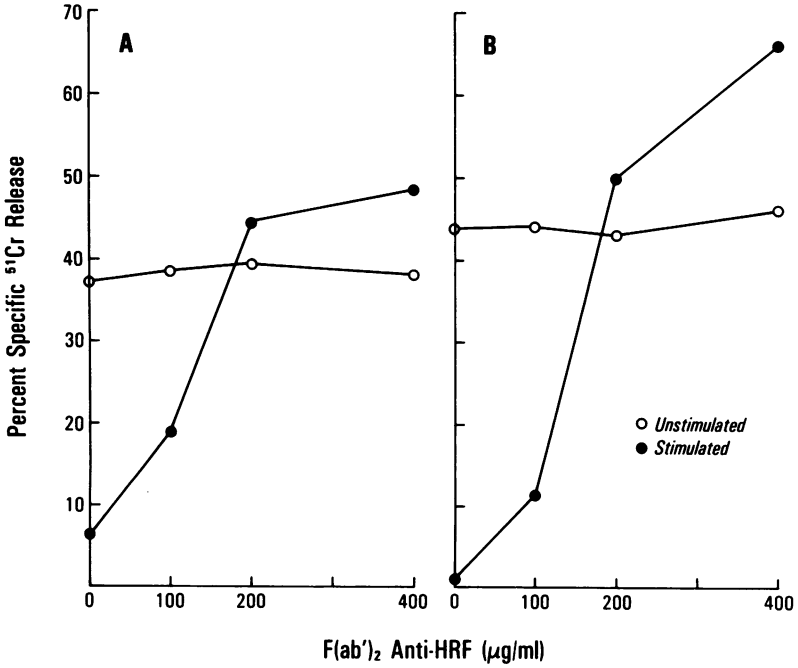

FIG. 4. C9RP-mediated lysis of OKT3-stimulated PBMC: Enhancement by treatment of cells with anti-HRF. ^{51}Cr-labeled unstimulated or OKT3-stimulated PBMC (10^5 cells), treated with F(ab')$_2$ anti-HRF, were incubated for 4 hr with 2 μg (*A*) or 3 μg (*B*) of purified C9RP and 10 mM Ca^{2+} in a total volume of 100 μl. (○) Unstimulated PBMC. (●) PBMC stimulated for 3 days with OKT3 (100 ng/ml). Data represent the average of duplicate determinations.

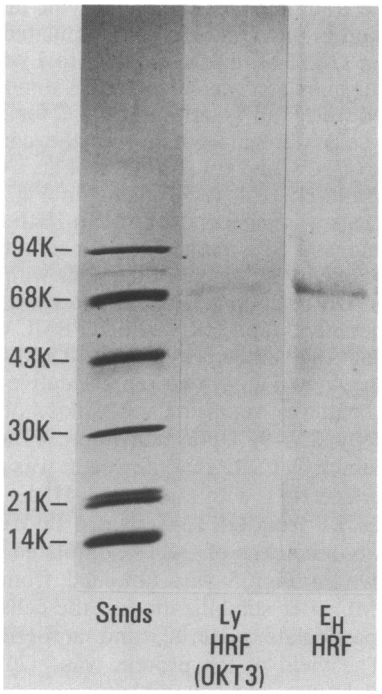

FIG. 5. HRF isolated from OKT3-stimulated PBMC analyzed by $NaDodSO_4$/polyacrylamide (1.7–17%) gel gradient electrophoresis. Lymphocyte (Ly) HRF (1.7 μg) was compared with human erythrocyte (E_H) HRF (1.5 μg). The gel was stained with Coomassie blue. K, kDa; stnds, standard molecular mass markers.

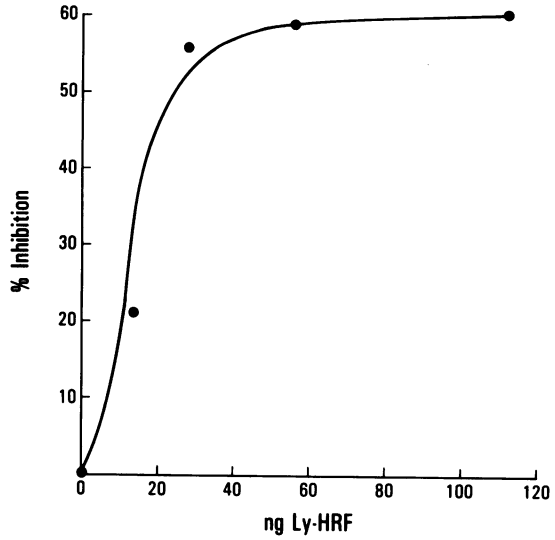

FIG. 6. Inhibition of reactive lysis by lymphocyte (Ly) HRF inserted into sheep erythrocytes. Erythrocytes (10^8 cells) were incubated with various amounts of isolated HRF (0–112 ng) in 1 ml of buffer containing 0.0016% DOC for 2 hr at 37°C. The cells were washed and subjected to reactive lysis by C5b–9. Each point represents the average of duplicate determinations. Control cells (not offered HRF, but incubated with detergent as above) gave 48% lysis with C5b–9.

whereas seven cytolytic T-cell lines were clearly resistant to lysis. The target cells had been coated with a monoclonal antibody to the T-cell receptor to trigger the cytolytic reaction. They found that the cells that were resistant to lysis were nevertheless capable of inducing discharge of the cytolytic granules from effector cells. These authors concluded that "CTL are largely resistant to the effects of cytolytic components released by activated CTL" (14).

The results of the present study might be used to explain the molecular basis of the resistance or self-protection exhibited by killing lymphocytes. They show that human peripheral T lymphocytes, which are largely resting cells, acquire the expression of HRF on their surface upon activation by anti-CD3. Unstimulated PBMC contained only a small proportion of HRF-positive cells, which were identified primarily as the Leu 19^+ subpopulation representing NK cell activity (20). In the OKT3-stimulated PBMC population, the $CD4^+$ and $CD8^+$ cells had become HRF-positive. HRF expression in the stimulated PBMC reached a maximum on day 2 and 3. Previous studies in this laboratory have shown that C9RP content (9) and cytolytic activity (9, 11) of PBMC also were maximal on day 2 and 3 of OKT3 stimulation. Thus, it appears that cells that are induced to acquire the ability to kill also acquire HRF on their surface, whereas NK cells that are ready to kill without further stimulation have HRF on their surface.

The potential importance of the appearance of HRF on T cells undergoing activation lies in the positive correlation of this phenomenon with the acquisition of resistance to lysis by isolated C9RP. Although HRF has been shown to be capable of inhibiting the action of C9RP on cell membranes (12), the resistance of activated T lymphocytes to C9RP-mediated lysis could be unrelated to HRF. The secretory granules of NK cells have been shown to contain proteoglycans of the chondroitin sulfate A type that are released during the cytotoxic reaction (21). It has been postulated (22) that these proteoglycans protect NK cells from autolysis by the secreted cytolytic lymphocyte protein. However, the demonstration that $F(ab')_2$ anti-HRF abrogated resistance of the cells to C9RP lysis suggests that resistance in the present experiments was due to membrane HRF. HRF, therefore, may be regarded as a candidate protein in the search for the molecular basis of the phenomenon of self-protection of cytotoxic lymphocytes.

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