Brief Definitive Report

Human Protectin (CD59), an 18–20-kD Homologous Complement Restriction Factor, Does Not Restrict Perforin-mediated Lysis

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Summary

Human protectin (CD59) is an 18–20-kD membrane glycoprotein that restricts lysis of human erythrocytes and leukocytes by homologous complement. By directly incorporating protectin into membranes of heterologous cells we observed that protectin did not prevent perforin-mediated killing, whereas complement killing was effectively restricted. Further, no significant enhancement of cell-mediated killing or target killing by purified perforin was observed with anti-protectin antibodies. Thus, in contrast with complement lysis, restriction of lysis by protectin does not apply to cell-mediated killing.

The cytolytic activity of the human complement (C) system has been shown to be restricted by a novel 18–20-kD glycoprotein, which is present on membranes of most cells physiologically in contact with human blood. This molecule has been called P-18 (1), MIRL (2), HRF20 (3), H-19 (4), MEM-43 (5), or CD59 (6). To clarify the complexity of used designations the functionally descriptive name "protectin" has recently been suggested (6a). Protectin is a glycosyl phosphatidyl inositol (GPI) anchored membrane glycoprotein, which at the primary sequence level is homologous to the mouse Ly-6 antigen family (6). Functionally, protectin has been shown to act at the final stages of C membrane attack complex (MAC) formation: by interacting with both the C8 and C9 components of MAC it was observed to inhibit the C5b-8 complex accelerated insertion and oligomerization of C9 in the lipid bilayer (6a).

Perforin isolated from cytotoxic T cells and NK cells has a lytic activity that resembles that of the C system. Both perforin and MAC have been shown to induce changes in membrane potential and ring-like "lesions" on the target cell membranes (7, 8). The lesions are believed to correspond to tubular polymers of perforin or C9 molecules. At the primary sequence level perforin shows a limited (17–21%) homology to C9 as well as to other terminal C components (C7, C8α, and C8β), and perforin appears to share the putative membrane spanning region and the cysteine-rich epidermal growth factor-type domain, but not the thrombospondin or the low density lipoprotein receptor type domains with the terminal C components (9).

In light of the structural and functional similarities between perforin and the MAC, it is of interest to know whether common mechanisms exist that inhibit both C- and cell-mediated cytotoxicity. The aim of this study was to discover whether lysis by human perforin is restricted by protectin. This question was addressed by studying the effects of antiprotectin antibodies on cell-mediated killing and on perforin lysis of protectin-positive human cells and by comparing the effect of human protectin incorporation on subsequent lysis of nonhuman targets by perforin and complement.

Materials and Methods

Purified Components and Antisera. Human protectin was isolated from detergent-solubilized human E membranes using a rat anti-CD59 mAb (YTH 53.1) Sepharose column (6). Partially purified, functionally active human perforin was extracted from granules of human large granular lymphocytes (9), and mouse perforin was purified from the granules of the murine cytotoxic T cell line CTLL-2B1/6 (10). Complement $C\overline{56}$ complexes and components C7, C8, and C9 (11) were isolated as described. The rabbit polyclonal anti-protectin antibody and the purified IgG fractions of the rat monoclonal antiprotectin (YTH53.1, IgG2b) and of the control anti-glycophorin A (YTH89.1, IgG2b) antibodies were prepared as described (6). Proteins were radiolabeled with ^{125}I using the Iodogen method (Pierce Chemical Co., Rockford, IL).

NK Cell-mediated Killing. The ability of the antiprotectin antibodies to alter the sensitivity to lysis of the human NK sensitive target K562 (a human erythroleukemia cell line; European Animal Cell Collection, Porton Down, Wilts., U.K.) was examined in a

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standard 4-h ^{51}Cr-release assay. Effector cells were isolated from PBMC by passage over a nylon-wool column to remove adherent cells and either used fresh or following stimulation for 6 d with 20 U/ml of rIL-2 (ICN Biochemicals, High Wycombe, UK). The K562 cells were maintained in culture in RPMI 1640 (Gibco Laboratories, Grand Island, NY) medium, which was supplemented with 10% (vol/vol) FCS. The cells were labeled with 100 μCi of ^{51}Cr in 0.2 ml RPMI-10% FCS for 1 h, washed, resuspended into 10 ml RPMI-10% FCS, and incubated for a further 1 h. After washing, 10^4 cells/50 μl were mixed with the appropriate antibody in a total volume of 150 μl and incubated for 15 min before the addition of 2 × 10^5 effector cells/50 μl. After 4 h at 37°C, 0.1 ml of the supernatant was removed and counted in a gamma counter to determine the percent cytotoxicity.

Lysis Assays. To study the effect of YTH53.1 and YTH89.1 IgG on human perforin-mediated lysis (9) of human erythrocytes, 10^7 cells in 100 μl TBS-Ca^{2+} (0.05 M Tris-HCl in physiological saline, 5 mM $CaCl_2$, pH 7.4) were mixed and preincubated (5 min at 37°C) with 50 μl of antibody dilution and subsequently incubated (30 min at 37°C) with increasing doses of perforin. Lysis was detected as hemoglobin release by absorbance at 412 nm. Lysis of K562 cells was tested after similar pretreatment with the antibody by incubating (60 min at 37°C) the cells in the presence of various concentrations of mouse perforin. To observe the effects of antibody on C lysis antibody (or buffer), pretreated K562 cells were mixed with dilutions of fresh human serum and CVF (25 μg/ml) and incubated for 1 h at 37°C. Cell lysis was determined as release of lactate dehydrogenase (LDH).

Incorporation of ^{125}I-Protectin into Cell Membranes. Purified protectin was incorporated into membranes of guinea pig (GPE), rabbit, and mouse erythrocytes by incubating (1 h at 37°C) 10^8 cells/tube with increasing amounts of ^{125}I-labeled protectin in TBS, 0.0005% Lubrol PX. Cells were washed twice with TBS and counted for radioactivity. The number of incorporated protectin molecules per cell was calculated on the basis of the known specific activity of the labeled preparation. Control cells of each species were treated similarly with radiolabeled sheep anti-rabbit IgG in an equivalent amount of detergent.

Perforin lysis of heterologous red cells with varying amounts of incorporated ^{125}I-protectin was performed by incubating (30 min at +37°C) 0.25 × 10^8 cells/tube with perforin in TBS-Ca^{2+}.

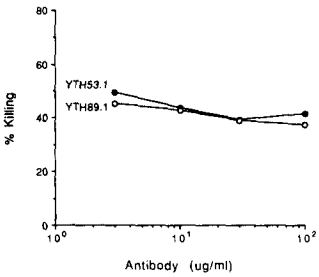

Figure 1. The effect of rat anti-CD59 (antiprotectin) mAb (YTH53.1) and anti-glycoprotein A antibody (YTH89.1) on killing of the ^{51}Cr-labeled K562 erythroleukemia cells by rIL-2-activated human PBMC. The E/T ratio was 20:1 and killing was determined after a 4-h incubation at 37°C.

The amount of the perforin preparation used was adjusted to give 50% lysis of unincorporated cells. For C-mediated killing (reactive lysis) 0.25 × 10^8 cells with varying amounts of incorporated ^{125}I-protectin were mixed with C56 (8.4 μg) and human EDTA plasma (final dilution 1:4 in a total volume of 100 μl) as a source of late C components (C7, C8, and C9). Possible nonspecific effects of protectin incorporation or of antibodies were controlled by studying red cell lysis by melittin and saponin (BDH, Poole, UK).

Results

The anti-CD59 (antiprotectin) mAb YTH53.1, which previously has been shown to enhance reactive lysis (6), did not have any detectable effect on killing of the K562 target cells by IL-2-activated human peripheral blood NK cells (Fig. 1). K562 cells, which by FACS analysis were found to express protectin on their surface, were lysed to the same extent in the presence of the antiprotectin or the isotype-matched control antibody, and no dose-response effects of the antibodies could be detected. The same result was obtained when fresh peripheral blood nonadherent mononuclear cells were used as effector cells or when the human NK insensitive B cell lymphoma Raji was used as a target (not shown).

NK cell cytotoxicity is not mediated by perforin alone but involves other factors. Therefore, we analyzed whether the YTH53.1 antibody has any effects in direct perforin killing assays. As shown in Fig. 2 *a*, the YTH53.1 IgG appeared

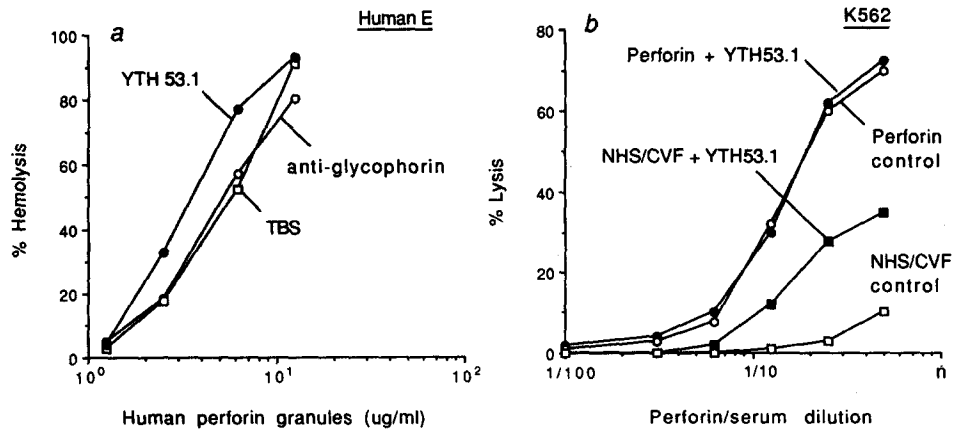

Figure 2. The effect of the YTH53.1 (antiprotectin) antibody on perforin-mediated lysis of human erythrocytes (*a*) or K562 erythroleukemia cells (*b*). (*a*) Human RBCs, 2 × 10^7 cells/tube (in TBS-Ca^{2+}), were mixed with increasing concentrations of partially purified human perforin in the presence of the YTH53.1 (●) or control (○; YTH89.1, antiglycophorin) antibody at 125 μg/ml or buffer (TBS; □). Lysis was assessed as hemoglobin release (A412) after a 30-min incubation at 37°C. Means of triplicate determinations are shown. The effect of YTH53.1 antibody on lysis of K562 cells by mouse perforin. The K562 cells were pretreated with the YTH53.1 antibody (●, ■) or Hepes buffer (○, □) and incubated in the presence of indicated dilutions of either mouse perforin (original, 1 μg/ml; ●, ○) or of fresh normal human serum (NHS) plus CVF (■, □). Lysis of K562 cells was determined as LDH release.

to slightly enhance human red cell lysis by partially purified human perforin as compared with the control antibody. When a rabbit polyclonal antiprotectin antibody was tested in a similar assay, no enhancement of human red cell lysis could be observed. Using highly purified mouse perforin, we could not see any effect of the YTH53.1 IgG on lysis of human red cells (not shown) or of the nucleated K562 cells (Fig. 2 *b*). In all these assays the amounts of antibodies used caused a clear-cut enhancement of C lysis.

To analyze directly the effects of protectin on perforin- and C-mediated killing, the purified membrane form of protectin was incorporated into membranes of erythrocytes from three different species: guinea pig (GPE), rabbit, and mouse. On the basis of the known specific radioactivity of ^{125}I-protectin it was calculated that maximally 6,500 molecules had become bound per cell. The degree of incorporation did not considerably differ between the three species.

As shown in Fig. 3 *a*, the incorporated human protectin converted the normally C-sensitive GPEs into C resistant cells, but no change in the sensitivity to lysis by human (Fig. 3 *a*) or mouse perforin (Fig. 3 *b*) was observed upon incorporation. The pattern was similar with cells from the two other species (rabbit and mouse) studied (not shown). Thus, protectin lacked a restrictive effect upon killing by perforin regardless of whether the latter was of human or murine origin.

Discussion

Protectin (CD59) is a recently discovered potent MAC inhibiting factor (1–3, 6). In this study we have analyzed whether protectin is also capable of preventing lysis by perforin, which is an important cytolytic mediator in cell-mediated killing.

Human perforin is nonspecific in its killing function (12). A possible explanation for this nonspecificity is that the target (or receptor) molecule for perforin is phosphorylcholine (13), an almost ubiquitous membrane constituent. Target recognition by C is more complex. The lytic molecule C9 does not by itself insert into the membrane, but requires the preassembly of the C5b-8 complex. Formation of the C5b-8 complex involves several steps, some of which are influenced by specific control factors on the membrane.

The rat anti-CD59 mAb YTH53.1 has proved a useful tool to neutralize the C lysis–restricting effect of protectin both on human red cells and on nucleated cells (6, 6a, Rooney, I.A., A. Davies, S. Meri, J.D. Williams, D. Griffiths, and B.P. Morgan; manuscript submitted for publication). In contrast to its effects in C lysis, the YTH53.1 IgG did not enhance NK cell–mediated cellular cytotoxicity (Fig. 1). In direct lysis assays with human, but not with mouse, perforin (Fig. 2) this antibody had a slight lysis-enhancing effect, although quantitatively this effect was much smaller than that observed in C lysis. The possibility of a nonspecific lysis-enhancing effect of the antibody was excluded in experiments using melittin or saponin as lytic agents.

To assess more directly the differential role of protectin in C- and perforin-mediated lysis, purified human protectin was incorporated into membranes of erythrocytes from three different heterologous species. Results of this experiment (Fig. 3) clearly illustrate the lack of any significant effect of protectin in perforin lysis. Also, neither restriction nor enhancement of melittin or saponin lysis was observed with incorporated protectin.

Taken together these results show that protectin is primarily a MAC inhibitor and has no significant protective role against perforin lysis. Our results thus confirm recent studies that suggest that GPI-linked glycoproteins are not involved in control of cell-mediated killing (9, 12, 14, 15). The mechanisms of protection of the killer cells themselves against perforin lysis are still unknown.

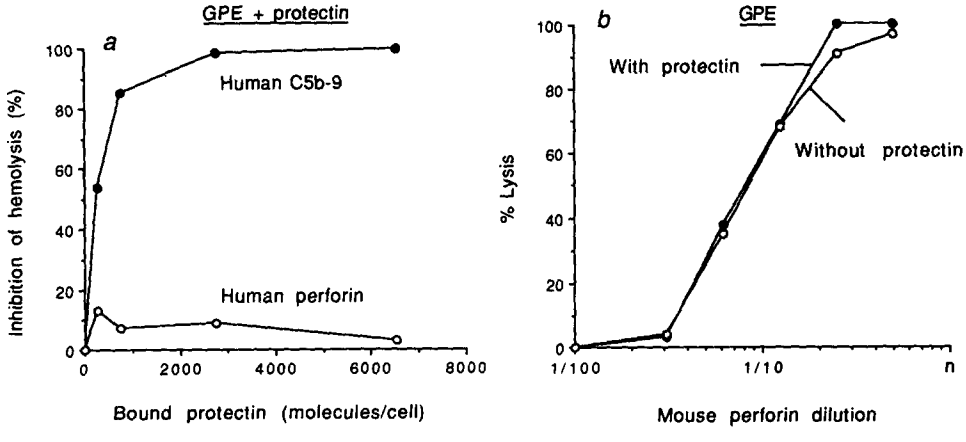

Figure 3. The effect of protectin incorporation on perforin and C lysis. (*a*) Purified human protectin was incorporated into membranes of guinea pig erythrocytes (GPE) as described in Materials and Methods. For C-mediated killing (●), 0.25 × 10^8 cells were mixed with C5$\bar{6}$ and human EDTA plasma (final dilution 1:4 in TBS) as a source of late C components (C7, C8, and C9). Cell lysis was determined as hemoglobin release (412 nm) after a 10-min incubation at 37°C. For perforin lysis (○) 0.5 μg of partially purified human perforin was mixed with 0.25 × 10^8 cells in TBS-Ca^{2+} and incubated for 30 min at 37°C. The effect of incorporated protectin on hemolysis is expressed as percentage inhibition of initial hemolysis in the absence of incorporated protectin. (*b*) The effect of protectin incorporation on GPE lysis by mouse perforin. The sensitivity of GPEs carrying ~2,500 protectin molecules per cell (●) to lysis by indicated amounts of mouse perforin was compared with GPE with no incorporated protectin (○). Original perforin concentration was 1 μg/ml. Each point represents a mean of triplicate determinations. Lysis was measured by Hb release at 412 nm.

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