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

By Seppo Meri,* B. Paul Morgan,† Mark Wing,* Jane Jones,† Alexandra Davies,† Eckhard Podack,§ and Peter J. Lachmann*

From the *Molecular Immunopathology Unit, Medical Research Council, Cambridge, CB2 2QH; the †Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, United Kingdom; and the §Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Florida 33136

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.

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 C56 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–glycoporin A (YTH89.1, IgG2b) antibodies were prepared as described (6). Proteins were radiolabeled with ¹²⁵I 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...
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 $\mu$Ci 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$^6$ cells/50 $\mu$l were mixed with the appropriate antibody in a total volume of 150 $\mu$l and incubated for 15 min before the addition of 2 x $10^4$ effector cells/50 $\mu$l. After 4 h at 37°C, 0.1 ml of the supernatant was removed and counted in a gamma counter to determine the percent cytolysis.

**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 $\mu$l 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 $\mu$l 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 $\mu$g/ml) and incubated for 1 h at 37°C. Cell lysis was determined as release of lactate dehydrogenase (LDH).

**Incorporation of $^{125}$I-Protection into Cell Membranes.** Purified protection was incorporated into membranes of guinea pig (GPE), rabbit, and mouse erythrocytes by incubating (1 h at 37°C) 10$^7$ cells/tube with increasing amounts of $^{125}$I-labeled protection in TBS, 0.0005% Lubrol PX. Cells were washed twice with TBS and counted for radioactivity. The number of incorporated protection 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-protection was performed by incubating (30 min at +37°C) 0.25 x $10^8$ cells/tube with perforin in TBS-Ca$^{2+}$.

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

**Results**

The anti-CD59 (antiprotection) 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
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 125I-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 pre-assembly 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.

Figure 3. The effect of incorporatoin 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 x 10^6 cells were mixed with C5b 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 hemo-
globin release (412 nm) after a 10-

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 perforin 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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