RESISTANCE OF CYTOLYTIC LYMPHOCYTES TO
PERFORIN-MEDIATED KILLING

Lack of Correlation with Complement-associated Homologous
Species Restriction

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The mechanisms of killing mediated by cytolytic lymphocytes have been extensively studied in recent years (1–10). One key cytolytic mediator, a pore-forming protein (PFP;[1] also termed perforin, cytolysin, or C9-related protein) has been identified both in CTL and NK cells. Perforin is released from the granules of cytotoxic lymphocytes onto target cell surfaces where it is thought to form transmembrane channel lesions (6–10). Recent work from this and other laboratories has shown that cytolytic lymphocytes, while killing their targets, do not undergo self-mediated lysis (11–15) and that both CTL and NK cells also resist lysis by purified granules and perforin (13–15). How lymphocytes protect themselves against the lytic effect of perforin is currently unknown.

Perforin is related structurally, functionally, and antigenically to the terminal components of the C cascade (16–19), which, like perforin, produce channel-like lesions on target cell membranes when activated. Erythrocytes are generally resistant to lysis mediated by C of the same species, a phenomenon known as homologous species restriction (20–23). Homologous restriction is thought to be mediated by a C8/C9-related erythrocyte surface protein (C8-binding protein [C8BP] or homologous restriction factor [HRF]) (24–26). C8BP/HRF has likewise been suggested to mediate protection of lymphocytes against lysis mediated by perforin (27, 28). Whereas these studies have suggested that protection against C- and perforin-mediated lysis depends on common mechanisms, the data presented here indicate that the two forms of protection are distinct and may require different mechanisms/polypeptides.

[1] *Abbreviations used in this paper:* C8BP, C8-binding protein; HRF, homologous restriction factor; HU, hemolytic unit; LAK, lymphokine-activated killer; PFP, pore-forming protein.

Materials and Methods

Animals. 6–10-wk-old CD_2F_1 mice were purchased from the Trudeau Institute (Saranac Lake, NY). Male Sprague Dawley rats ($\sim$400 g) were purchased from the Charles River Breeding Laboratories, Inc., (Wilmington, MA).

Cell Lines. Murine CTL lines CTLL-R8 and CTLL-1 were maintained in αMEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS and 10% IL-2-containing leukocyte-conditioned medium. The target cell specificities of these lines have been reported elsewhere (29). WEHI-164 (methylcholanthrene-induced murine fibrosarcoma), EL-4 (mouse T cell lymphoma), and K562 (human erythromyloid leukemia cell line) were maintained in culture in RPMI 1640 (Gibco Laboratories) supplemented with 5% FCS (RPMI/FCS).

Antibodies. Polyclonal anti-RBC antisera (goat anti–rabbit RBC, rabbit anti–mouse RBC) and goat anti–mouse IgG Fc fragment antisera were purchased from Organon Teknika Corp. (West Chester, PA). Polyclonal anti-asialo GM1 and anti-laminin antisera were purchased from Wako Chemicals (Dallas, TX) and from Biodesign Inc. (Kennebunkport, ME), respectively. mAbs to human CD3 (OKT3, IgG2a) and to murine Thy-1.2 (TIB 99, IgM) were produced in our laboratories from hybridomas obtained from American Type Culture Collection (Rockville, MD). Antibodies to rat OX8, OX19, and OX39 were purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). Production and characterization of the anti-CD16 antibody B73.1 (IgG1) has been described previously (30). FITC-labeled goat $F(ab)'_2$ fragments directed against IgG H and L chains were used for indirect immunofluorescence and were purchased from Tago Inc. (Burlingame, CA).

A polyclonal antiserum specific for murine CTLL-R8 cells was prepared in rabbits immunized subcutaneously with 3×10^7 live CTLL-R8 cells suspended in PBS, and given five boosts, one every 3 wk. Serum was collected 5 d after each boost. This antiserum reacts with >95% of R8 cells as analyzed in a FACS (Becton Dickinson & Co., Mountain View, CA) and was used at 1:40 dilution.

Complements. The sera used as source of C were from guinea pig, rabbit, and mouse (CD_2F_1). Sera from more than one animal were rapidly processed and pooled.

RBCs. RBC of goat, cow, sheep, swine, rabbit, rat, chicken, and goose were purchased from Carolina Biological Supply Co., (Burlington, NC). All RBC preparations were maintained in sterile Alsever's solution (Gibco Laboratories) at 4°C and used before the expiration date. Fresh mouse and human RBC were collected, stored in Alsever's solution at 4°C, and used within 2 wk.

Murine Thymocytes. Thymuses were removed aseptically from CD_2F_1 mice. Single cell suspensions were prepared by mincing with the hub of a syringe in RPMI 1640. The cell suspensions were passed through 100-gauge steel mesh, washed three times in RPMI 1640, and resuspended in the same medium.

Rat and Human LAK/NK Cells. Rat LAK cells were prepared according to a procedure modified from a published protocol (31). Rat spleens were aseptically removed and single cell suspensions were prepared in RPMI 1640. Contaminating RBC were lysed with NH_4Cl (0.88%) solution, followed by two washes in serum-free RPMI 1640 medium. After passage through a nylon wool column, the nonadherent cells were cultured in RPMI 1640/FCS medium containing 1,000 U/ml rIL-2 (generously supplied by Cetus Corp., Emeryville, CA). After a 2-d incubation the culture plates were washed with RPMI 1640 containing 2% FCS, and the adherent cells were incubated with rIL-2-containing medium for an additional 2 d. On day 4, typically, >95% of the cells were positive for the NK markers OX8, asialo GM1, and laminin, and <8% of them were positive for the T cell markers OX19 and OX39, as determined by FACS analysis.

Human lymphokine-activated killer (LAK) cells were prepared according to a published protocol (32, 33) with minor modifications. Briefly, human PBMC were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient centrifugation from plateletpheresis leukocyte concentrates obtained from the New York Blood Center, New York. PBMC were depleted of monocytes and B lymphocytes by adherence to plastic dishes and to nylon wool columns, respectively (34). Plastic- and nylon wool–nonadherent lymphocytes were washed and resuspended in RPMI 1640/FCS. A negative selection procedure (panning with anti-CD3 OKT3 antibodies) was used to enrich for NK cells (35). Briefly, cells were

incubated with OKT3 hybridoma supernatant (at 1:3 dilution) for 1 h at 4°C, washed, and resuspended (3 × 10⁶ cells/ml) in RPMI 1640/FCS. Washed cells were then plated in petri dishes previously coated with goat anti-murine IgG (120 μg IgG per 60 × 15-mm dish). Plates were then centrifuged at 40 g for 4 min and incubated at 4°C for 60 min, after which, the nonadherent cells were collected and washed three times with serum-free RPMI 1640. This procedure yielded a cell population enriched for cells with NK cell phenotype, with 95% of cells staining positive for the NK cell marker B73.1, as determined by FACS analysis. Cells were resuspended (2 × 10⁶ cells/ml) in RPMI 1640, supplemented with 10% FCS, 2 mM glutamine, 5 × 10⁻⁵ M 2-ME (Sigma Chemical Co., St. Louis, MO), penicillin (50 U/ml), and streptomycin (50 μg/ml). Human rIL-2 was added to a concentration of 1,000 U/ml. After 4 d of incubation at 37°C in 5% CO_2, cells were harvested and used as LAK cells.

In some experiments, large numbers of homogeneous human NK cell populations were purified from short-term cultures of PBMC with B lymphoblastoid cell line RPMI 8866 as described (36, 37). Briefly, PBMC from healthy donors were cultured with 30 Gy-irradiated RPMI 8866 cells. The cells were collected on day 9, and homogeneous preparations of CD16⁺/NKH1⁺/CD3⁻/CD5⁻ NK cells were negatively selected and purified by density gradient centrifugation after sensitization of the lymphocytes with a mixture of anti-CD3 (OKT3), anti-CD5 (B36.1), and anti-monocyte (B52.1) mAbs, and indirect rosetting with $CrCl_3$-treated goat anti-mouse Ig-coated erythrocytes (36). The purity of NK cells, as tested by indirect immunofluorescence with a panel of anti-NK and anti-T mAbs, always exceeded 95%. The purified NK cells were kept in culture (10⁶ cells/ml) with rIL-2 (100 U/ml) for 4 d. The cells were then collected, washed three times with PBS, and kept frozen as a pellet at −70°C until used to prepare extracts enriched for PFP/perforin.

Preparation of Cell Extracts Enriched for Perforin from Human and Rat LAK/NK Cells. IL-2-activated human NK cells were resuspended (2 × 10⁷/ml) in relaxation buffer (130 mM KCl, 5 mM NaCl, 1 mM disodium ATP, 2 mM $MgCl_2$, 10 mM Hepes, 1 mM EGTA, and 1 mM PMSF, pH 6.8) and disrupted by nitrogen cavitation, as previously described (29). After centrifugation at 800 g for 10 min, the pellet, containing hemolytic activity, was resuspended in PBS (2 × 10⁸ cell equivalents/ml) and subjected to three cycles of freezing and thawing. After sedimentation of debris in a microfuge for 5 min, the supernatant, containing all the hemolytic and cytolytic activity, was collected. This material was stored frozen (−20°C) until used as a source of perforin-containing extract from human NK cells.

Perforin-containing extracts from rat LAK/NK cells were prepared by directly resuspending cells in PBS (2 × 10⁷ cells/ml) followed by three cycles of freezing and thawing. The nucleus-free supernatant after a 5-min centrifugation in a microfuge was collected and kept at −20°C until use. A granule-enriched material from CTLL-R8 cells (29) was used as source of mouse perforin.

Purification of Murine Perforin. Murine perforin was purified from CTLL-R8 cells, following modifications made on a previously published protocol (29, 38). Briefly, nucleus-free lysates of R8 cells (5–10 × 10⁹) were centrifuged at 39,000 g for 20 min. The organelle-enriched pellets were resuspended in relaxation buffer (10⁸ cell equivalents/ml) to which the same volumes of 2× extraction buffer (4 M $NaKHPO_4$, pH 6.5, 1 mM EGTA, 0.2 mM diisopropyl fluorophosphate [Sigma Chemical Co.]) were added and the mixtures were incubated on ice for 1 h. After centrifugation at 39,000 g for 30 min, the upper fluffy liquid phases containing most of the hemolytic activity were collected and resuspended in 400 mM NaCl/20 mM Tris-HCl, pH 8. After another centrifugation (39,000 g, 30 min), the PFP-enriched supernatant was diluted fivefold in buffer A (20 mM Tris HCl, 1 mM EGTA, pH 7.2) and subjected to ion-exchange chromatography using an FPLC system (Pharmacia Fine Chemicals). The starting material was first applied to a DEAE-Sepharose column equilibrated with buffer A and eluted with a 0.1–1 M NaCl linear gradient (in 150 min) at 3 ml/min. The fractions containing hemolytic activity were pooled, diluted 10-fold in buffer A, loaded on a Mono Q column, and eluted at 0.5 ml/min (gradient, 0.1–1 M NaCl in 45 min). The active fractions from the Mono Q column were further fractionated on a Superose 12 column, equilibrated, and eluted with 300 mM NaCl, 20 mM Tris-HCl, pH 7.2, and 1 mM EGTA at 0.3 ml/min. Active fractions were pooled and used as a source of purified perforin. Purification of ∼1,000-fold was achieved with this modified protocol. On a SDS-polyacrylamide gel, a single band

migrating with M_r of 70,000 could be visualized by staining with silver nitrate. Since the hemolytic activity of perforin is labile and has a short half-life (29), this purification fold probably represents an underestimate of the actual extent of purification achieved.

Complement-mediated Hemolysis.   Mouse and rabbit RBC were washed and resuspended (5 × 10^8/ml) in dextrose/gelatin-containing veronal buffer (DGVB^{2+}) (Diamedix Corp., Miami, FL) as described (39). Mouse or rabbit RBC suspension was mixed (1:1, vol/vol) with rabbit anti–mouse RBC antiserum diluted 1:200 in gelatin-containing veronal (GVB^{2+}) with 10 mM EDTA (Diamedix Corp.) or with goat anti–rabbit RBC antiserum diluted 1:20, respectively. The cell mixtures were incubated on ice for 30 min, after which cells were washed three times and resuspended (5 × 10^8/ml) in GVB^{2+}. Sera from mice, rabbits, and guinea pigs were diluted 1:10, 1:20, and 1:40, respectively, in GVB^{2+}. 150 μl of diluted sera were added in triplicates to a 96-well plate, followed by addition of 20 μl of a suspension of antibody-coated mouse or rabbit RBC. After shaking on a plate minishaker (Dynatech Laboratories, Inc., Alexandria, VA) for 1 min, the plate was incubated at 37°C for 60 min, after which it was centrifuged at 200 g for 10 min. 50 μl of the cell-free supernatant from each well was transferred to a new plate. Release of hemoglobin was determined spectrophotometrically at 410 nm using an automated microplate reader (model MR700; Dynatech Laboratories, Inc.). Hemolysis was calculated according to the formula: percent hemolysis = 100 × (OD_{410} experimental release – OD_{410} spontaneous release)/(OD_{410} total release – OD_{410} spontaneous release). Spontaneous or total release of hemoglobulin was determined in wells in which RBC suspension was incubated with 150 μl of GVB^{2+} or water, respectively.

Perforin-mediated Hemolysis.   Purified perforin or perforin-containing extracts diluted in PBS were added, 140 μl per well in triplicates, to 96-well plates; 20 μl of RBC suspension (5 × 10^8/ml PBS) and 10 μl of 20 mM $CaCl_2$ solution were then added sequentially. The plates were shaken for 1 min and incubated at 37°C for 30 min. Hemolysis was measured as described above. The titer of each perforin preparation used in these assays was determined before the test: one hemolytic unit (HU) of PFP was defined as the amount of material required to achieve 50% hemolysis of 10^7 murine RBC in a volume of 170 μl during a 30-min incubation at 37°C. Because perforin activity is labile, there is usually a slight change in the final hemolytic activities when experiments are performed after HU of perforin activity have been determined. In most experiments, therefore, one predetermined HU of perforin does not lead to exactly 50% hemolysis of murine RBC, as would be expected otherwise. To ensure validity of all analyses, experiments in which the activities of both C and perforin were compared were all performed simultaneously, using the same batches of lytic reagents and cells.

C- and Perforin-mediated Cytolysis of Nucleated Cells.   Nucleated cell targets were washed 3 times with RPMI 1640 and resuspended (8 × 10^6 cells/ml) in 0.25 ml FCS containing 0.05 mCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA). After a 60-min incubation at 37°C with occasional shaking, cells were washed three times, resuspended in serum-free RPMI 1640, and stored on ice until use.

For C-mediated lysis, the ^{51}Cr-labeled cells were resuspended in RPMI 1640 containing specific antibodies at the indicated dilutions and incubated on ice for 30 min, after which the cells were washed three times and resuspended in RPMI 1640 to 10^5 cells/ml for cell lines and 2 × 10^5 cells/ml for primary cells. 100 μl of cell suspension was added in triplicates to round-bottomed microtiter wells and mixed with 40 μl of C-containing fresh sera diluted 1:10, 1:20, or 1:40 in RPMI 1640. After a 60 min incubation at 37°C, 70 μl of the supernatant in each well was collected for determination of radioactivity. The percent of specific ^{51}Cr release was determined as described (13).

In experiments using perforin-containing material, the indicated amount of perforin in 40 μl of RPMI 1640 (without $CaCl_2$) was mixed with 100 μl of ^{51}Cr-labeled target cells in a microtiter well in triplicates and incubated at 37°C for 3 h, after which the ^{51}Cr release was measured as before.

Results

Homologous Species Restriction Associated with C and Perforin.   Most studies describing the phenomenon of homologous species restriction were performed using the reac-
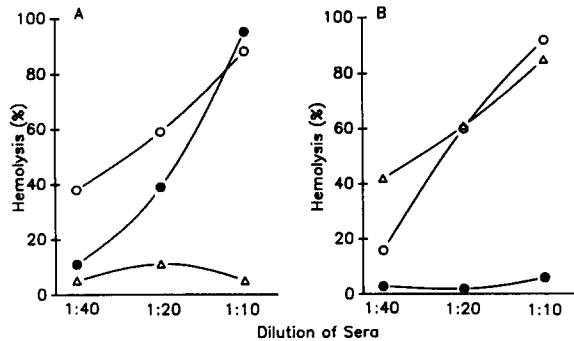
FIGURE 1. Hemolysis of rabbit and murine RBC by homologous and heterogous C. Antibody-coated mouse (A) and rabbit (B) RBC were incubated with the indicated dilutions of guinea pig (O), rabbit (●), and mouse (△) sera at 37°C for 1 h. Hemolysis was determined as described in Materials and Methods. Mouse and rabbit RBC were coated with rabbit anti–mouse RBC antisera and goat anti–rabbit RBC antisera, respectively.

tive lysis method, that is, by incubating RBC with defined quantities of purified C5b-6, C7, C8, and C9; this phenomenon, however, can also be easily visualized treating antibody-coated RBC with fresh sera as sources of C. In the experiment shown in Fig. 1 A, rabbit, guinea pig, and mouse C-mediated lysis was tested using mouse RBC sensitized with rabbit anti–mouse RBC antibodies. Rabbit and guinea pig, but not mouse, sera readily induced hemolysis. Conversely, rabbit RBC coated with goat anti–rabbit antibodies were efficiently lysed by mouse and guinea pig sera but poorly by rabbit sera (Fig. 1 B). These complementary results indicate that all sources of C used in our experiments were active, and furthermore, that the resistance of RBC to C-mediated lysis is independent of the source of antibody (goat vs. rabbit).

We next tested whether homologous species restriction was also applicable to PFP/perforin of lymphocytes. Perforin purified from mouse CTLL-R8 was tested against RBC from 10 different species (Fig. 2). RBC from all 10 species tested (cow, goat, sheep, swine, goose, chicken, rabbit, rat, mouse, and man) were susceptible to mouse perforin-mediated lysis, though at different degree. Similar results were obtained using partially purified granule-enriched material from CTLL-R8 (data not shown), consistent with the earlier observations that the hemolytic activities associated with cytolytic lymphocytes can be accounted for entirely by perforin. Several different batches of RBC and perforin yielded similar results. Although RBC from the different species were somewhat variable in their sensitivity to perforin-mediated lysis, no clear pattern of restriction could be observed, and the absolute
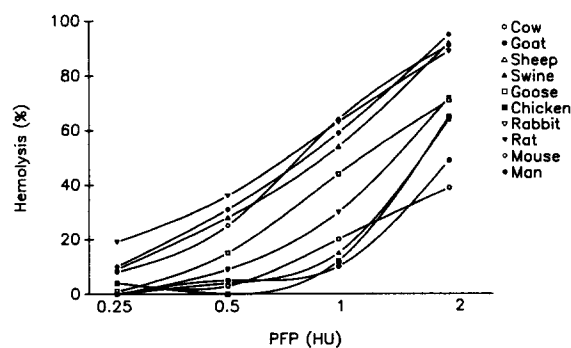

FIGURE 2. Hemolysis mediated by purified PFP/perforin. RBC from the 10 indicated species were incubated with purified murine PFP/perforin in the presence of 1 mM CaCl₂ at 37°C for 30 min. The PFP HU were defined using mouse RBC as targets (see Materials and Methods).
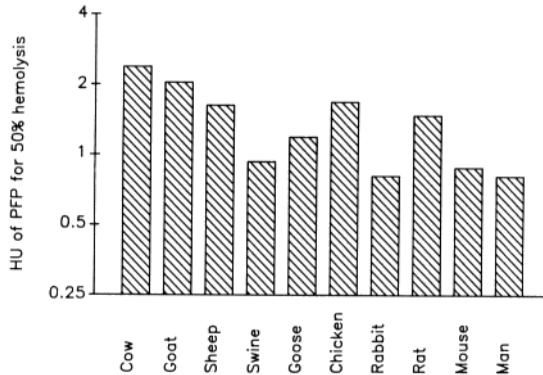
FIGURE 3.   Amount of murine PFP/perforin required to achieve 50% hemolysis of an equivalent number of RBC. HU is defined using mouse RBC as targets (see Materials and Methods). Data were interpolated from Fig. 2. The HU for 50% hemolysis of murine RBC is not exactly 1, as expected, mainly because perforin is labile and determination of HU of activity was performed before each comparative experiment (see Materials and Methods).

amount of murine PFP/perforin required to lyse 50% of the RBC from any of the species tested did not vary significantly (Fig. 3).

The lack of homologous species restriction for perforin-mediated lysis of RBC was confirmed using perforin-enriched material obtained from LAK/NK cells of rat (Fig. 4) and human (Fig. 5) origin. Perforin-containing cell extracts obtained from these cell types lysed both homologous and heterologous RBC (Figs. 4 and 5). However, some differences in target cell susceptibility were noted when rat and human perforin were compared with mouse perforin. Perforin from all three sources were capable of lysing homologous RBC. Whereas mouse perforin lysed significant proportions of RBC from all species tested, 1 HU of rat perforin-containing extract lysed only 6% of swine RBC and none of cow, goat, and sheep RBC (Fig. 4), and no hemolysis of swine, sheep, goat, and cow RBC was detected with 1 HU of human perforin (Fig. 5). It should be noted that HU in all instances was defined using mouse RBC as target cells (Materials and Methods).

C- and Perforin-mediated Lysis of Nonerythroid Nucleated Target Cells.   All the original observations on homologous species restriction have been obtained with RBC (20–23). Fig. 6 shows results from an experiment indicating that the same phenomenon occurs with nucleated targets. C-dependent lysis mediated by sera from different species was tested on EL-4, a murine T lymphoma cell line bearing Thy-1.2 antigen (40),
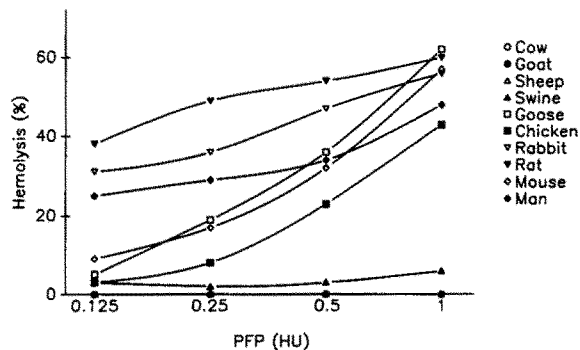

FIGURE 4.   Hemolysis mediated by rat PFP/perforin on RBC from different species. PFP-containing fractions obtained from rat spleen LAK cells were tested against the 10 indicated species of RBC. No lysis was obtained for cow, goat, and sheep RBC.
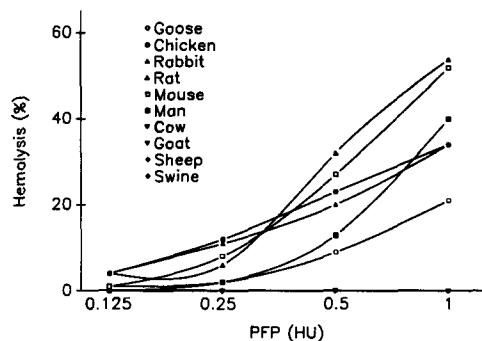
FIGURE 5. Hemolysis mediated by human PFP/perforin on RBC from different species. PFP-containing fractions were prepared from human peripheral blood NK cells that had been stimulated with 1,000 U/ml of rIL-2 for 4 d (see Materials and Methods). The different species of RBC used as target cells are indicated in the figure. No lysis was obtained for cow, goat, sheep, and swine RBC.

coated with mouse anti-Thy-1.2 antibodies. EL-4 cells were lysed by rabbit and guinea pig C, but not by mouse C (Fig. 6 A). However, EL-4 cells were efficiently lysed by mouse perforin (Fig. 6 B).

To further investigate the sensitivity of nucleated targets of different species to lysis by perforin, we challenged cell lines of either murine or human origin with mouse perforin. Fig. 7 illustrates representative results for two cell lines, human K562 and murine WEHI-164 tumor cells. Both cell lines were equally susceptible to either mouse or human perforin. To date, a panel of 15 tumor cell lines of human, simian, or murine origin has been tested with mouse perforin; no discernible pattern of species-related restriction has been detected (data not shown).

In contrast to the tumor cell lines tested, mouse CTLL were markedly resistant, whereas human IL-2-activated NK cells were relatively resistant to both human and mouse perforin used in equivalent amounts (Fig. 7, data shown only for CTLL-R8). Similar results were obtained with CTLL-1 (not shown). We next studied the response of CTLL to C-mediated lysis. Fig. 8 illustrates representative results obtained with CTLL-R8. CTLL-R8 cells, coated with anti-CTLL-R8 cell antibodies, were incubated with mouse, rabbit, or guinea pig sera as a source of C. As shown in Fig. 8, both rabbit and guinea pig C lysed R8 cells effectively, but murine C did not lyse R8 cells even at the highest serum concentrations tested (1:10). These results show that although homologous species restriction protects CTL effectively against lysis mediated by C, the same mechanism does not appear to be responsible for pro-
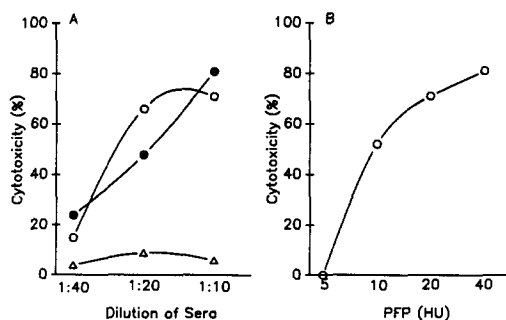

FIGURE 6. C- and PFP/perforin-induced lysis of EL-4 cells. This was determined in ^{51}Cr release assays using ^{51}Cr-labeled EL-4 cells (A) coated with anti-Thy-1.2 mAb and incubated with the indicated dilutions of guinea pig (O), rabbit (●), and mouse (△) sera for 1 h at 37°C or (B) incubated with mouse-purified PFP/perforin (O) for 3 h at 37°C.
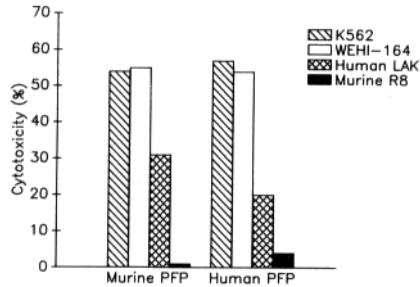
FIGURE 7.   Lysis of nucleated cells mediated by murine and human PFP/perforin. Murine PFP was obtained from CTLL-R8 cells; human PFP, obtained from IL-2-stimulated NK cells, was that used in Fig. 5. ^{51}Cr-labeled CTLL-R8, K562, and Wehi-164 cells in log phase and peripheral blood LAK cells (see Materials and Methods) were used as targets. 12.5 HU of murine and human PFP each were used. ^{51}Cr release was determined after a 3-h incubation at 37°C.

tecting CTL against perforin-mediated injury, since CTL are equally resistant to both homologous and heterologous perforin.

Human LAK cells derived from primary cultures were only partially resistant to both mouse and human perforin (Fig. 7). However, the extent of their resistance was comparable regardless of whether murine or human perforin was used (Fig. 7). These cells were consistently more resistant than human T and B lymphocyte subsets (not shown). The resistance of human LAK cells to either form of perforin varied directly with the duration of IL-2 stimulation (Liu, unpublished observations).

Sensitivity of Primary Cells to C- and Perforin-mediated Lysis.   Thymocytes freshly obtained from CD$_2$F$_1$ mice were coated with anti-Thy-1.2 antibodies and treated with C from different species (Fig. 9). Thymocytes treated this way were lysed effectively by both rabbit and guinea pig C, but not by mouse C (Fig. 9 *A*). However, both mouse and rat perforin lysed the mouse thymocytes equally well (Fig. 9 *B*).

Discussion

Since PFP/perforin is structurally related to the terminal components of C (16–19), it is reasonable to hypothesize that the mechanisms by which cells are protected against the two forms of cytolysis may be related or identical. Moreover, since isolated human C8BP/HRF incorporated into SRBC were shown to protect these cells from lysis both by human C and by human perforin, suggestions were made that HRF may be active also in protecting lymphocytes from lysis induced by either lytic mediator (27, 28). HRF is thought to be structurally related to C8 and C9 (10), and by analogy, it is speculated that HRF may be homologous to perforin. By complexing rapidly
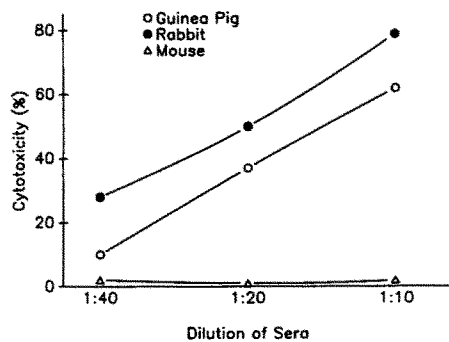

FIGURE 8.   Susceptibility of CTLL to lysis by homologous and heterologous C. ^{51}Cr-labeled, anti-R8 antibodies-coated CTLL-R8 cells (see Materials and Methods) were incubated with guinea pig, rabbit, and mouse sera at the indicated dilutions for 1 h at 37°C before ^{51}Cr release determination.
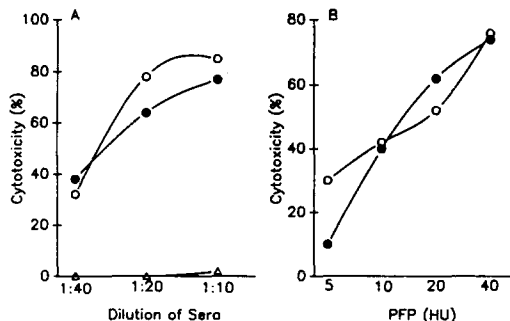
FIGURE 9. Cytolysis of primary cells by C and PFP/perforin: comparative analysis. (*A*) Thymocytes from CD_2F_1 mice were ^{51}Cr-labeled and coated with anti-Thy-1.2 mAb as described (see Materials and Methods). [51]Cr release was tested after incubation (3 h, 37°C) of the cells with the indicated dilutions of guinea pig (●), rabbit (O), and mouse (△) sera for 3 h at 37°C. Triplicate samples of 2 10^4 cells were used per data point. (*B*) ^{51}Cr-labeled mouse thymocytes were incubated with mouse purified PFP/perforin (O) and rat PFP-containing fractions (●).

with attacking C8 and C9 molecules, HRF is thought to interrupt the C polymerization process that leads to channel formation.

Here, we report a detailed comparative analysis on the characteristics of C- and perforin-mediated lysis of several types of cells from different species. Our results indicate that not only RBC, as previously described by other investigators (20–23), but also nucleated target cells, including primary and long-term culture cells, are resistant to lysis by homologous, but not heterologous, C. These data support the notion of homologous species restriction for C-mediated lysis, extending a previous observation to indicate that HRF is present on a wide variety of cell types. In fact, C8BP-HRF has been identified in human RBC and some nucleated cells, including human polymorphonuclear leukocytes, Raji cells, and large granular lymphocytes (10, 24–28). HRF-mediated protection is, thus, likely to have a role in sparing the host from developing injuries in response to circulating autoantibodies or other inflammatory factors that otherwise activate autologous C.

We show here that, unlike C, perforin preparations obtained from three different species are all capable of target cell lysis across a variety of species, including cell types that are resistant to homologous C, such as RBC and nucleated long-term and primary target cells. Only RBC from cow, goat, sheep, and swine, of all species tested, are insensitive to rat or human perforin, although they are sensitive to mouse perforin.

Because, as shown here, no correlation is demonstrable between protection of targets against C- and PFP-mediated cytolysis and because susceptibility of target cells to perforin is independent from the species of origin of perforin used, our data indicate that the phenomenon of homologous species restriction does not operate in perforin-induced lysis of either RBC or nucleated, noncytotoxic, target cells. Based on these observations, we conclude that the resistance of cells (including cytolytic lymphocytes) to perforin-inflicted injury can not be mediated by C8BP/HRF. If HRF were the molecule responsible for protection of lymphocytes and other cells against perforin-mediated killing, then it would be difficult to explain why perforin lyses HRF-containing target cells. We cannot, however, rule out the possibility that HRF may have some affinity for perforin and that, at high concentrations, such as in the case of reconstitution experiments performed by Zalman et al. (27), it may exert some protective function against perforin-mediated lysis.

CTL and NK cells to date have been shown to be the only cell types resistant

to perforin-dependent lysis (11–15). Although mouse CTL lines have been shown to be sensitive to heterologous C but not to perforin (11, 13), the role of HRF in mediating resistance of CTL to perforin was not addressed in those initial studies. Our data support the possibility that CTL carry HRF on their surface, since these cells are protected from homologous but not heterologous C. However, the observation that CTL are equally resistant to perforin from different species further supports our conclusion that the protective mechanism(s) of these cells is not species restricted, as in the case of C-mediated lysis, and that distinct mechanisms are involved in protection of cytotoxic cells against C- and perforin-mediated lysis. Our studies indicate that primary LAK/NK cell cultures, like CTL, are also partially resistant to perforin. Preliminary studies show that the resistance of NK cells to perforin is acquired and inducible by IL-2 (Liu, unpublished observations). We noticed that primary LAK/NK cells are relatively resistant to perforin attack when compared with B and T cell subsets. However, these cells are less resistant to perforin than mouse CTLL grown in long-term cultures in the presence of IL-2, suggesting the possibility that, in long-term cultures, cytotoxic cells may have been selected continuously for the perforin-resistant phenotype.

At the moment, it is difficult to explain why some species of RBC tested are resistant to the rat and human forms of perforin while being susceptible to mouse perforin. SRBC are among the resistant cells. These data are in agreement with a previous report (41) in which we were unable to detect measurable lysis of SRBC using NK cell extracts containing human perforin, as detected biochemically and on the basis of its ability to insert channels in lipid bilayers, and with that of Zalman et al. (27) showing that a human form of PFP/perforin induced only weak lysis of SRBC after a 5-h incubation at 37°C. The observation that RBC from four species are insensitive to lysis by rat and human perforin only is difficult to explain at the moment but is important to be kept in mind when interpreting data obtained using only hemolysis to test for the presence of PFP/perforin in cell types from different species. This cautions us to choose appropriate RBC targets for experimental studies of perforin.

The nature of the self-protective mechanism(s) of cytolytic lymphocytes is unknown. Our observations do not rule out, for example, the possibility that a molecule (protectin?) closely related to but not identical to HRF is involved in protecting lymphocytes from perforin-mediated damage. On the other hand, since lymphocytes may deploy multiple pathways of target cell damage, it is also possible that multiple mechanisms (and polypeptides) are involved in mediating protection of lymphocytes against self-inflicted injury. Further experiments are warranted to substantiate these and any other speculations.

Summary

CTL and NK cells resist self-mediated killing and lysis by their own pore-forming protein (PFP; perforin). Perforin, like C, lyses RBC. Efficient C-mediated lysis of RBC occurs when both C and RBC are from different species (homologous species restriction). A protective surface protein (C8-binding protein, homologous restriction factor) has been reported to mediate both homologous species restriction in C-dependent cytolysis and protection of some target cells against perforin-induced lysis.

We show here that perforin, unlike C, lyses target cells across a variety of species,

including the homologous one, while the same target cell populations resist the attack by homologous C. Perforin-containing extracts of CTL and LAK/NK cells from three species (rat, mouse, and human) and purified mouse perforin were tested against RBC from 10 different species, several nucleated target cell lines, and one primary cell population (thymocytes). While resisting lysis by homologous C, most of these cell types were lysed effectively by perforin without any homologous restriction pattern. CTL and NK cells, like other nucleated targets, are resistant to lysis by homologous but not heterologous C; however, these cell types are resistant to both homologous and heterologous perforin. Together, our results suggest that the protective mechanisms associated with C- and perforin-mediated lysis are distinct.

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