RESISTANCE OF CYTOLYTIC LYMPHOCYTES TO PERFORIN-MEDIATED KILLING

Induction of Resistance Correlates with Increase in Cytotoxicity

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Several lytic mediators of CTL and NK cells have been described, including a pore-forming protein (PFP, perforin or cytolysin)[1] contained in their cytoplasmic granules (1-4). PFP/perforin is known to lyse a variety of cellular targets with little cell type specificity.

After lysing their targets, CTL and NK cells can recycle to kill additional targets without undergoing self-injury (5, 6), indicating that these cells may be resistant to their own cytolytic mediators. Recent work performed in this and other laboratories has already shown that CTL clones are resistant to the cytotoxic activity mediated not only by other CTL clones (7-10), but also by isolated CTL granules and perforin (9-12). The molecular basis for this self-protection mechanism is still unclear. It is also unknown whether the resistance of cells to perforin-mediated lysis is restricted only to CTL and NK cells that have been maintained in long-term cultures. In this study, we investigated the susceptibility of primary cytotoxic cell populations to the lytic activity of perforin and examined whether it correlates with induction of cytolytic potential. In addition, we have used CTL hybridomas with inducible cytolytic activity and an IL-2-independent CTL line to examine whether activation of perforin resistance is dependent on induction of cell proliferation. We find that, like cytotoxicity, the resistance of these cells can be activated without induction of cell proliferation.

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[1] *Abbreviations used in this paper:* actD, actinomycin D; CHX, cycloheximide; LAK, lymphokine-activated killer; PEL, peritoneal exudate lymphocytes; PFP, pore-forming protein.

Materials and Methods

Animals. 8-wk-old BALB/c ($H2^d$), CD_2F_1 ($H2^d$) and C57BL/6 ($H2^b$) mice were purchased from The Charles River Breeding Laboratories (Wilmington, MA) and maintained in our animal facility.

Antibodies. The mAbs against murine L3T4 (GK 1.5) and Lyt-2 (TIB 150) and human CD3 (OKT3), CD4 (OKT4), and CD8 (OKT8) were generated in our laboratories from hybridomas obtained from American Type Culture Collection (ATCC, Rockville, MD). The mAb B73.1 against human NK marker CD16, a generous gift from Dr. Bice Perussia from The Wistar Institute (Philadelphia, PA), was produced as described (13). Polyclonal antisera against the NK markers asialo-GM1 and laminin were obtained from Accurate Chemical & Scientific Corp. (Westbury, NY) and Collaborative Inc. (Bedford, MA), respectively.

Cells. Murine CTLL-R8 and CTLL-1 were maintained in IL-2-containing medium as previously described (14, 15). CTLL-R8 was grown to large numbers and used as a source of PFP/perforin (15).

An IL-2-independent subclone of CTLL-R8 (R8i) was derived by serial passages of cells in growth medium without IL-2, followed by subcloning using limiting dilution.

Peritoneal exudate lymphocytes (PEL) were harvested from BALB/c mice ($H2^d$) that had been injected with EL-4 ($H2^b$) cells intraperitoneally 10 d before (16). PEL were collected by peritoneal lavage and passed through a nylon-wool column. Recovery after nylon-wool passage was 5–7 × 10^6 cells/mouse. The cells were washed, resuspended in modified MEM (αMEM; Gibco Laboratories, Grand Island, NY), supplemented with 10% FCS, and used immediately either as effector cells, or for ^{51}Cr labeling for use as target cells.

The CTL hybridomas were derived from BALB/c (H-$2D^d$) CTL as described previously (17). Hybrid clones Md90 and M8.23 (referred in the text as M-hybridomas) were derived from MLC-CTL, and the clone PMM1 (P-hybridoma) was derived from PEL. Nonfunctional hybrid clone Md26.9 was isolated by limiting dilution from an active clone (Md26). Its characteristics will be reported in detail elsewhere (Kaufmann, Y., et al., manuscript in preparation). All functional hybridomas demonstrate inducible killing activity and lymphokine production. They can be activated by T cell mitogens (Con A or PMA), interferons, or by cells expressing H-$2D^b$ antigen (18). The hybridomas were maintained in Dulbecco's modified minimal essential medium (DMEM, Gibco Laboratories) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, and 15% FCS.

The A.Sn ($H2^a$) lymphoma YAC-1, C57BL/6 lymphoma EL-4, DBA/2 mastocytoma P815, and human erythromyeloid leukemia K562 were grown in suspension in RPMI 1640 (Gibco Laboratories) supplemented with 5% FCS.

Mouse Spleen Cytotoxic Cells and LAK/NK Cells. Spleens were removed from CD_2F_1 mice and minced to a single cell suspension. Contaminating erythrocytes were lysed with 0.88% NH_4Cl (5-min incubation at room temperature). After three washes in αMEM, cells were suspended in αMEM/5% FCS to 2.5 × 10^6 cells/ml. To prepare murine antigen-nonspecific cytotoxic lymphocytes, spleen cells were resuspended in the medium supplemented with 10% IL-2-containing, leukocyte-conditioned medium and cultured at 37°C in a 5% CO_2 humidified incubator for the indicated times. The leukocyte-conditioned medium was obtained by stimulating rat spleen cells with 5 μg/ml Con A and 10 ng/ml PMA for 24 h before harvesting the cell spent medium. Lymphocyte subsets were separated by a negative-selection panning procedure using anti-L3T4, anti-Lyt-2, and anti-Ia mAbs. The spleen cells were resuspended in αMEM/5% FCS to 5 × 10^6 cells/ml and incubated with appropriate hybridoma supernatants (1:3 vol/vol dilution) on ice for 1 h. The antibody-coated cells were washed, resuspended to 3 × 10^6 cells/ml, and plated onto petri dishes precoated with either goat anti-rat or goat anti-mouse IgG (120 μg in 9 ml of PBS per 10 × 60 mm plate), which were then used with either anti-L3T4-coated cells or anti-Lyt-2-coated cells, respectively. After a 4-min centrifugation at 40 *g* and an additional 20-min incubation on ice, plates were washed three times and the nonadherent cells were removed and resuspended in αMEM/5% FCS. The cells were enriched for either L3T4$^+$ or Lyt-2$^+$ cells as determined by FACS (Becton Dickinson & Co., Mountain View, CA) analysis.

Allospecific murine CTL were generated from MLC. Briefly, nylon wool-nonadherent spleen cells obtained from C57BL/6 ($H2^b$) or C57BL/1 ($H2^k$) mice were mixed with irradi-

ated (3,000 rad) spleen cells obtained from CD_2F_1 ($H2^d$) at a responder/stimulator ratio of 1:1. The mixed cell populations at 5×10^6/ml were cultured in αMEM/5% FCS/5×10^{-5} M 2-ME for the indicated times, after which cells were washed three times and resuspended in αMEM/5% FCS and used as effector cells. In some experiments, mixed cell populations were harvested, resuspended in serum-free medium, and then treated with perforin or control buffer before their use as effector cells.

To prepare murine lymphokine-activated killer (LAK)/NK cells, spleen cells were prepared as before, with the exception that the incubation medium contained 1,000 U/ml of human rIL-2 (generously provided by Cetus Corp., Emeryville, CA). NK cells were obtained as plastic-adherent cells after rIL-2 stimulation, essentially as described (19).

Human Lymphocyte Subsets. Human PBMC were isolated by Ficoll-Hypaque gradient centrifugation from leukocyte concentrates obtained in plateletpheresis bags (New York Blood Center, New York, NY). Monocytes and B lymphocytes were removed by adherence to plastic dishes and to nylon-wool columns, respectively (20). Nylon wool–nonadherent lymphocytes were washed and resuspended in RPMI 1640/10% FCS. A panning procedure was used to enrich for NK, $CD4^+$, and $CD8^+$ cells. Briefly, cells were incubated with OKT3 hybridoma supernatant (1:3 dilution) for 1 h at 4°C, washed three times, and resuspended to 3×10^6 cells/ml in RPMI 1640/FCS. Cells were then plated in petri dishes precoated with goat anti-murine IgG (120 μg per 60 × 15 mm petri 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 RPMI 1640. This cell population was enriched for NK cell phenotype and typically contained >95% of cells staining positive for the NK cell marker B73.1, as determined by FACS analysis. The plates containing adherent cells were incubated for an additional 3 h at 37°C, after which plate-adherent cells were collected. Stimulation of human LAK/NK cells with rIL-2 was performed as for murine cells, at 1,000 U of rIL-2 per milliliter.

Purification of PFP/Perforin. Perforin was purified from CTLL-R8 cells by a procedure outlined elsewhere (21). Briefly, perforin from granule-enriched material was purified sequentially on DEAE-Sepharose, Q Sepharose, mono Q, and Superose 12 columns adapted to an FPLC system (Pharmacia Fine Chemicals, Uppsala, Sweden). The purified protein migrated as a single band of 70 kD when analyzed on SDS-polyacrylamide gels performed under disulfide-reducing conditions and stained with silver nitrate (not shown). Human perforin activity was obtained from peripheral blood NK cells essentially as described (21).

Hemolysis Assay for Perforin. A hemolysis microassay (15) was used to measure perforin activity. SRBC were used as targets at 10^8 cells/ml in PBS; 200 μl of SRBC suspension per microtiter well was tested in triplicates against 20 μl of lytic reagent in the presence of 1 mM $CaCl_2$ (14). Hemolysis was determined after a 30-min incubation at 37°C by spectrophotometric reading of the plates at 700 nm. The extent of hemolysis was given as percent hemolysis by $[1 - (b\text{-}c)/(a\text{-}c)] \times 100$, where *a* represents A_{700} of the intact SRBC suspension, *b* the A_{700} of the lysed erythrocyte suspension, and *c* the A_{700} of the SRBC suspension completely lysed with water. One hemolytic unit (HU) is defined as the amount of perforin required to lyse 50% of 2×10^7 SRBC in a total volume of 220 μl. The specific activity of purified perforin used in most of the experiments was ~150 HU/μg protein.

^{51}Cr-Release Assay. Target cell lines at 2×10^6/ml were incubated in 1 ml of FCS containing 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) at 37°C for 2 h with occasional mixing. For primary cells, labeling was done in αMEM/5% FCS for 5 h at 37°C. Labeled cells were then washed in αMEM three times and resuspended to 10^5 cells/ml in serum-free αMEM. For measurement of perforin-induced cytotoxicity, 10^4 ^{51}Cr-labeled cells were incubated with the indicated amounts of perforin in a final volume of 200 μl per microtiter well, followed by a 4-h incubation at 37°C. After the incubation, the plates were centrifuged at 200 *g* for 5 min and 100 μl of the supernatant was removed from each well for determination of the radioactivity in a gamma counter. To measure the cytotoxic activity of CTL hybridomas or primary killer cells, 10^4 ^{51}Cr-labeled target cells resuspended in αMEM/10% FCS were mixed with appropriate effector cells at indicated E/T ratios in a round-bottomed microwell in a total volume of 200 μl, followed by a 5-h incubation at 37°C. In some experiments, the cytotoxic reaction mediated by primary spleen cytotoxic cells was carried out in medium containing 5 μg/ml of Con A. The percent cytotoxicity is calculated

as: 100 × [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(total ^{51}Cr release − spontaneous ^{51}Cr release)]. ^{51}Cr release by target cells cultured in medium alone was taken as spontaneous release, while ^{51}Cr release by target cells lysed with 1% NP-40 was measured as total release.

Induction of Cytotoxic Activity in CTL Hybridomas. For induction of cytotoxic activity, Md90 cells were treated with 5 μg/ml of Con A for the indicated periods of time at 37°C, washed three times in αMEM containing 1 μg/ml α-methyl manoside, and immediately used either as effectors or as targets after ^{51}Cr labeling as described above. For antigenic stimulation, hybridomas M8.23, PMM1, and Md26.9 were cocultured with 4,000-rad irradiated EL-4 cells at the hybridoma/target ratio of 2:1 for 20 h (18); cells were then washed and used as effectors against ^{51}Cr-labeled EL-4 target cells.

Treatment of CTL with Protein and RNA Synthesis Blockers. Md90 hybridomas were activated as described above in the presence of 10 μg/ml of cycloheximide (CHX) (Sigma Chemical Co., St. Louis, MO). To ensure complete termination of protein synthesis before the activation event, Md90 cells were incubated with CHX for 30 min before stimulation with Con A or EL-4 cells and for the duration of the subsequent cytotoxicity assays in which Md90 cells were tested. In experiments using emetine (Sigma Chemical Co.), an irreversible protein synthesis inhibitor, CTL hybridomas, and cloned CTLs were pretreated with 5×10^{-6} M of emetine for 30 min, washed three times for removal of any residual emetine, and then subjected to induction. ^{51}Cr labeling and cytotoxic reaction were performed exactly as described above. In some experiments, emetine was present throughout the induction period and the cytotoxicity assay. Alternatively, cells were induced in the presence of actinomycin D (actD; Sigma Chemical Co.), an RNA synthesis inhibitor, and processed for functional assays exactly as described above. Viability of cells was given by trypan blue exclusion.

[^{35}S]Methionine Labeling of Cells. [^{35}S]Methionine incorporation was determined in CTL hybridomas and cloned CTL that have been treated with metabolic inhibitors. 10^7 cells were incubated for 4 h in 40 ml of methionine-free RPMI/5% FCS (using a Select amine kit [Gibco Laboratories] and FCS that had been dialyzed extensively against PBS) containing 200 μCi of [^{35}S]L-methionine (New England Nuclear). Cells were washed three times in PBS, resuspended in 1 ml of PBS containing 1% NP-40 and 1 mM PMSF. After a 30-min incubation on ice and centrifugation in a microfuge for 5 min, the cytosol supernatant was collected. Proteins were then precipitated by extraction with 10% (wt/vol) TCA. The protein pellet was washed with diethyl ether and resuspended in PBS. Aliquots were taken from each sample and counted in a β scintillation counter in triplicate. The percent of inhibition of label incorporation was calculated as: 100 × [(control cpm − experimental cpm)/(control cpm)], where cpm of cells cultured in medium alone was taken as control and cpm of cells cultured in medium containing inhibitors was measured as experimental data.

Results

Effect of Purified Perforin on Cloned CTL. Although previous studies indicated that cloned CTL are resistant to granule-mediated killing, it was not clear whether primary cytotoxic cells are also resistant to the lytic effect of the pore-forming protein perforin. For the present study, purified perforin was obtained from murine CTLL-R8 after several steps of liquid chromatography (Materials and Methods). The membrane-lytic activity of this purified material was ascertained through hemolytic assays (used to define lytic activity in hemolytic units) and through ^{51}Cr-release cytotoxicity assays for nucleated target cells. Fig. 1 illustrates the typical dose-dependent cytotoxic effect of purified perforin on P815 mastocytoma and YAC-1 lymphoma cells. While both targets were susceptible to perforin, in parallel assays, perforin lysed poorly CTLL-1 (a perforin nonproducer) and did not produce any measurable cytotoxicity on CTLL-R8 (Fig. 1). For comparison, at concentrations that produced at least 70% lysis of tumor targets, <10% of CTLL-1 were lysed while CTLL-R8 were completely refractory to lysis. These results extend our previous findings made

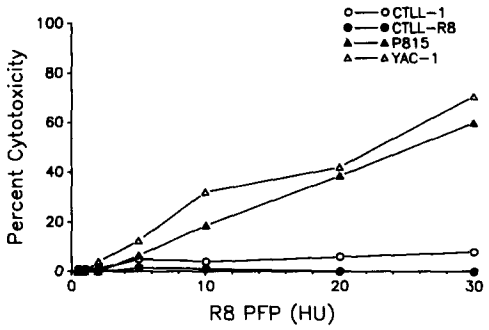

FIGURE 1. Cytotoxic effects of perforin on cloned CTL and non-CTL cell lines. ^{51}Cr-labeled YAC-1, P815, CTLL-R8, or CTLL-1 cells were plated at 10^4 cells per microtiter well, in triplicate, and incubated with the indicated amounts of perforin (given in hemolytic units) for 4 h at 37°C before determination of ^{51}Cr release. The spontaneous release in these experiments was <15%.

with perforin-enriched granules (10), suggesting that the CTL lines tested are also resistant to purified perforin. Purified perforin was next used to assess the resistance of primary cytotoxic cells to perforin-mediated killing.

Effect of Purified Perforin on Primary Cytotoxic Cells. Primary cytotoxic lymphocyte populations were generated by culturing spleen cell populations obtained from CD_2F_1 mice in the presence of IL-2-containing leukocyte-conditioned medium (Materials and Methods). Cells stimulated for 1–5 d in this conditioned medium were tested as effectors against EL-4 targets in a lectin-dependent cell-mediated cytotoxicity (LDCC) assay (Fig. 2 *a*). This assay measures the development of nonspecific effector cells that are triggered to lyse targets to which they bind via a lectin-dependent mechanism. The results showed clearly that cytotoxicity increased with the number of days that spleen cells were stimulated with leukocyte-conditioned medium. The relatively high E/T ratio required to achieve any substantial amount of cell lysis (Fig. 2 *a*) was probably due to the fact that bulk unfractionated spleen cell populations containing also non-killer cells were used for these experiments.

Spleen cells stimulated with leukocyte-conditioned medium were labeled with ^{51}Cr and used as target cells for purified perforin. A marked increase in cellular resistance against perforin-mediated lysis was observed 24 h after stimulation with

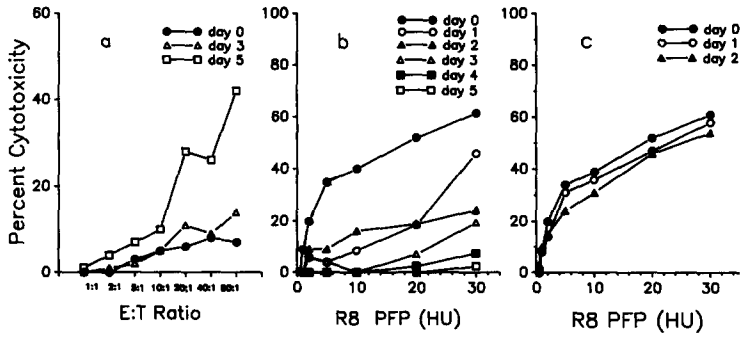

FIGURE 2. Lytic efficiency of primary splenic killer cells correlates with their resistance to perforin-mediated lysis. Murine spleen nylon wool-nonadherent cells were grown in leukocyte-conditioned medium (*a*, *b*) or in control medium (*c*) for the indicated number of days. These cells were then either (*a*) used as effectors in LDCC assays against ^{51}Cr-labeled EL-4 cells, or (*b*, *c*) were labeled with ^{51}Cr and used as targets for purified perforin. 4-h cytotoxicity assays were performed, each one in triplicate. The spontaneous ^{51}Cr release was <20%.

conditioned medium, and resistance increased steadily from day 1 to day 5 of culture (Fig. 2 *b*). After 5 d of in vitro stimulation, <10% of spleen cells were lysed by 30 HU of perforin (Fig. 2 *b*), corresponding to the resistance level observed with cloned CTLL. In parallel experiments in which spleen cells were cultured in the absence of leukocyte-conditioned medium, cells remained highly susceptible to perforin-mediated lysis (Fig. 2 *c*). However, the viability of cells in the absence of leukocyte-conditioned medium decreased to <30% on the third day of culture, as determined by trypan blue exclusion; moreover, the remaining viable cells labeled poorly with ^{51}Cr (not shown). Thus, a direct comparison between the resistance of stimulated and unstimulated spleen cells could only be made during the first 2 d of culture (Fig. 2, *b* vs. *c*).

The cell type distribution of spleen cells cultured in vitro was studied by FACS analysis using mAbs directed against L3T4 (CD4) and Lyt-2 (CD8) (not shown). After stimulation with leukocyte-conditioned medium, the percentage of Lyt-2^+ cells increased from 18% (range 9–27%) on day 0 (before treatment) to 66% (range 41–90%) on day 5, corresponding to approximately a fivefold increase. The percentage of L3T4^+ cells, on the other hand, decreased from 57% (45–69%) on day 0 to 13% (range 8–17%) on day 5. Unstimulated cells, however, did not show significant change during the first 2 d of culture in their relative distribution of Lyt-2 and L3T4 phenotypes. Since Lyt-2^+ cells include CTL, our results suggest further that an increase in cell resistance to perforin may correlate with an increase in the number of CTL in the bulk spleen population.

Primary CTL were also obtained through an MLC reaction ($H2^b$ anti-$H2^d$ and $H2^k$ anti-$H2^d$). MLC-derived bulk spleen cell populations were either treated with perforin or control buffer (Table I). The rationale of these experiments was based on the premise that if primary alloimmune CTL were resistant to perforin, then perforin-treated cells would retain cytotoxic activity toward allospecific targets. Thus, perforin-treated and control cells were used as effectors against P815 mastocytoma targets ($H2^d$). The data summarized in Table I show that although 30–69% of spleen cells were lysed in different experiments by various doses of perforin, perforin-treated cells could still lyse P815 as effectively as untreated MLC-activated cells, suggesting that the perforin-resistant cells were also the cells with cytotoxic capability. Perforin-resistant cells must have represented mostly allospecific CTL since they killed MHC-imcompatible target cells poorly (e.g., only up to 8% of YAC-1 [$H2^a$] were killed at an E/T ratio of 50:1).

To further verify the phenotype of the perforin-resistant cells, a panning technique was used to separate B, L3T4^+, and Lyt-2^+ lymphocytes, which were then tested for their sensitivity to perforin-mediated lysis. As shown in Fig. 3, B and L3T4^+, but not Lyt-2^+, lymphocytes were readily lysed by perforin, indicating that even unstimulated CTL (e.g., cells of Lyt-2^+ phenotype) were the least sensitive to perforin-mediated killing when compared with other lymphocyte subsets.

In another set of experiments, perforin was also tested against PEL obtained from mice undergoing rejection of MHC-incompatible tumors (Materials and Methods). PEL were also significantly more resistant to perforin-mediated lysis when compared with peritoneal macrophages or B cells (data not shown).

Resistance of Primary NK/LAK Cells to Perforin-mediated Killing. Primary NK/LAK cells from mouse spleen were obtained by stimulation of spleen cells with high con-

TABLE I
Treatment of Allospecific CTL with Perforin Does Not Decrease their Cytotoxic Activity

Effector cells*	Target cells	PFP treatment	Cytotoxic activity‡
			%
MLC ($H2^b$ anti-$H2^d$), 3rd day	P815	–	31.6
	P815	+§	38.8
MLC ($H2^b$ anti-$H2^d$), 4th day	P815	–	68.5
	P815	+§	54.1
MLC ($H2^b$ anti-$H2^d$), 5th day	P815	–	69.4
	P815	+§	59.2
MLC ($H2^k$ anti-$H2^d$), 5th day	P815	–	81.2
	P815	+‖	75.8
	P815	+¶	69.0
MLC ($H2^k$ anti-$H2^d$), 5th day	YAC-1	+	8.0

* MLC-generated cells were harvested on the indicated day of MLC and used as effectors against allospecific $H2^d$-bearing P815 cells or $H2^{a+}$ YAC-1 cells (control).

‡ MLC-derived effector cells were tested against ^{51}Cr-labeled P815 cells at E/T ratio of 40:1 in a 5-h cytotoxicity assay. Effector cells that had been treated with PFP/perforin were washed and resuspended in fresh medium to initial volume before their use in cytotoxicity assays.

§ MLC-derived cells (5×10^6/ml) were incubated for 3 h with purified PFP at a dose that in parallel experiments killed 90% of equivalent numbers of YAC-1 cells. At this dose, 69% of MLC-generated cells were killed, as determined by trypan blue exclusion.

‖ PFP was used at a dose that killed 30% of MLC cells.

¶ PFP was used at a dose that killed 55% of MLC cells.

centrations of rIL-2 (1,000 U/ml), following a published protocol (19). Rat NK/LAK cells were previously found to proliferate and to adhere to plastic after short-term stimulation with high doses of IL-2 (19). We found that mouse NK/LAK cells acquired similar adherence properties under these conditions, thereby allowing their separation from other lymphocyte subsets by a single plastic-adherence step. Using this protocol (outlined in Materials and Methods), we typically obtained >95% NK cells, as judged by enrichment for the NK cell marker asialo-GM1 (Materials and

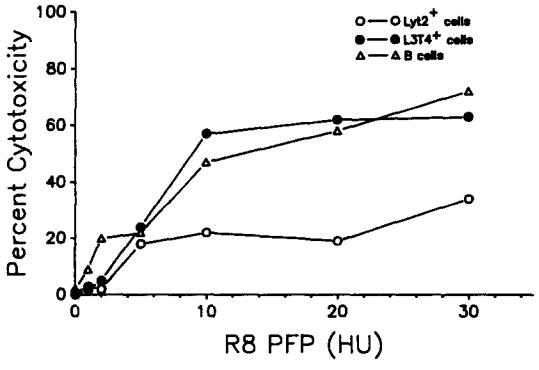

FIGURE 3. Murine Lyt-2^+ lymphocytes are least susceptible to perforin-mediated lysis. Murine spleen cells were fractionated by a panning procedure (see Materials and Methods), labeled with ^{51}Cr, and incubated in triplicate for 4 h with varying doses of perforin. The spontaneous release was <15%.

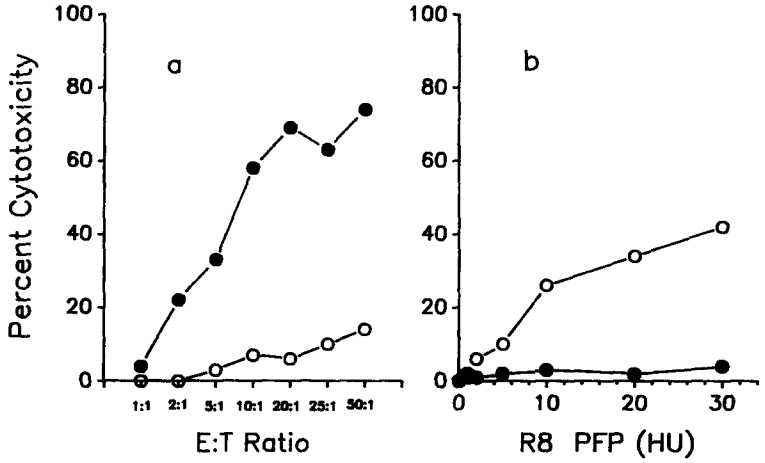

FIGURE 4. Murine LAK/NK cells are efficient killers but remain refractory to perforin-mediated lysis. (*a*) Murine plastic-adherent LAK/NK cells (●) or plastic-nonadherent lymphocytes (○) obtained from rIL-2-stimulated spleen cells were used as effectors against ^{51}Cr-labeled YAC-1 cells at the indicated E/T ratios in a 4-h cytotoxicity assay. (*b*) Murine LAK/NK cells (●) or plastic-nonadherent cells (○) (same batch as in *a*) were labeled with ^{51}Cr and tested as targets for purified perforin. Data points represent averages of triplicates. The spontaneous ^{51}Cr release was <15%.

Methods). IL-2-stimulated NK cells, but not plastic-nonadherent cells, effectively killed NK-sensitive YAC-1 cells (Fig. 4 *a*). In parallel experiments, stimulated NK cells, but not plastic-nonadherent cells, were shown to be markedly resistant to perforin-mediated killing (Fig. 4 *b*; only data for cells stimulated with rIL-2 for 4 d shown here). These experiments suggest that both primary CTL and NK cells are resistant to perforin-mediated lysis.

We also studied the resistance of human peripheral blood-derived primary CTL and NK cells to lysis mediated by human perforin. Human NK cells isolated by a panning procedure (Materials and Methods) were stimulated with 1,000 U of rIL-2 for 2 or 4 d. This treatment increased significantly the cytotoxicity of stimulated cells against the K562 target, an NK-sensitive tumor (Fig. 5 *a*). IL-2-stimulated cells also became progressively resistant to the lytic effect of human perforin when tested

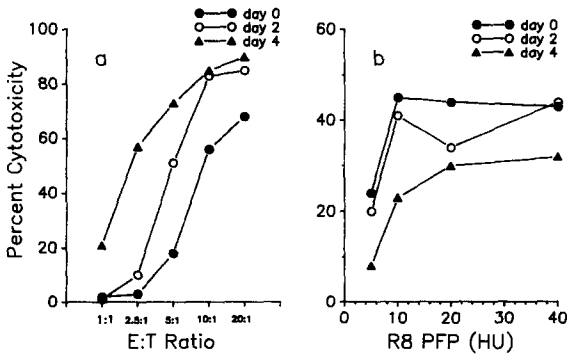

FIGURE 5. Cytotoxicity and resistance of human LAK/NK cells as a function of days of rIL-2 stimulation. (*a*) Human peripheral blood LAK/NK cells, stimulated with rIL-2 (1,000 U/ml) for the indicated number of days, were used as effectors against ^{51}Cr-labeled K562 cells at the indicated E/T ratios in a 4-h cytotoxicity assay. (*b*) Human LAK/NK cells (same batch as in *a*) were labeled with ^{51}Cr and tested as targets for purified murine perforin. Data points represent averages of triplicates.

on days 0, 2, and 4 after IL-2 treatment (not shown). In parallel experiments, purified murine perforin was tested against human NK cells (Fig. 5 *b*). The pattern of increase in resistance was comparable to that observed with murine NK cells. This cross-species experiment revealed that human NK cells were also markedly more resistant to lysis by murine perforin than several other cell types tested ($CD4^+$, $CD8^+$, and B lymphocytes; data not shown). However, human LAK/NK cells were in general less resistant than murine NK cells after IL-2-stimulation (compare Figs. 4 *b* and 5 *b*, with cells on day 4 of rIL-2-stimulation).

Resistance to Perforin-mediated Killing Is Inducible. The results presented above would suggest that the lymphocyte resistance to perforin is an inducible phenotype. However, since lymphocytes proliferate vigorously after antigen or IL-2 stimulation, one cannot dissociate induction of the resistance phenotype from cell proliferation. Since primary killer cells could not be used to address this issue, we instead studied two IL-2-independent CTL clones: (*a*) CTLL-R8i, derived originally from CTLL-R8 and (*b*) murine CTL-T cell hybridomas. These CTL hybridomas had previously been shown to acquire cytotoxicity under certain conditions of stimulation without enhancement of cell proliferation (22, 23). We found that both these cell types could be induced to become resistant to purified perforin under conditions in which proliferation was not enhanced.

The proliferation of R8i cells observed by direct cell counting after 48 h of stimulation with either rIL-2 (100 U/ml) or leukocyte-conditioned medium (20% final medium volume, see Materials and Methods) was not significantly different from that of control cells grown in medium alone. Unstimulated R8i cells, unlike the parent CTLL-R8, were partially susceptible to lysis by perforin (Fig. 6 *a*). Upon stimulation for 24 h with rIL-2 or leukocyte-conditioned medium, as before, R8i cells became markedly resistant to perforin (Fig. 6 *a*).

R8i cells were found to produce measurable levels of perforin-mediated hemolytic and BLT-esterase activities (two granule markers) before any stimulation: for per-

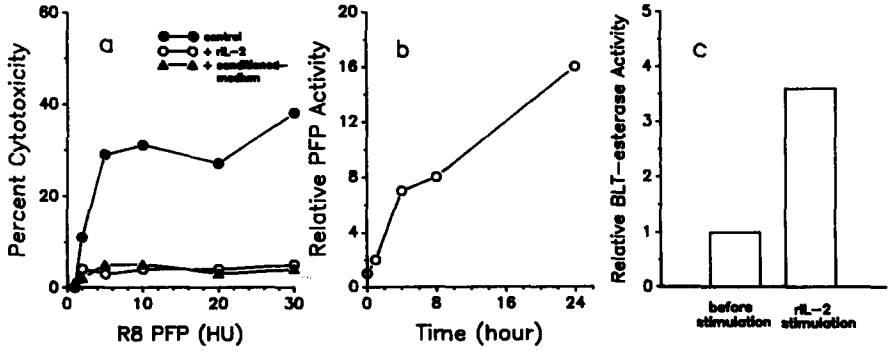

FIGURE 6. The production of perforin and BLT-esterase and the cellular resistance to perforin-mediated lysis are inducible. (*a*) Untreated CTLL-R8i (*control*) or CTLL-R8i treated either with rIL-2 (100 U/ml) or leukocyte-conditioned medium (20% final volume) for 24 h were tested for their sensitivity to PFP-mediated lysis in a 4-h cytotoxicity assay. The amounts of PFP used are given in hemolytic units. (*b*, *c*) CTLL-R8i was stimulated with rIL-2 (100 U/ml) for the indicated times (*b*) or for 24 h (*c*) after which they were assayed for either perforin (*b*) or BLT-esterase (*c*) contents (see Materials and Methods). Results are given in relative units, with the amounts of activity measured at time 0 taken as unity.

forin, 3 HU per 10^5 R8i cells compared with 25 HU per 10^5 parental R8 cells; for BLT-esterase, 0.12 and 0.55 A_{410} units for same numbers of R8i and R8 cells, respectively (Fig. 6, *b* and *c*). A marked increase in PFP/perforin and BLT-esterase content was found in the R8i cell lysates (15–20-fold increase of PFP and 4-fold increase of BLT-esterase after stimulation for 24 h; Fig. 6, *b* and *c*, shows data only for rIL-2 stimulation). These observations suggest that, like the resistance phenotype, the production of granule contents in perforin-containing cells may also be induced without concomitant cell proliferation.

The IL-2-independent CTL hybridoma Md90 has previously been shown to acquire cytotoxicity gradually in 4–20 h after stimulation with the lectin Con A (18). This time-dependent activation of Md90 cells was confirmed in the present studies using Con A at 5 μg/ml (not shown). In parallel experiments, Md90 cells were shown to acquire partial resistance to perforin after Con A stimulation (Fig. 7). The time courses for development of cytotoxicity and resistance were comparable.

Two other IL-2-independent CTL hybridomas, M8.23 and PMM 1, were also tested. Upon incubation with irradiated EL-4 cells, both CTL hybridomas became more efficient killers as well as more resistant to perforin (Table II). However, a subclone of M-hybridomas, Md26.9, which could not be induced to become cytotoxic (Kaufmann, Y., et al., manuscript in preparation), did not become significantly more resistant to perforin-mediated lysis under stimulation (Table II).

Protein Synthesis Is not Required for Induction of Resistance. Resistance induction in R8i and CTL-T hybrids was next carried out in the presence of either CHX (10 μg/ml for Md90, and 2 μg/ml for CTLL-R8i) or emetine (50 μM), an irreversible protein synthesis blocker (Table III). In the presence of either one of the two inhibitors, induction of both resistance and cytotoxicity was unaltered (Table III). To ascertain that CHX and emetine were effective at the doses used, cells were biosynthetically labeled with [^{35}S]methionine either in the presence or absence of the inhibitor (Table IV). The amount of radiolabeled proteins was then determined by TCA precipitation. These experiments revealed that CHX or emetine blocked 81–95% (range of three experiments) of ^{35}S incorporation into cellular proteins. To further ensure that conditions used here were optimal for inhibition of protein synthesis, the inhibitor was added 30 min before the addition of cell induction reagents (IL-2 in the case of R8i and Con A for Md90 cells). This type of treatment was also ineffective in blocking resistance induction.

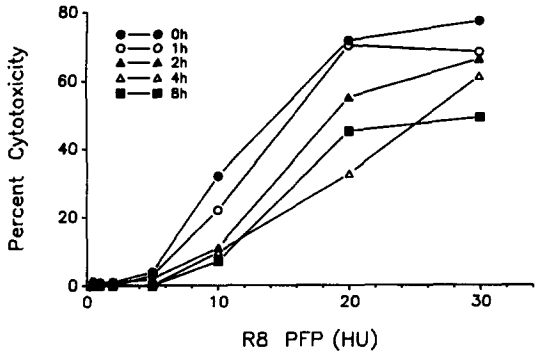

FIGURE 7. The CTL hybridoma Md90 acquires partial resistance to perforin-mediated lysis upon activation with Con A. Md90 cells were stimulated with 5 μg/ml of Con A for the indicated periods of time, after which cells were labeled with ^{51}Cr and used as targets for purified perforin in a 4-h cytotoxicity assay. Each data point represents average of triplicates.

TABLE II

Functional CTL Hybridomas Become More Resistant to Perforin-mediated Lysis upon Antigenic Stimulation

Hybridomas	Stimulants	Cytotoxic activity*	Lytic sensitivity‡
		%	%
PMM 1	–	5.2	73.5
	EL-4 cells	37.0	36.0
M8.23	–	8.0	76.0
	EL-4 cells	27.3	48.0
Md26.9	–	0	78.0
	EL-4 cells	0	65.6

* CTL-T cell hybridomas were co-cultured with irradiated (4,000 rad) EL-4 cells at the cell ratio of 2:1 for 20 h. Treated or untreated control hybridomas were then used as effectors against ^{51}Cr-labeled EL-4 cells at E/T of 20:1 in a 5-h cytotoxicity assay.

‡ Stimulated or control hybridomas were ^{51}Cr labeled and tested as target cells using 30 HU of perforin in a 4-h cytotoxicity assay.

TABLE III

Protein and RNA Neosynthesis Are Not Required for Resistance Induction

Cells	Inducers*	Inhibitors‡	Lytic sensitivity§
			%
Md90	–	–	77
	+	–	42
	+	CHX	34
	+	Emetine	40
	+	ActD	39
CTLL-R8i	–	–	34
	+	–	3
	+	CHX	8
	+	Emetine	4
	+	ActD	11

* CTL hybridoma Md90 was stimulated with 5 μg/ml of Con A for 8 h, while CTLL-R8i was treated with rIL-2 for 24 h.

‡ CHX was used at 10 μg/ml for Md90 cells and 2 μg/ml for CTLL-R8i. Emetine was used at 5×10^{-5} M. ActD was used at 1 μg/ml.

§ After treatment, Md90 or CTLL-R8i cells were labeled with ^{51}Cr and used as targets for purified perforin (30 HU) in a 4-h assay.

RNA synthesis also appeared not to be required for the induction of resistance in cytotoxic cells. This conclusion was inferred from experiments in which actD was added at 1 μg/ml, while CTL hybridoma Md90 and R8i were induced as before (Table III). Although actD effectively interrupted [^{3}H]thymidine incorporation into treated R8i or Md90 cells (90–95% inhibition), this treatment did not affect the resistance increase in these cells seen after activation (Table III).

Discussion

Both CTL and NK cells in culture are known to produce granules that contain the pore-forming protein perforin. While there is still some controversy regarding

the distribution of this protein in various cytolytic cell types (24–26), there is little doubt that perforin is among the more potent mammalian cytotoxins isolated to date. By growing large numbers of CTL and NK cells in vitro, it is possible now to obtain purified perforin in amounts sufficient for biochemical and functional studies. Using purified perforin, we have shown here that both cloned CTL and primary killer cell populations, including allospecific CTL, NK/LAK cells, and MHC-non-restricted CTL, are more resistant to perforin-mediated killing than other lymphocyte populations. The resistance of killer cells to perforin appears to correlate with their cytolytic capability, i.e., cells that are or have become competent killers are also the more resistant cells. For a given cytolytic cell population, resistance and cytotoxicity are shown to increase simultaneously after stimulation with certain reagents such as IL-2 or leukocyte-conditioned medium. The acquisition of resistance to perforin-mediated lysis is independent of the induction of cell proliferation, since IL-2-independent CTL lines and hybridomas, when triggered to become cytotoxic, acquire perforin resistance. It should be pointed out that the acquired resistance to perforin is not complete and that the resistance can be overcome with high doses of perforin.

Whether cells are required to produce perforin in order to become perforin resistant is not clear yet. Of the primary cytolytic populations tested, only cells that have been activated directly with IL-2 acquire measurable levels of perforin (our unpublished observations; see also references 24–27). CTL activated through MLC do not appear to produce any measurable levels of perforin (reference 25 and our unpublished observations), while they do become partially perforin resistant. The various CTL hybridomas tested also acquire resistance upon stimulation with Con A and APCs. However, our preliminary studies show that they do not produce any measurable amounts of perforin upon activation. Several perforin-nonproducer CTLL, such as CTLL-1 shown here, have remained perforin resistant. Experiments with PEL also indicate that while these cells do not produce measurable amounts of perforin, they become resistant to killing induced by other CTL and by perforin (data not shown). Another more recent study by Nagler-Anderson et al. (28) also showed that primary $CD8^+$ T cells elicited in vivo or in vitro are more resistant than $CD4^+$ T cells and non-T cells to lysis mediated by CTL granules. Since primary $CD8^+$ cells are now known to be perforin-negative under the conditions studied by these authors, together these results imply that the acquisition of resistance to perforin may be dissociated from the production of perforin in the same cells. More experiments are required, however, to substantiate this statement.

Our analysis of the IL-2-independent CTL variant R8i indicates that IL-2 may supply the necessary signal in this case to induce cells to produce perforin as well as to acquire the perforin-resistant phenotype. How this could occur without concomitant protein and/or RNA neosynthesis remains a puzzling finding. These results do not rule out, however, that a post-translational event or modification may be involved in the development of the resistance phenotype.

While perforin-treated primary killer cells were shown here to retain their capability to kill targets, it should be pointed out that in another study (29) we have shown that CTL granules contain an inhibitory activity, distinct from perforin, that is capable of inactivating reversibly the lytic activity of CTL. Accordingly, we confirmed here that perforin is not responsible for this inhibitory activity present in the granules.

We are currently attempting to dissect further the molecular details underlying the lymphocyte resistance to perforin. In two previous reports (30, 31), human lymphocytes were shown to acquire resistance to perforin upon stimulation with anti-CD3 antibodies. The reports by the same group also showed an augmented expression of homologous restriction factor (HRF) (31, 32) (also named C8/C9-binding protein [32, 33]) on lymphocyte surface after stimulation with antibodies specific for CD3, and the authors suggested that HRF is the molecular species responsible for self-protection of lymphocytes (34). This conclusion is not supported by our own studies, which have demonstrated that the phenomenon of homologous species restriction, while applicable to complement-mediated lysis, is not observed in the lymphocyte perforin system (12). Thus, although murine perforin lyses poorly, or not at all, homologous and heterologous cytotoxic lymphocyte populations, it lyses very well noncytotoxic cell targets of various species (including the homologous species) that nevertheless contain HRF. Here we have also shown that both murine and human LAK/NK cells, upon rIL-2 stimulation, become more resistant to murine perforin, further indicating that the lymphocyte resistance to perforin is not restricted to the perforin of homologous species. We hypothesize that other molecules/mechanisms must be involved in conferring resistance to lymphocytes against perforin-mediated lysis. Current studies in our laboratories are directed towards elucidating these mechanisms.

Summary

CTL and NK cells cultured in vitro are known to produce a cytolytic pore-forming protein (PFP, perforin) localized in their cytoplasmic granules. Using purified perforin, we showed here that both cloned CTL and primary killer cell populations, including allospecific CTL, NK/lymphokine-activated killer cells, and MHC-nonrestricted CTL, were more resistant to perforin-mediated killing than other lymphocyte populations and cell types. Similar results were obtained with both murine and human cytolytic lymphocyte populations. Resistance of killer cells to perforin correlated in general with their cytolytic capability. Thus, cells that have acquired competence to kill after stimulation with Con A, IL-2, or leukocyte-conditioned medium, were also the more resistant cells. IL-2-independent CTL lines and hybridomas derived in our laboratories could be triggered to become cytotoxic and perforin resistant by short-term stimulation with various cytokines, indicating that the acquisition of resistance to perforin-mediated lysis was independent of cell proliferation. Activation of one IL-2-independent CTL line with IL-2 also resulted in enhanced production of perforin and in enhanced serine esterase activity. The acquisition of cell resistance to perforin by these IL-2-independent cell lines after activation with stimulatory reagents was independent of protein and RNA neosynthesis: emetine, cycloheximide, and actinomycin D, while effectively blocking the incorporation of [^{35}S]methionine into cell proteins, did not affect the induced increase in perforin resistance.

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References

1. Henkart, P. A. 1985. Mechanism of lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* 3:31.
2. Podack, E. R. 1985. The molecular mechanism of lymphocyte-mediated tumor cell lysis. *Immunol. Today.* 6:21.
3. Young, J. D.-E, and Z. A. Cohn. 1987. Cellular and humoral mechanisms of cytotoxicity: structural and functional analogies. *Adv. Immunol.* 41:269.
4. Masson, D., and J. Tschopp. 1985. Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. *J. Biol. Chem.* 260:9069.
5. Berke, G. 1983. Cytotoxic T-lymphocytes. How do they function? *Immunol. Rev.* 72:5.
6. Martz, E. 1976. Multiple target cell killing by the cytolytic T lymphocytes and the mechanism of cytotoxicity. *Transplantation (Baltimore).* 21:5.
7. Kranz, D. M., and H. N. Eisen. 1987. Resistance of cytotoxic T lymphocytes to lysis by a clone of cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 84:3375.
8. Skinner, M., and J. Marbrook. 1987. The most efficient cytotoxic T lymphocytes are the least susceptible to lysis. *J. Immunol.* 139:985.
9. Verret, C. R., A. A. Firmenich, D. M. Kranz, and H. N. Eisen. 1987. Resistance of cytotoxic T lymphocytes to the lytic effects of their toxic granules. *J. Exp. Med.* 166:1536.
10. Blakely, A., K. Gorman, H. Ostergaard, K. Svoboda, C.-C. Liu, J. D.-E Young, and W. R. Clark. 1987. Resistance of cloned cytotoxic T lymphoctes to cell-mediated cytotoxicity. *J. Exp. Med.* 166:1070.
11. Shinki, T., H. Ishikawa, M. Hattori, and K. Okumura. 1988. Resistance of mouse cytolytic cells to pore-forming protein-mediated cytolysis. *Eur. J. Immunol.* 18:29.
12. Jiang, S., P. M. Persechini, A. Zychlinsky, C.-C. Liu, B. Perussia, and J. D.-E Young. 1988. Resistance of cytolytic lymphocytes to perforin-mediated killing: lack of correlation with complement-associated homologous species restriction. *J. Exp. Med.* 168:2207.
13. Perussia, B., S. Starr, S. Abraham, V. Fanning, and G. Trinchieri. 1983. Human natural killer cells analysed by B73.1, a monoclonal antibody blocking Fc receptor functions. I. Characterization of the lymphocyte subset reactive with B73.1. *J. Immunol.* 130:2133.
14. Palladino, M. A., Y. Obata, E. R. Stockert, and H. F. Oettgen. 1983. Characterization of interleukin 2-dependent cytotoxic T-cell clones: specificity, cell surface phenotype, and susceptibility to blocking by Lyt antisera. *Cancer Res.* 43:572.
15. Young, J. D.-E, H. Hengartner, E. R. Podack, and Z. A. Cohn. 1986. Purification and characterization of a cytolytic pore-forming protein from granules of cloned lymphocytes with natural killer activity. *Cell.* 44:849.
16. Berke, G., and D. B. Amos. 1973. Lymphocyte-mediated cytolysis. The LMC cycle and its role in transplantation immunity. *Transplant. Rev.* 17:71.
17. Kaufmann, Y., G. Berke, and Z. Eshhar. 1981. T lymphocyte hybridomas that mediate specific tumor-cell lysis in vitro. *Proc. Natl. Acad. Sci. USA.* 78:2502.
18. Kaufmann, Y., and G. Berke. 1983. Monoclonal CTL-hybridomas, capable of specific killing activity, antigenic responsiveness and inducible interleukin(s) secretion. *J. Immunol.* 131:50.
19. Vujanovic, N. L., R. B. Herberman, A. L. Maghazachi, and J. C. Hiserodt. 1988. Lymphokine-activated killer cells in rats. III. A simple method for the purification of large granular lymphocytes and their rapid conversion into lymphokine-activated killer cells. *J. Exp. Med.* 167:15.
20. Julius, M. H., E. Simpson, and A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3:645.
21. Persechini, P. M., and J. D.-E. Young. 1988. The primary structure of the lymphocyte pore-forming protein perforin: partial amino acid sequencing and determination of isoelectric point. *Biochem. Biophys. Res. Commun.* 156:740.

22. Kaufmann, Y., T. Silverman, B.-Z. Levi, and K. Ozato. 1987. Induction of c-ets and c-fos gene expression upon antigenic stimulation of a T cell hybridoma with inducible cytolytic capacity. *J. Exp. Med.* 166:810.
23. Kaufmann, Y., A. E. Chang, R. J. Robb, and S. A. Rosenberg. 1984. Mechanism of action of Cyclosporin A: inhibition of lymphokine secretion studied with antigen-stimulated T cell hybridomas. *J. Immunol.* 133:3107.
24. Berke, G., and D. Rosen. 1987. Are lytic granules and perforin 1 involved in lysis induced by in vivo-primed peritoneal exudate cytolytic T lymphocytes?. *Transplant. Proc.* 19:1.
25. Dennert, G., C. G. Anderson, and G. Prochazka. 1987. High activity of N-alpha-benzyloxycarbonyl-L-lysine thiobenzyl ester serine esterase and cytolytic perforin in cloned cell lines is not demonstrable in in-vivo-induced cytotoxic effector cells. *Proc. Natl. Acad. Sci. USA.* 84:5004.
26. Berke, G., and D. Rosen. 1988. Highly lytic in vivo primed cytolytic T lymphocytes devoid of lytic granules and BLT-esterase activity acquire these constituents in the presence of T cell growth factors upon blast transformation in vitro. *J. Immunol.* 141:1429.
27. Garcia-Sanz, J. A., F. Velotti, H. R. MacDonald, D. Masson, J. Tschopp, and M. Nabholz. 1988. Appearance of granule-associated molecules during activation of cytolytic T-lymphocyte precursors by defined stimuli. *Immunology.* 64:129.
28. Nagler-Anderson, C., C. R. Verret, A. A. Firmenich, M. Berne, and H. N. Eisen. 1988. Resistance of primary $CD8^+$ cytotoxic T lymphocytes to lysis by cytotoxic granules from cloned T cell lines. *J. Immunol.* 141:3299.
29. Gorman, K., C.-C. Liu, A. Blakely, J. D.-E. Young, B. E. Torbett, and W. R. Clark. 1988. Cloned cytotoxic T lymphocytes as target cells. II. Polarity of lysis revisited. *J. Immunol.* 141:2211.
30. Jung, G., D. E. Martin, and H. J. Muller-Eberhard. 1987. Induction of cytotoxicity in human peripheral blood mononuclear cells by monoclonal antibody OKT3. *J. Immunol.* 139:639.
31. Martin, D. E., L. S. Zalman, and H. J. Muller-Eberhard. 1988. Induction of expression of cell-surface homologous restriction factor upon anti-CD3 stimulation of human peripheral lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:213.
32. Zalman, L. S., L. M. Wood, and H. J. Muller-Eberhard. 1986. Isolation of a human erythrocyte membrane protein capable of inhibiting expression of homologous complement transmembrane channels. *Proc. Natl. Acad. Sci. USA.* 83:6975.
33. Shin, M. L., G. Hansch, V. W. Hu, and A. Nicholson-Weller. 1986. Membrane factors responsible for homologous species restriction of complement-mediated lysis: evidence for a factor other than DAF operating at the stage of C8 and C9. *J. Exp. Med.* 136:1777.
34. Zalman, L. S., L. M. Wood, and H. J. Muller-Eberhard. 1988. Inhibition of antibody-dependent lymphocyte cytotoxicity by homologous restriction factor incorporated into target cell membranes. *J. Exp. Med.* 166:947.