RESISTANCE OF CYTOTOXIC T LYMPHOCYTES TO THE LYTIC EFFECTS OF THEIR TOXIC GRANULES

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Considerable evidence suggests that when cytolytic T lymphocytes (CTLs) adhere to and recognize surface antigens on other cells (target cells), the CTLs are stimulated to release toxic cytoplasmic granules (1, 2). The granules contain a cytolytic protein, termed perforin (3) or cytolysin (4), that forms ion channels in cell membranes (5); the resulting perturbations in intracellular ion concentrations of the adherent target cells, particularly of Ca^{2+} (6, 7), are probably responsible for their death. Fragmentation of target cell DNA is another lethal change induced by CTLs (8, 9), and this change is also evoked in target cells when they interact with the toxic granules isolated from CTLs (7). CTLs are evidently able to escape all of these potentially lethal effects, since they can interact successively with many target cells, lysing each in turn (10, 11).

How does a CTL avoid being destroyed by the cytolytic components it releases? To search for an answer, we previously compared several CTL cell lines with a variety of other cells as targets for another CTL cell line (12). The comparison was complicated by antigenic diversity of the cells used as targets and the critical role of antigen recognition in triggering the expression of cytolytic activity. Nevertheless, the results as a whole indicated clearly that, when tested as targets, the cloned CTL cell lines were less susceptible to destruction than the other cells examined under the same conditions. The findings implied that CTLs may be more resistant than other cells to the cytotoxic molecules released during a CTL–target cell encounter. To test this possibility directly, we have here measured the extent to which isolated cytotoxic granules lyse CTL and a variety of other cell lines. The cell lines tested included tumor cells, cultured fibroblasts, and representatives of the two principal kinds of cloned murine T cell lines: $CD4^+$ T cells ($L3T4^+$, $Lyt\text{-}2^-$) and $CD8^+$ T cells ($L3T4^-$, $Lyt\text{-}2^+$). All of the $CD8^+$ T cell lines were cytolytic; of the $CD4^+$ T cell lines, five were cytolytic and three were not. We refer to the $CD8^+$ cells as CTLs, to the noncytolytic $CD4^+$ cells as T helpers, and to the cytolytic $CD4^+$ cells as T helper/killers. Of the 26 cell lines tested, the $CD8^+$ CTLs stood out as being strikingly resistant; they were

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resistant even after they were subjected to prolonged exposure to reagents that deplete cells of ATP, an exposure that enhanced the susceptibility of all the other cells tested to granule-mediated lysis.

Materials and Methods

Cytolytic Granules. All procedures for isolating cytolytic granules from cloned CTL cell line 3H2 by Percoll-density gradient centrifugation (3) were performed at 4°C or on ice. Cells (1–2 × 10^9) were washed twice in HBSS, once in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 1 mM ATP, 10 mM Pipes, pH 6.8 [13]), resuspended in relaxation buffer at 10^8 cells/ml, and disrupted by nitrogen cavitation (20 min at 350 psi). Nuclei were removed by centrifugation at ~1,000 *g* for 5 min, and the supernatant (combined with a washing from the pelleted nuclei) was applied to a discontinuous Percoll gradient in relaxation buffer at ~10^9 cell-equivalents per 25 ml gradient (10 ml, 90% Percoll; 5.5 ml, 60%; 9.5 ml, 39%). Gradients were centrifuged at 20,000 rpm in a Beckman SW 28 rotor (g_{av} = 58,000 *g*) for 30 min. Fractions were collected and hemolytic activity was assayed as described below. Fractions with peak hemolytic activity were pooled; their density was raised by adding one-half volume of 90% Percoll, and the Percoll was removed by centrifugation at 100,000 *g* for 90 min. The granules, which sedimented as a fine layer above the clear Percoll pellet, were resuspended in a small volume of relaxation buffer (0.5–1 ml) and stored at −70°C in small aliquots. These granule preparations not only lyse target cells but also induce DNA fragmentation in YAC-1 cells (7).

Protein content of the granule preparations was measured by the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). To 1–20 μl of sample was added 1 ml of BCA reagent. After 30 min at 37°C, optical density at 600 nm was measured and compared against BSA and ovalbumin standards.

Assay for Hemolysis. Lytic activity of granules was assayed by a modification of the protocol of Masson and Tschopp (14). Sheep erythrocytes (SRBC) (Colorado Serum Co., Denver, CO) were washed twice in Hepes-buffered saline (HBS)[1] (155 mM NaCl, 10 mM Hepes, pH 7.2) and resuspended at 3.3 × 10^8 cells/ml in HBS, 5 mM $CaCl_2$, 0.4 mg/ml BSA. 1–10 μl of granule suspension, appropriately diluted in relaxation buffer, was added to 1.5 ml Eppendorf centrifuge tubes at 0°C, and then 100 μl of SRBC suspension was introduced. After 15 min at 37°C, 1 ml of cold 1 mM EDTA in PBS was added to stop the reaction and the samples were centrifuged for 1 min at 8,000 *g*. Hemoglobin in the supernatant was determined from the optical density at 414 nm of 150 μl aliquots measured on a Titertek Multiskan plate reader.

Cytolytic Activity of Isolated Granules. Cells were labeled with ^{51}Cr by incubating them with 100 μCi $Na_2{}^{51}CrO_4$ in 200 μl of 1:1 saline and K medium (K medium: RPMI 1640, 10% FCS, 10 mM Hepes, 50 μM 2-ME, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin) for 1 h at 37°C. They were then washed once and resuspended in K medium. For ATP depletion experiments, ^{51}Cr-labeled cells were preincubated at ~10^6 cells/ml for 2 h at 37°C in K medium supplemented with 1 mM KCN and 1.5 mM NaN_3, inhibitors of oxidative phosphorylation. Control cells were incubated, in parallel, without the inhibitors. In some experiments 2-deoxyglucose (at 5 mM), an inhibitor of glycolysis, was included with the KCN and NaN_3. All cells were finally washed twice in cold HBS, counted, and resuspended at 2.5 × 10^5 cells/ml in HBS, 5 mM $CaCl_2$, 0.4 mg/ml BSA; in addition, 1 mM KCN, 1.5 mM NaN_3, and 5 mM 2-deoxyglucose were included for cells that had been preincubated with these inhibitors.

Granules from frozen (−70°C) stocks were diluted serially in relaxation buffer, and 10 μl of diluted granules were added at 4°C to duplicate wells of U-bottom, 96-well microtiter plates. In some experiments, 10 μl aliquots of serial dilutions of melittin was added in place of the granules. To measure total release of ^{51}Cr from the labeled target cells, 10 μl of 10% NP-40 was added; to measure nonspecific (spontaneous) release, 10 μl of relaxation buffer was added. 100 μl of ^{51}Cr-labeled cell suspension was then added to the granules,

[1] *Abbreviation used in this paper:* HBS, Hepes-buffered saline.

or melittin, or the control solutions. After 1 h at 37°C, 150 μl of 5 mM EDTA in PBS were added to each well. The plates were then centrifuged at 1,500 *g* for 5 min, and 150 μl of the supernatants were counted in a Packard gamma scintillation counter. Percent specific lysis was determined as 100 × [(cpm released in the presence of granules) − (cpm released in absence of granules)]/[(total cpm released by NP-40) − (cpm released without granules)]. Background ^{51}Cr release did not exceed 15% of total ^{51}Cr released with NP-40.

Inhibition of SRBC Lysis by Isolated Granules. Increasing numbers of competing cells (P815 and CTL clone 2C) in 50 μl of HBS, 5 mM $CaCl_2$, 0.4 μg/ml BSA were added to 100 μl of SRBC (3.3×10^7 cells) in the same buffer. Then 10 μl of diluted granules were added, selecting a granule dilution (in relaxation buffer) that, in the absence of competing cells, yielded ~50% lysis of 3.3×10^7 SRBC. Lysis of SRBC was measured as described above.

Cell Lines. Cell lines (see Table I) were cultured in K medium (described above). The T cells received, in addition, rIL-2, irradiated (4,500 rad) stimulator cells, and in some instances, soluble protein antigens (see below). Cell lines P815, A20, YAC-1, EL4, BW 5147, S49, and BALB/3T3 clone A.31 are described in the American Type Culture Collection (Rockville, MD) catalogue (15). RDM4 was kindly provided by Dr. D. Raulet (16). Line X63-Ag8.653 cells has been described (17). We derived the primary fibroblast culture from 14-d BALB/c fetuses and maintained it in K medium for 7 d.

The noncytolytic $CD4^+$, $CD8^-$ clone D.10 (18) and the cytolytic $CD4^+$, $CD8^-$ T cell clones 5.5 and 5.9 (19, 20) were gifts of Drs. C. Janeway and B. Jones. D.10 was grown in K medium (see above), supplemented with 10 U/ml of rIL-2, 50 μg/ml conalbumin (Sigma Chemical Co., St. Louis, MO) and 10^6 cells/ml irradiated (4,500 rad) BALB.K spleen cells. Clones 5.5 and 5.9 were supplemented with 10 U/ml rIL-2, 50 μg/ml ovalbumin (Sigma Chemical Co.), and 10^6 cells/ml irradiated BALB/c spleen cells. Cytolytic ($CD4^+$, $CD8^-$) T cell lines L3C1, L2C4, and L3C4 were generously provided by Dr. M. Gefter (20); they were supplemented with 5 U/ml rIL-2, 10 μl/ml λ phage repressor protein, and 10^6 cells/ml irradiated BALB/c spleen cells.

Alloreactive $CD4^-$, $CD8^+$ cytolytic T cell clones 2C, 3H2, 2.1.1, 4K3, 3C11.1, and G4 were anti-L^d or anti-D^d CTLs derived from BALB.B mice as described (22, 23), and maintained in medium supplemented with 20 U/ml rIL-2 and 2×10^5 cells/ml irradiated P815 cells. Clone 18.1 ($CD4^+$, $CD8^-$) was derived from a BALB/c anti-BALB.B mixed lymphocyte reaction and maintained with 20 U/ml rIL-2 and 10^6 cells/ml irradiated BALB/c spleen cells; though formerly cytotoxic, this clone no longer has cytolytic activity. Clone TdH-1 ($CD4^+$, $CD8^-$) was derived by George Sigal from spleen cells of BALB/c mice that had been immunized with 2,4,6-trinitrochlorobenzene; it has the T helper phenotype, i.e., it secretes IL-2 in response to TNP–BALB/c spleen cells and lacks cytotoxicity. CTLL-2, originally a cytolytic T cell line (24), is devoid of cytolytic activity, though it contains cytolytic granules (3). It was grown in K medium with 20 U/ml rIL-2.

Phenotypes of T cells were established by cytofluorographic analysis (Model 50H; Ortho Diagnostics, Raritan, NJ) of cells labeled with the mAbs GK15 and 3.168.8, generously provided by Dr. F. Fitch. Cells were incubated at 0°C for 1 h with mAb diluted in K medium, washed, and then incubated with FITC-labeled goat anti–rat Ig. Finally, they were washed with cold PBS and fixed in PBS with 1% formaldehyde.

Cytotoxicity Assays of Intact T Cells. Cytotoxic activity of T cell clones was determined against P815 cells in lectin-mediated assays. ^{51}Cr-labeled P815 cells (2×10^4) were mixed with varying numbers of T cells in 200 μl of K medium and 10 μg/ml Con A (Pharmacia Fine Chemicals, Piscataway, NJ). After 4 h at 37°C, the reaction mixtures were centrifuged at 1,000 *g* for 5 min, and 100 μl of supernatant were counted. Maximum release was established from cells incubated with 1% NP-40. Percent specific lysis was calculated in a manner analogous to that described for assay of isolated granules. Antigen-specific cytotoxicity was also measured (in absence of lectin), using P815 cells as targets for class I–specific anti-$H2^d$ ($CD8^+$, $CD4^-$) CTLs and antigen-primed A.20 cells as targets for class II–restricted ($CD4^+$, $CD8^-$) T cells (17).

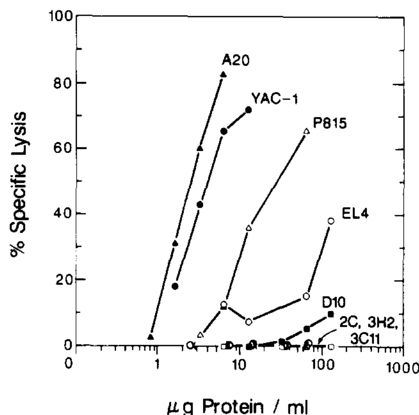

FIGURE 1. Lysis of representative cell lines by isolated toxic granules. Tumor cell lines (A20, YAC-1, P815, and EL4), a T helper clone (D10), and CTL clones (2C, 3C11, and 3H2) were labeled with ^{51}Cr and incubated for 1 h at 37°C with increasing concentrations of toxic granules (see Materials and Methods).

Results

Susceptibility of CTLs and Other Cell Lines to Lysis by Isolated Cytotoxic Granules. Marked differences in the susceptibility of various cell lines to lysis were seen when they were labeled with ^{51}Cr and incubated for 1 h at 37°C with cytotoxic granules at diverse concentrations. Fig. 1 illustrates titrations with representative cell lines that were either highly susceptible to lysis (A-20, YAC-1), moderately susceptible (P815, EL-4), or resistant ($CD8^+$ CTL clones 2C, 3H2, and 3C11, and a T helper clone D10). In Table I, the results with all cell lines are summarized by listing (*a*) the concentration of granule protein required to lyse 30% of each cell line (termed the L_{30} value) and (*b*) the percent lysis observed at the maximum concentration of granules tested (130 μg granule protein per milliliter).

All of the tumor cell lines were extensively lysed, as were the two fibroblast cell lines, one an established 3T3 cell line and the other a primary fibroblast culture. In contrast, the three noncytolytic $CD4^+$ (T helper) cells and one of the cytolytic $CD4^+$ cells were lysed only to a slight extent. The other cytolytic $CD4^+$ cell lines and all seven of the CTL ($CD8^+$) cell lines were not lysed to a detectable extent (<2% specific lysis).

The Effects of ATP-depleting Reagents. One possible explanation for the resistance of the T cell lines is that they may be able to rapidly clear their cell surface membranes of inserted perforin, just as many nucleated cells reduce their susceptibility to complement-mediated lysis by removing membrane lesions due to inserted late-acting complement components (25, 26). Another possibility is that the T cell lines might have unusually active ion pumps that prevent or promptly restore the marked changes in intracellular ion concentrations, especially of Ca^{2+} (7), that result from insertion of perforin channels. All of these mechanisms are expected to be dependent upon cellular ATP levels. Accordingly, we determined whether reagents that deplete cells of ATP (cyanide, azide, 2-deoxyglucose) affected cell lysis. As illustrated in Fig. 2*A* and summarized in Table II, exposure of the prospective target cells to these reagents for 2 h resulted in the two representative tumor cell lines tested becoming much more sensitive to the granules and in all of the $CD4^+$ T cell lines (both cytolytic and noncytolytic) shifting from minimal to substantial susceptibility to granule-me-

TABLE I
Lysis of Various Cell Lines by Toxic Granules from CTL and by Melittin, an Ion Channel–forming Polypeptide

Cell lines	Specific lysis by: CTL granules		Melittin
	L_{30}*	Maximum	L_{30}*
	μg granule protein/ml	%	*μg protein/ml*
A20, B lymphoma	1.6	>90	3
YAC-1, T lymphoma	2.7	>90	3
L-10, B lymphoma	2.2	>90	3
RDM-4, T lymphoma	5.4	85	6
X63.653, myeloma	6.5	70	ND
BW5147, T lymphoma	8.7	75	6
S49, T lymphoma	13	63	6
P815, mastocytoma	13‡	74‡	6
EL-4, T lymphoma	105	30	12
A_{31}, 3T3 fibroblast	130	30	6
Primary fibroblast culture	65	50	6
TdH-1, T helper	—	12	6
18.1, T helper	—	8	ND
D10, T helper	—	8	6
L3C1, T helper/killer	—	<2	ND
L2C4, T helper/killer	—	<2	ND
L3C4, T helper/killer	—	7	ND
5.9, T helper/killer	—	<2	12
5.5, T helper/killer	—	<2	ND
2C, CTL	—	<2	6
3H2, CTL	—	<2	6
2.1.1, CTL	—	<2	6
3C11.1, CTL	—	<2	6
G4, CTL	—	<2	ND
4K3, CTL	—	<2	ND
CTLL-2, CTL	—	<2	6

* L_{30} values are concentrations of granule protein or melittin at which 30% specific lysis was observed. —, 30% lysis was not reached, even at the highest granule concentration (130 μg protein/ml).
‡ In another pair of titrations, with what may have been another subline of P815 cells, the L_{30} value and maximum percent lysis were 95 and 37, respectively.

diated lysis. In contrast, after exposure to these reagents under identical conditions all of the $CD8^+$ CTL cell lines were still resistant (Fig. 2*B*).

Are CTLs Resistant to Other Polypeptides that Form Ion Channels in Cell Membranes? To determine whether the resistance of CTLs to cytotoxic granules reflects a general property that enables these cells to resist the lytic effects of other toxic, ion channel–forming polypeptides, we tested some of the cell lines with melittin, a cytolytic polypeptide derived from bee venom. Representative titrations with melittin are shown in Fig. 3, and all results are summarized in Table I as L_{30} values (i.e. concentration of melittin required for 30% specific

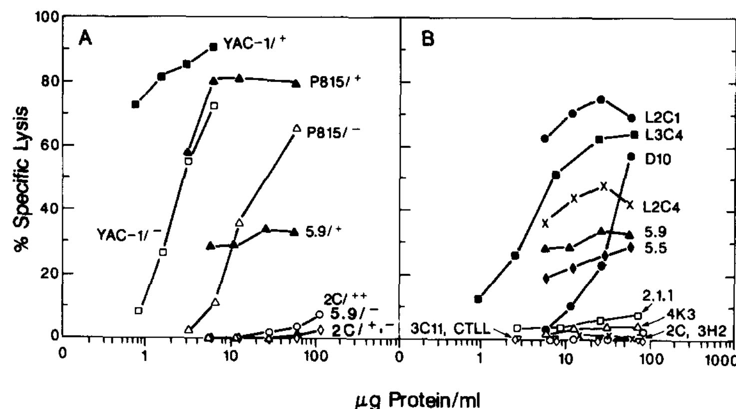

FIGURE 2. Effect of inhibitors of energy metabolism on the susceptibility of cell lines to granule-mediated lysis. *A*. Cells were first incubated for 2 h with (*closed symbols*) or without (*open symbols*) NaN_3 and KCN, and then for 1 h with toxic granules at varying concentrations. When inhibitors were present in the first incubation, they were present in the second one. (+) and (−) signs after the names of cell lines also indicate that both incubations were carried out in the presence (+) or absence (−) of NaN_3 and KCN. 2C/+,− indicates overlapping curves of 2C incubated with and without the inhibitors NaN_3 and KCN; the curve 2C/++ represents 2C cells incubated and maintained in 2-deoxyglucose, as well as in NaN_3 and KCN (see Materials and Methods). *B*. The effect of NaN_3 and KCN on the susceptibility of cloned T cell lines to lysis by toxic granules. $CD4^+$ T cells (*closed symbols*: D10, 5.9, 5.5, L3C4, L2C1 and ×, L2C4) are compared to $CD8^+$ CTLs (*open symbols*: 2C, 3H2, 2.1.1, 3C11-1, 4K3, and CTLL). All cell samples were incubated for 2 h with NaN_3 and KCN and then for 1 h with lytic granules plus the metabolic inhibitors.

release of ^{51}Cr). No significant difference between CTLs and the other cells was seen. The absence of a significant difference agrees with a previous observation that CTLs as a whole are probably not more resistant than other cells to the lytic effects of antibody-activated complement (12), which are also due to the formation of ion channels in cell membranes. Thus CTLs appear to be selectively resistant to the lytic components (perforin and perhaps others) they release.

Inhibition of Granule-mediated Lysis of Red Blood Cells by CTL and Other Cell Lines. CTLs may be less sensitive to granule-mediated lysis than other cells because perforin does not insert into the CTL surface membrane, or because it inserts but is then effectively removed or inactivated. In a preliminary attempt to distinguish between these possibilities, a CTL cell line (clone 2C) and a tumor cell line (P815) were compared for their ability to inhibit lysis of SRBC by the granules. For this purpose, a fixed number of SRBC (3×10^7) were mixed with increasing numbers of the CTL or P815 cells; the granules were then added and hemolysis determined. As shown in Fig. 4, lysis of the SRBC was inhibited by ~50% by 2×10^4 P815 cells, and almost completely by 2×10^5 P815 cells. In contrast, the same numbers of CTL (2C cells) had only minimal inhibitory effects. That granule-sensitive P815 cells were inhibitory, but not the granule-resistant CTLs suggests that granule-sensitive cells take up perforin and that the granule-resistant cells do not, or do so to a far lesser extent. Many more cell lines will have to be examined in this way to determine whether or not marked resistance to lysis by the toxic granules correlates with inability to inhibit granule-mediated hemolysis.

TABLE II
Effect of Cyanide and Azide on Lysis of Various Cell Lines by Cytotoxic Granules

Cell lines‡	L_{30}*		Maximum lysis	
	Without N_3^- and CN^-	With N_3^- and CN^-	Without N_3^- and CN^-	With N_3^- and CN^-
	μg granule protein/ml		%	
Tumor cells				
YAC-1	1.6	<0.1	>90	>95
P815	13	1.6	80	80
$CD4^+$ T cells				
TdH-1	—	130	12	30
D10	—	33	8	50
L3C1	—	<8	<2	76
L2C4	—	<8	<2	48
L3C4	—	<8	7	72
5.5	—	130	<2	23
5.9	—	130	<2	32
$CD8^+$ T cells				
2C	—	—	<2	3
3H2	—	—	<2	14
2.1.1	—	—	<2	7
3C11.1	—	—	<2	<2
4K3	—	—	<2	5
CTLL-2	—	—	<2	<2

* See Table I; —, 30% lysis was not reached, even at the highest concentration of toxic granules (130 μg protein/ml).
‡ See Table I for more information on the cell lines.

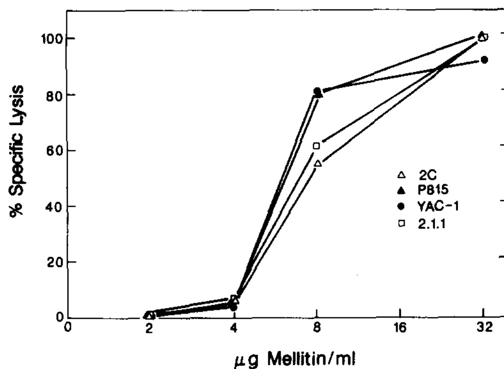

FIGURE 3. Lysis of cell lines by melittin. Representative tumor cell lines (P815 and YAC-1) and cloned CTL cell lines (2C, 2.1.1) were incubated 1 h with varying concentrations of the bee venom peptide melittin (see Table I).

Discussion

The main point that emerges from this study is that cloned murine CTL ($CD8^+$, $CD4^-$) cell lines are more resistant than other cells to toxic granules isolated from CTLs. The findings extend several other observations that CTLs make poor targets for other CTLs (12, 27). Yet many other studies have demonstrated that, under some circumstances, a CTL can be lysed when it is recognized by other CTLs (28–30), a phenomenon referred to as one-way killing

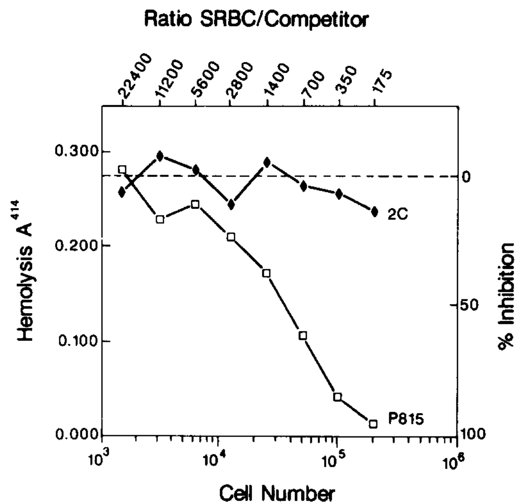

FIGURE 4. Inhibition of granule-mediated lysis of erythrocytes by a tumor cell line and a CTL cell line. A constant number of SRBC (3.3×10^7) was incubated with a fixed amount of toxic granules (75 ng protein) in the presence of varying numbers of a tumor cell line (P815) and a CTL cell line (2C). Lysis of the SRBC is indicated by absorbance at 414 nm (see Materials and Methods).

(30). Thus, the polyclonal CTL population in a mixed lymphocyte culture (MLC) prepared by incubating spleen cells from BALB.K mice (H-2 haplotype, H-2^k) with irradiated spleen cells of BALB.B mice (H-2^b) was previously shown (12) to lyse several of the granule-resistant CTL cell lines studied here (clones 2C, 2.1.1, and G4, all with the H-2^b haplotype); however, 5–10 times more cytolytic anti-H-2^b cells were required to kill these CTL cell lines than an H-2^b tumor (EL-4). Thus, it would not be surprising if CTLs could be lysed by higher concentrations of active granule components than we have so far been able to prepare. From all of these observations we conclude that, although CTLs are not totally resistant to cytolytic attack by other CTLs, they are more resistant than all other cells so far tested.

The pronounced but less than total resistance of CTLs provides the basis for a coherent view that can reconcile (*a*) the ability of these cells to kill multiple target cells in succession without themselves being damaged, and (*b*) their susceptibility to lysis when recognized by other CTLs. According to this view, at each encounter with a target cell a CTL releases only a small proportion of its toxic granules; the amount released is sufficient to kill most target cells but not to kill the CTL. Besides enabling the CTL to avoid killing itself, the release of a limited number of granules has the additional advantage that the CTL is left, after attacking one target cell, with a sufficient supply of granules to kill other target cells in a time interval (e.g., 4 h) that is probably too brief for the CTL to replenish its supply of granules.

This limited-release model is consistent with one-way killing of target CTLs by other (aggressor) CTLs, especially in an allogeneic MLC where the fraction of aggressor CTLs is not defined (30). At high aggressor/target ratios, several aggressor CTLs might be able simultaneously to adhere to and deliver their individually controlled release of toxic granules on a single hapless target CTL. The latter (i.e., the recognized CTL) would therefore be the recipient of multiple doses of granules that, in aggregate, would be lethal. However, the aggressor CTLs would be undamaged, because each of them, according to this model,

would be exposed only to the limited number of granules it releases. Under these circumstances, one-way recognition would result in one-way killing.

It should also be noted that there are additional mechanisms that may damage CTLs in MLC. For instance, such cultures also contain activated macrophages, which destroy susceptible cells by means of secreted reactive oxygen intermediates (31, 32). It is also possible that various CTL clones differ in the amount of granules they release per target cell encounter, and those that release a larger number (i.e., the more aggressive CTLs) might be able to kill the target CTLs they recognize, even in 1:1 agressor/target cell conjugates. But in this event we expect that the more aggressive CTLs will also prove to be more resistant to granule-mediated lysis than less aggressive ones.

An essential feature of the model is that only a portion of a CTL's granules are released on reacting with a target cell. An indication of the proportion is suggested by the amount of a serine esterase (termed BLT-serine esterase after the synthetic substrate it cleaves) released into the culture medium when CTLs attack target cells (33). Because the enzyme seems to be located in the same cytoplasmic granules that contain perforin (33–35), the amount of secreted enzyme probably approximates the number of toxic granules that are released. Secreted perforin, in contrast, is not detectable in the culture medium, perhaps because of a strong propensity to bind to cell membranes. As previously described, 30% of the BLT-serine esterase present initially in CTLs was found in the extracellular medium after a 3-h incubation with an excess of target cells (33). Because multiple CTL–target cell encounters must have occurred during this period, a preliminary estimate suggests that no more than ~10% of a CTL's toxic granules are released per target cell encounter.

The exposure of prospective target cells to reagents (azide, cyanide, 2-deoxyglucose) that deplete cells of ATP enhanced the susceptibility of most cells to granule-mediated lysis, but left the $CD8^+$ CTLs resistant. The difference between these CTLs and the other cells tested leads us to distinguish two modes of resistance to lysis: one ATP dependent (active resistance), the other ATP independent (passive resistance). As noted above, the ATP-dependent mechanism may reflect the clearing of cell membranes of inserted perforin, much the way many nucleated cells reduce their susceptibility to lysis by activated complement by eliminating the inserted complement attack complexes (C5b-8), possibly through endocytosis of affected plasma membranes (25, 26). The ATP-dependent mechanism seems to be well developed in the $CD4^+$ T cell lines (both cytolytic and noncytolytic) studied here, as their susceptibility to lysis increased greatly on incubating them with azide and cyanide. However, the cytolytic $CD4^+$ T cells (clones 5.5, 5.9, L2C1, L3C4, and L2C4) were only weakly cytolytic (e.g., they caused only ~20% lysis of their specific target cells at a $CD4^+$ T cell/target cell ratio of 20:1). It is possible that more highly cytolytic $CD4^+$ cells would also exhibit ATP-independent resistance. Nevertheless, since several of the $CD4^+$ T cells tested here were readily lysed by intact CTLs (12) it seems clear that the ATP-dependent mechanism does not confer a high level of resistance.

The mechanism responsible for the ATP-independent (passive) resistance of the $CD8^+$ CTLs is unknown. But the tentative finding that granule-mediated lysis of erythrocytes was inhibited by a tumor cell line and not by a $CD8^+$ CTL

cell line suggests that these CTLs may not bind the granules' cytolytic components. If borne out by future studies, this inference could mean that surface membranes of cloned murine CTL cell lines differ in some significant manner from those of other cells, including $CD4^+$ T cells.

The cloned CTL cell lines used for this study have been maintained in culture for several years as nontransformed, essentially diploid cells (23), and their antigen-specific cytolytic activity depends upon periodic exposure to their specific target cells. Whether the corresponding cells in vivo are also resistant is not clear. To explore this issue we carried out preliminary tests on two spleen populations that were derived from a 7-d MLC (BALB/c spleen cells incubated with irradiated BALB.B spleen cells). One population, subjected to panning to enrich for $CD4^+$ T cells, was readily lysed by the granules. The other population, subjected to panning to enrich for $CD8^+$ T cells (i.e., for CTLs), was lysed only slightly, and only after preincubation in azide and cyanide; though this population, which is expected to contain recently differentiated CTLs, appeared to be somewhat resistant, the results are ambiguous because it is not clear that all of the $CD8^+$ T cells in a MLC are CTLs. To determine directly whether CTLs are more resistant than other cells in such cultures, it should be possible to add isolated granules to a MLC and determine if the surviving cells are significantly enriched for CTLs. We are currently accumulating the quantities of isolated granules required for such a test.

Finally, we note that human cloned cytotoxic T cell lines were readily lysed by toxic granules derived from murine CTLs (L_{30} = 20–65 μg granule protein per milliliter). Because these human CTL cell lines also lacked the hemolytic activity that is characteristic of perforin (36), it is possible that, in contrast to most murine CTLs, the human CTLs do not depend upon perforin for lysis of their target cells.

Summary

A cytotoxic T lymphocyte (CTL) characteristically kills target cells one after the other by releasing toxic granules that contain one or more cytolytic components. To determine how CTLs avoid destroying themselves when they release granules and lyse target cells, 7 murine $CD8^+$ CTL cell lines were compared with 19 other cell lines for susceptibility to lysis by the isolated toxic granules. Murine $CD8^+$ CTLs were clearly the most resistant cells: granules did not lyse them even after they were exposed to azide, cyanide, and 2-deoxyglucose, conditions that were found to enhance the susceptibility of all the other cells tested, including other T cells. Thus, resistance of $CD8^+$ CTLs to cytotoxic granules appears to be independent of cellular ATP. To reconcile these findings with other observations that, under some circumstances, CTLs can be lysed by other CTLs, we suggest a model in which a CTL releases only a limited proportion of its toxic granules at each antigen-specific encounter with a target cell; the amount released is sufficient to kill most target cells but to leave the CTL undamaged and with enough granules to attack other target cells.

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