

## RESISTANCE OF CLONED CYTOTOXIC T LYMPHOCYTES TO CELL-MEDIATED CYTOTOXICITY

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CTLs are known to kill their targets by a contact-dependent mechanism (see references 1 and 2 for review). The CTL binds to its target presumably through interaction of the TCR with class I antigens on the target cell surface, perhaps stabilized by subsidiary interactions involving lymphocyte function-associated antigen (LFA-1)<sup>1</sup> and Lyt-2 (3, 4). After conjugation, the CTL delivers effectively a lethal hit, the nature of which has been the subject of intensive research in several laboratories (see references 1, 2, and 5–8 for review). According to one model, CTL may synthesize, store, and secrete a pore-forming protein (PFP, perforin or cytolysin) that inserts into the target membrane in a fashion at least operationally similar to the complement system (6–8).

After a CTL has damaged its target cell, it is known that the same CTL can recycle to lyse new targets (9, 10). Throughout the expression of this repetitive lytic activity, the CTL spares itself from lysis. This observation suggests a mechanism by which a CTL may protect itself from inflicting self-injury. However, experiments first reported by Golstein (11), and subsequently confirmed by other laboratories (12–15), have shown that under certain circumstances CTLs are lysed by other CTLs, implying that CTLs must themselves be sensitive to whatever mechanism it is that they use to kill other targets. If this is true, then it is difficult to imagine how CTLs would escape being killed by any granule polypeptide they secrete into the intercellular spaces between the effector and target cells. Here, we report that cloned CTLs are in fact highly resistant to lysis mediated by primary and cloned CTLs and by isolated cytotoxic CTL granules. These experiments provide the basis for a protection mechanism by which CTLs may avoid self-inflicted destruction during the cytolytic reaction.

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<sup>1</sup> *Abbreviations used in this paper:* LAK, lymphokine-activated killer; LDCC, lectin-dependent, cell-mediated cytotoxicity; LFA, lymphocyte function-associated antigen; PFP, pore-forming protein; PK, promiscuous killer; TCGF, T cell growth factor.

## Materials and Methods

**Animals.** 6–8-wk-old CBA/J and C57BL/6 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. 6–8-wk-old CBA/cum mice were purchased from Cumberland View Farms, Clinton, TN. C57BL/6 and DBA/2 retired breeder mice were purchased from Simonsen Laboratories, Inc., Gilroy, CA. BALB.B mice were bred and maintained in our breeding facility.

**Preparation of T Cell Growth Factor (TCGF).** IL-2 producing EL-4 cells were grown to a density of  $10^6$  cells/ml in DME, supplemented with 0.55 mM L-arginine HCL, 0.27 mM L-asparagine, 1.47 mM L-glutamine, 2 g/L NaHCO<sub>3</sub>,  $5 \times 10^{-5}$  M 2-ME, antibiotics, and 5% FCS (medium referred as supplemented DME). The cells were cultured in the presence of PMA at a final concentration of 10 ng/ml. After 40 h, the cell free supernatant was collected, passed through a 0.22- $\mu$ m filter, and stored at  $-20^\circ\text{C}$ . Alternatively, TCGF was prepared from rat spleen cell cultures stimulated for 40 h in the presence of 10  $\mu$ g/ml Con A (Pharmacia Fine Chemicals, Uppsala, Sweden) in RPMI with 2% FCS. The supernatant was collected and stored at  $-20^\circ\text{C}$ .

**Cell Lines.** Clones AB.1 and AB.2 were derived in our laboratories by limiting dilution from a line that originally started as a BALB/c anti-C57BL/6 (H-2<sup>d</sup> anti-H-2<sup>b</sup>) MLC. AB.1 and AB.2 were selected for their ability to specifically lyse EL-4 target cells, and the clones were expanded into 2-ml flat-bottomed wells (Costar, Cambridge, MA) on a feeder layer of  $6.5 \times 10^6$  irradiated cells per each 2-ml well. The clones are maintained in 2-ml wells with weekly stimulation with  $6.5 \times 10^6$  irradiated feeder cells in supplemented DME containing 5% EL-4-conditioned medium as a source of TCGF. On the third day after stimulation, the cultures were split 1:3 and given fresh medium and TCGF (without stimulating cells). Clones KB1.24 and KB1.33 were derived in our laboratory in the same fashion as AB.1 and AB.2, except from a CBA anti-C57BL/6 (k anti-b) MLC.

Clone 83.4 was derived from a C57BL/6 anti-DBA/2 MLC line kindly provided by Dr. Michael Bevan (Scripps Clinic and Research Foundation, La Jolla, CA), and cloned and subcloned by limiting dilution in our laboratory. It was maintained by weekly stimulation with irradiated DBA/2 splenocytes in RPMI 1640 supplemented with 5% FCS and 5% rat spleen cell Con A supernatant as a source of TCGF.  $10^5$  83.4 cells were restimulated with  $5 \times 10^6$  DBA/2 spleen cells in 2-ml flat-bottomed wells (Costar). The cells were usually split 1:2 on day 3 with fresh medium and TCGF, but without addition of fresh stimulator cells.

Clone B3.3.1 was also provided by Dr. Michael Bevan. It was derived in his laboratory by limiting dilution from a C57BL/6 anti-BALB.B MLC-generated line. It is maintained in our laboratory exactly as described above for clone 83.4, except that irradiated BALB.B spleen cells are used for stimulation.

Clone L3 (C57BL/6 anti-DBA/2; b anti-d) was obtained from Dr. Andrew Glasebrook (Eli Lilly Research Labs, La Jolla, CA); clone OE-4 (C57BL/6 anti-BALB/c; b anti-d) was a gift of Dr. Osami Kanegawa (Eli Lilly Research Labs).

The C57BL/6 T cell leukemia EL-4, and the DBA/2 mastocytoma P815, are carried as ascites tumors by weekly injections of  $2 \times 10^6$  washed cells into the peritoneal cavities of syngeneic hosts.

CTL R8 cells, obtained originally from Dr. Michael A. Palladino, Genentech, Inc., So. San Francisco, CA, have been shown to react to a unique determinant on R1 $\delta$ 1 cells (16). They are maintained in  $\alpha$ MEM supplemented with 1 mM L-glutamine, nonessential amino acids, 15% Con A/PMA-stimulated rat spleen cell supernatant, and 10% FCS. These cells were used as a source of cytotoxic granules.

The H-2<sup>k</sup> lymphoma R1.1 (17), obtained from American Type Culture Collection (Rockville, MD), and the mastocytoma P815 cells are maintained *in vitro* in MEM/FCS. The EL-4 T cells are maintained by weekly passages in C57BL/6 mice.

**MLCs and Promiscuous Killer (PK) Cells.** For MLCs, spleens of 6–8-wk-old mice to be used as the responding cell population were teased and passed through a stainless steel screen into sterile PBS. The cells were treated with 0.85% NH<sub>4</sub>Cl at room temperature for 4 min to remove erythrocytes, washed twice in cold PBS, and resuspended in supplemented DME. Retired breeder mice were used as a source of stimulating cells;

spleen cells were prepared as just described but were irradiated with 2,500 rad from a 60 kV x-ray source. After washing, the responding cells were mixed with the stimulators at a ratio of 3:1. The MLCs were cultured for 5–6 d in DME with 5% FCS at a final concentration of  $2.5 \times 10^6$ /ml in 25-cm<sup>2</sup> tissue culture flasks (Costar).

PK cells were generated by incubating C57BL/6 naive spleen cells for 5 d in DME supplemented with 5% FCS and 15% EL-4-conditioned medium. Promiscuity was established by the ability to lyse self target cells in the absence of lectin. A second source of nonspecific killer cells was generated from cloned CTL by preincubation of the CTL with  $10^{-7}$  M PMA and  $4 \times 10^{-6}$  M ionomycin for 30 min at 37°C. The cells were washed three times with PBS before use in <sup>51</sup>Cr-release assays.

*Preparation of CTL R8 Granules.* CTL R8 cells were grown in TCGF-containing medium in large Petri dishes, and were collected after reaching confluence.  $2\text{--}3 \times 10^9$  cells were washed three times with PBS to remove residual serum, and then disrupted by nitrogen cavitation, as previously described (18). Granules were prepared by centrifugation through Percoll gradients, essentially as reported (18). Granule-enriched fractions were pooled and the Percoll was removed by sedimentation at 180,000 g for 2 h in an SW41 rotor (Beckman Instruments, Inc., Fullerton, CA) at 4°C. Isolated granules were extracted with high phosphate buffer as described, and soluble granule proteins were obtained by sedimentation of granule membranes by high-speed centrifugation (18). The final concentration of granules was thereafter adjusted with extraction buffer so that 1 μl of extract was equivalent to granules isolated from  $10^6$  cells. The granule extract was stored at -20°C and thawed immediately before use.

*Serological Detection of Class I Molecules.* Class I antigen density on the surface of various cells was determined by means of their ability to absorb cytotoxic activity from a standard aliquot of directly cytotoxic anti-class I mAb. Varying numbers of test cells were pelleted and resuspended directly in the standard aliquot of anti-class I mAb, and incubated 30 min on ice. The cells were repelleted, and a portion of the overlying antibody was tested in a precalibrated complement-dependent <sup>51</sup>Cr-release assay. Alternatively, the density of class I antigens was determined by using a cellular ELISA, essentially as described (19).

*Cytotoxicity Assays.* Appropriate target cells were suspended in 0.3 ml FBS containing 0.1 mCi Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> for 45 min at 37°C with occasional mixing. Target cells were washed three times with cold PBS and stored on ice until use. Effector cells recovered from culture were washed twice in cold PBS, resuspended in culture medium, and serially diluted to give the desired effector/target cell ratios upon mixture with  $10^4$  target cells in 96-v-well plates in a total volume of 0.2 ml/well. The plates were centrifuged at 300–500 g for 5 min before incubation for 3–4 h in a CO<sub>2</sub> incubator.

After incubation, the microtiter plates were centrifuged at 1,000 g, and 0.1 ml of the sample supernates were counted in a gamma counter (Gamma 5500; Beckman Instruments, Inc.). Cytotoxicity was calculated according to the formula: percent cytotoxicity =  $100 \times [(\text{cpm experimental release}) - (\text{cpm spontaneous release})] / [(\text{total cpm}) - (\text{cpm spontaneous release})]$ .

In experiments using soluble granule proteins as the cytotoxic effector reagent, specified amounts of the granule extract were plated out in 96-well plates kept on ice. <sup>51</sup>Cr-labeled targets were added to a final concentration of  $10^4$  cells/200 μl/well in quadruplicate. Serum-free medium was used to prevent inactivation of granule cytotoxicity by serum. The plates were incubated at 37°C for 3 h and <sup>51</sup>Cr released was assayed as before. The release associated with incubation of targets with extraction buffer alone was subtracted from each point.

*Conjugation Assays.* Target cells were washed and incubated in PBS containing 0.1 mg/ml FITC. Effector and target cells were mixed together either at 10:1 (primary effector cells) or 1:1 (cloned CTL effector cells), and centrifuged at 300 g for 5 min. Pelleted cell mixtures with cloned CTL effector cells were resuspended immediately after centrifugation to reduce extensive cell clumping. In both cases, resuspension was accomplished with a fixed number of strokes through a plastic disposable pipette tip. The cell mixture was analyzed by direct visualization through a fluorescence microscope. A total

TABLE I  
*Cloned CTLs Are Highly Resistant to Direct CTL-mediated Lysis*

Effector cell	Target cell	Net percent <sup>51</sup> Cr release at E/T ratio of:					
		20:1	10:1	5:1	2.5:1	1.2:1	0.6:1
83.4 (b anti-d)	P815 (H-2 <sup>d</sup> )	89	81	77	69	62	41
	EL-4 (H-2 <sup>b</sup> )	-1	-3	0	0	2	1
	AB.2 (d anti-b)	2	3	-1	0	-2	-2
	AB.1 (d anti-b)	2	-1	-2	1	ND	ND
AB.2 (d anti-b)	EL-4 (H-2 <sup>b</sup> )	93	96	91	87	83	72
	P815 (H-2 <sup>d</sup> )	0	0	-3	-3	-1	-2
	83.4 (b anti-d)	-1	0	0	0	3	-1
	B3.3.1 (b anti-BALB.B)	0	3	-5	-1	2	1
	OE4 (b anti-d)	-2	0	-2	-1	ND	ND
	L3 (b anti-d)	4	3	4	4	ND	ND
KB1.24 (k anti-b)	EL-4 (H-2 <sup>b</sup> )	70	72	63	54	40	41
	P815 (H-2 <sup>d</sup> )	2	3	0	-1	ND	ND
	A20.2J (H-2 <sup>d</sup> )	2	-1	-1	-2	ND	ND
	83.4 (b anti-d)	-3	0	-11	-4	ND	ND
	L3 (b anti-d)	2	2	1	0	ND	ND
	H7.1 (b anti-BALB.B)	ND	3	1	0	-1	ND

CTL clones 83.4 (H-2<sup>b</sup> anti-H-2<sup>d</sup>), AB.2 (H-2<sup>d</sup> anti-H-2<sup>b</sup>), and KB1.24 (H-2<sup>k</sup> anti-H-2<sup>b</sup>) were used as effector cells in 3-h <sup>51</sup>Cr release assays against the indicated target cells. Spontaneous release values for P815 and EL-4 targets were <10%; for AB.2, B3.3.1, 83.4, OE4, H7.1, and L3, 17, 27, 29, 20, 27 and 30%, respectively.

of 300 target cells was counted, and the percentage having one or more effector cells bound was recorded.

*Pretreatment of Target Cells with Lectin for LDCC Assays.* P815 mastocytoma, EL-4 leukemia tumor, and CTL clones were used as target cells in lectin-dependent cellular cytotoxicity (LDCC) assays. Target cells first labeled with <sup>51</sup>Cr as described above, were washed three times with PBS, and then resuspended in RPMI containing 5% FCS at a cell density of 5–7 × 10<sup>6</sup> cells/ml. To this, 13 μg/ml Con A was added, and allowed to incubate 30 min at 37°C. The cells were washed twice with RPMI (with 5% FCS), resuspended to the desired concentration and used in the <sup>51</sup>Cr-release assay described above.

## Results

*CTL Clones are Highly Resistant to Direct CTL-mediated Lysis.* Table I shows the results of several experiments in which we used a variety of cells, including several cloned CTLs, as targets for direct, antigen-specific killing by various cloned CTL effector cells. In every case, the effector cells lysed the normal specific targets very well, and the “normal” nonspecific target cells not at all, as expected, since effector clones were initially selected on the basis of this antigen-specific lytic capability (see Materials and Methods). However, in parallel experiments, we were completely unable to lyse any cloned CTL as a target cell, even though the target CTLs used displayed the same class I antigens as the other normal lysis-sensitive targets. Lack of lysis between cloned CTLs was observed whether recognition was bidirectional or unidirectional (Table I).

We also tested the sensitivity of cloned CTLs (as targets) to lysis by primary

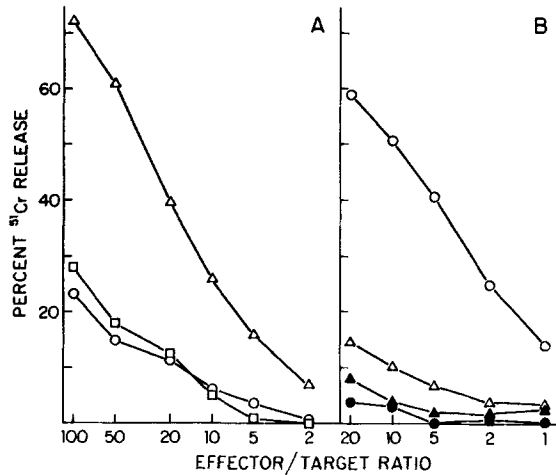


FIGURE 1. Cloned CTL are poorly susceptible to lysis by primary effector CTL. Primary CTLs were generated in MLCs as described in Materials and Methods. CBA anti-DBA/2 (A) or CBA anti-C57BL/6 (B) CTLs were harvested at 5 d and assayed against the following target cells. (Δ) P815; (○) EL4; (□) AB.2; (▲) 83.4; (●) B3.31. Assays were for 3 h at the indicated E/T ratios.

CTLs generated in MLC. The MLC cultures were harvested on day 5 or 6, and tested against the specific target, a third party (nonspecific) target, and a CTL clone syngeneic to the specific target. The results are shown in Fig. 1. The two experiments shown are representative of the maximal (A) and minimal (B) levels of lysis of cloned CTL target cells we have seen in 20 such experiments. Taking the number of effector cells required to give a comparable level of lysis of different targets as a measure of relative target cell sensitivity, we find that cloned CTLs are on the average between 10 and 20 times more resistant to killing by primary CTLs compared with target cells normally used to measure cytolysis. In most cases, the level of killing of a cloned CTL target for which the MLC-generated effectors are specific is about the same as that displayed toward a third-party target cell. This pattern of response is true for primary CTL effectors generated either in MLCs or by in vivo immunization (data not shown).

*Cloned T Cells Display as much Class I Antigen as Other Target Cells.* Since direct CTL-mediated lysis is specific for and absolutely dependent upon the presence of class I MHC antigens, it was important to show that these antigens are present in normal quantities on our cloned T cell targets. This was accomplished by comparing the ability of cloned T cells and normal (lysis-susceptible) target cells to absorb class I-specific cytolytic antibodies. Sample results are shown in Fig. 2. The CTL clones AB.2 and 83.4 show the same levels of anti-class I antibody-absorbing capability as the respective H-2 syngeneic target cells P815 and EL-4. Similar results were found for all our CTL clones and other normal target cells using both the antibody absorption technique and an ELISA assay for cell surface antigens (data not shown). As shown in Fig. 3, the low level of lysis of cloned CTL target cells mediated by primary (MLC-generated) effector cells can be effectively blocked by target cell class I antibodies. We therefore conclude that the low level of antigen-specific lysis observed is carried out by CTLs and not by NK or PK cells.

Although cloned CTLs display normal levels of class I MHC antigens, it is still possible that other CTLs are not able to bind properly to them. Analyses were therefore carried out to verify conjugate formation using both cloned and primary CTLs as effector cells, and cloned CTLs as targets. Analysis of the

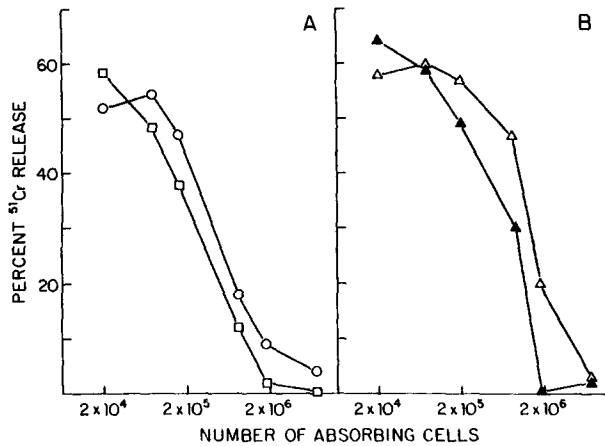


FIGURE 2. Cloned CTL display as much serologically detectable class I antigen as lysis-susceptible target cells. Cloned CTLs were compared with H-2-identical lysis-susceptible target cells for their ability to absorb class I-specific cytotoxic mAbs as described in Materials and Methods section. (A) The H-2<sup>d</sup> CTL clone AB.2 (□) compared with the H-2<sup>d</sup> target L10.A (○). (B) The H-2<sup>b</sup> CTL clone 83.4 (△) compared with the H-2<sup>b</sup> target EL-4 (▲).

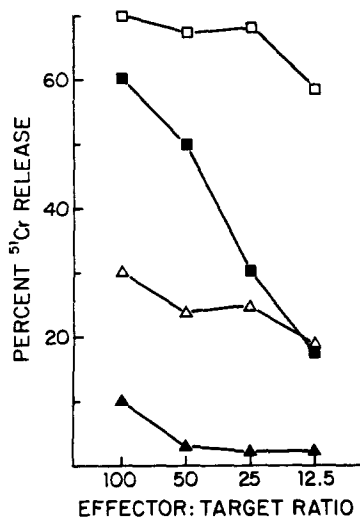


FIGURE 3. Low levels of lysis of cloned CTL by primary effector cells is class I-dependent. Primary CTLs generated in a CBA anti-C57BL/6 primary MLC (k anti-b) were tested against EL-4 target cells (□, ■) or the bad CTL clone 83.4 (△, ▲). Assays were carried out in the presence of normal serum (*open symbols*), or an antiserum specific for H-2<sup>b</sup> class I proteins (*closed symbols*).

binding of both primary and cloned CTL effectors to cloned CTL targets was difficult, due to excessive nonspecific aggregation particularly in clone/anticlone combinations. The clumps formed between cloned CTLs and their specific targets obviously contained both effector cells and fluoresceinated targets, but precise quantitation was difficult. Because of technical problems in conjugate scoring, it was not possible to reach a definitive conclusion about specificity of binding of either primary or cloned CTL effectors to cloned T cell targets.

*Sensitivity of Cloned CTL to Lysis in LDCC.* To further assess the extent of resistance displayed by CTLs to lysis by other CTLs, we tested the ability of cloned CTLs to kill cloned T cell targets in LDCC. Lectins are known to activate CTLs to lyse targets to which they adhere, irrespective of the target specificity. Representative results of such experiments are shown in Table II. The CTL clone 83.4 kills the syngeneic target cell EL-4 (H-2<sup>b</sup>) very efficiently if the latter is treated first with Con A. However, when tested against five different CTL clones, 83.4 was unable to kill any of them, whether they were treated with lectin

TABLE II  
*Cloned CTLs Are Not Lysed in LDCC*

Effector cell	Target cell	Con A	Net percent <sup>51</sup> Cr release at E/T ratio of:				
			20:1	10:1	5:1	2.5:1	1.2:1
83.4 (b anti-d)	EL4 (H-2 <sup>b</sup> )	-	4.0	2.2	1.9	3.3	0.6
		+	66.2	73.1	72.8	69.6	60.4
	AB.2 (H-2 <sup>d</sup> )	-	0.3	1.6	3.4	-1.2	2.2
		+	1.4	1.4	0.2	4.1	1.6
	83.4 (H-2 <sup>b</sup> )	-	2.1	2.7	1.6	0	0.2
		+	0.8	0.4	2.1	2.0	2.4
	42 (H-2 <sup>k</sup> )	-	-0.2	-3.2	-3.1	-3.0	ND
		+	2.8	-3.3	2.5	-1.6	ND
	OE4 (H-2 <sup>b</sup> )	-	-3.7	-4.6	-2.5	-3.4	ND
		+	-0.5	-1.5	-1.6	-1.5	ND
	KB1.24 (H-2 <sup>k</sup> )	-	2.7	6.3	2.2	1.8	2.4
		+	0.9	1.1	0.6	4.2	0.1
CBA anti-B6 primary CTL (k anti-b)	P815 (H-2 <sup>d</sup> )	-	8.3	4.2	4.6	0.2	0.4
		+	61.8	60.6	52.3	31.2	8.6
	AB.2 (H-2 <sup>d</sup> )	-	10.7	6.6	3.0	2.8	2.9
		+	9.0	8.6	13.1	1.9	4.8
	EL-4 (H-2 <sup>b</sup> )	-	59.6	51.7	42.8	29.4	13.0
		+	50.2	39.3	46.1	28.1	19.5

Target cells were assayed for lysis directly, or after pulsing with the lectin Con A as described in Materials and Methods. Assay time was 3 h; values shown are averages of triplicate samples corrected for spontaneous release.

or not. Identical results were obtained (a) with all CTL clones as effectors or targets; (b) whether the lectin was added free in the assay or precoated onto the target cell; (c) with both PHA and Con A as the mediating lectin. We also tested the ability of primary CTLs to lyse normal and cloned CTL target cells in LDCC. A sample of these results is also shown in Table II. MLC-generated effector cells lysed third-party targets very well in the presence of Con A, but were unable to lyse cloned CTL targets effectively. We found in every case that the relative sensitivity of cloned CTLs to lysis in LDCC is about the same as their relative sensitivity to lysis in direct CTL killing; e.g., between 5 and 10% that of normal target cells.

*Susceptibility of Cloned CTLs to Lysis by PK Cells.* Several laboratories have described the generation of cytotoxic nonspecific effector cells from lymphocytes and/or CTL lines in the presence of high levels of TCGF or IL-2, but in the absence of antigen or mitogen (20-25). They are distinguished from normal, allospecific CTLs in that they kill target cells in a class I-independent fashion (21-23). They may be distinguished from NK cells in that they kill untransformed self target cells as well as transformed self and nonself cells (23).

The susceptibility of cloned CTLs to lysis by PK cells generated from naive C57BL/6 spleen cells is shown in Fig. 4. Both the allogeneic target P815 and the syngeneic target EL-4 were killed effectively by TCGF-generated promiscuous C57BL/6 killer cells. However, the cloned CTL targets AB.2 and 83.4, which are H-2 identical to P815 and EL-4, respectively, were only poorly killed by

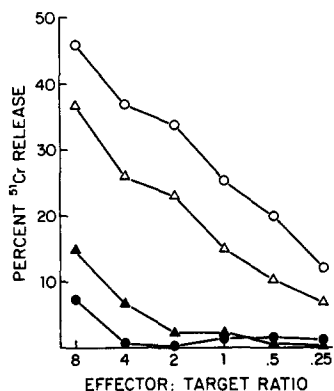


FIGURE 4. Cloned CTL are poorly lysed by PK cells. C57BL/6 spleen cells were incubated for 6 d with DME, 5% FCS, supplemented with 15% TCGF generated from EL4 cells (see Materials and Methods). The cells were washed and incubated for 3 h with  $^{51}\text{Cr}$ -labeled target cells in the absence of exogenous TCGF. (O) EL4; ( $\Delta$ ) P815; ( $\bullet$ ) 83.4; ( $\blacktriangle$ ) AB.2.

C57BL/6 PK cells. In our hands, cloned CTLs are even less susceptible to lysis by PK cells than they are to direct CTL killing or LDCC, using MLC-generated effector cells.

*PMA- and Ionomycin-induced Cloned CTLs Kill Normal Target Cells but not Cloned CTL Target Cells.* Using the method of Russell (26) to induce promiscuous killing in cloned CTLs, we tested PMA- and ionomycin-induced CTL clones against various target cells in a  $^{51}\text{Cr}$ -release assay. As shown in Table III, CTL clone 83.4 (b anti-d) lyses its specific target P815 very well, but does not normally lyse the syngeneic target cell EL-4 or the allogeneic (H-2<sup>k</sup>) target R1.1. However, after a 30-min exposure to PMA and ionomycin, 83.4 cells were very effective in lysing both of these target cells. On the other hand, PMA/ionomycin-induced 83.4 could not kill the H-2<sup>b</sup> CTL clone AB.2. This pattern of reactivity is exactly the same as that seen for direct killing of target cells by untreated cloned CTL effector cells: normal targets are lysed, but cloned CTL targets are not. This conclusion was supported with results obtained with all possible combinations of cloned CTLs (as effectors and targets) described in this paper.

*CTL Clones are Resistant to Lysis by Granule Proteins.* Recent studies in several laboratories have suggested that cloned CTLs and NK cells contain cytoplasmic granules/PFP that are lytic to a variety of tumor targets (reviewed in references 6–8). We next determined whether the CTL clones studied here could also be resistant to the contents of cytotoxic granules. As shown in Fig. 5, cloned CTLs when used as targets are highly resistant to lysis by granule proteins. CTLs were either absolutely refractory to lysis (R8, syngeneic to the source of granules) or weakly susceptible (AB.2, 83.4, and L3), while non-CTL targets (YAC-1, EL-4, P815, and R1.1) were highly susceptible to granule-mediated lysis. These data indicate that cloned CTLs resist cytolysis not only at the intact effector cell level but also when soluble effector proteins are used.

*Sensitivity of CTL Clones to Complement.* The sensitivity of several of our CTL clones to lysis by complement is shown in Fig. 6. The cells were first sensitized with an IgM anti-Thy-1 antibody, and then titrated with guinea pig complement. In several such experiments, we were unable to detect any difference in sensitivity to complement-mediated lysis between any of our CTL clones and target cells normally used to assay cytotoxicity.



TABLE III  
*PMA/Ionomycin-induced Cloned CTLs Lyse Third-party Normal Target Cells, but not Third-party Cloned CTL Target Cells*

Effector cell	Treatment	Target cell	Net percent <sup>51</sup> Cr release at E/T ratio of:			
			10:1	5:1	2.5:1	1.2:1
83.4	None	P815	64.2	66.1	63.0	42.4
		EL-4	0	0	0	0
		A20.2J	34.6	31.0	32.8	26.9
		KB1.24	0	0	0	0
		AB.2	2.8	2.1	1.9	0
83.4	PMA/iono	P815	58.3	34.8	27.2	13.6
		EL-4	50.5	38.8	35.1	28.7
		A20.2J	53.1	41.6	20.9	13.3
		KB1.24	3.9	2.2	0	0
		AB.2	0	0	0	0
KB1.24	None	P815	1.3	0.8	0	0
		EL-4	74.4	70.8	69.2	66.0
		A20.2J	1.1	0	0	0
		83.4	0	0	0	0
		AB.1	0	0	0	0
		AB.2	1.8	1.0	0	0
KB1.24	PMA/iono	P815	17.4	9.2	4.1	3.0
		EL-4	64.2	67.2	52.8	41.7
		A20.2J	39.7	20.1	9.8	3.9
		83.4	0	0	0	0
		AB.1	2.8	0	0	0
		AB.2	0	0	0	0

CTL clones 83.4 (b anti-d) and KB1.24 (d anti-b) were used as effector cells either directly or after 30 min incubation with PMA (10<sup>-7</sup> M) plus ionomycin (4 × 10<sup>-6</sup>) at 37°C. Assays were carried out for 3 h at 37°C. The <sup>51</sup>Cr-release values at the E/T ratios shown are the average of triplicate samples, corrected for spontaneous release.

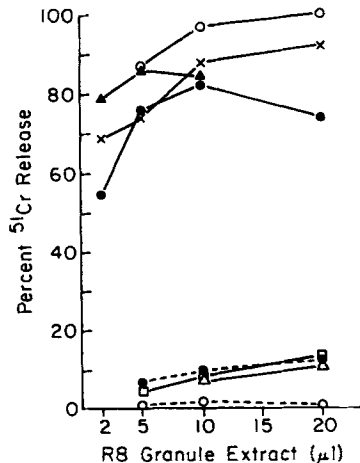


FIGURE 5. Susceptibility of cloned CTLs and other targets to granule-mediated lysis. <sup>51</sup>Cr-labeled targets were incubated with granule extracts in a 3-h cytotoxicity assay, as described in Materials and Methods. Targets were Yac-1 (○), EL-4 (●), P815 (X), R1.1 (▲), and CTLs AB.2 (Δ), 83.4 (—●—), R8 (—○—), L3 (□). Points shown represent means of quadruplicate samples.

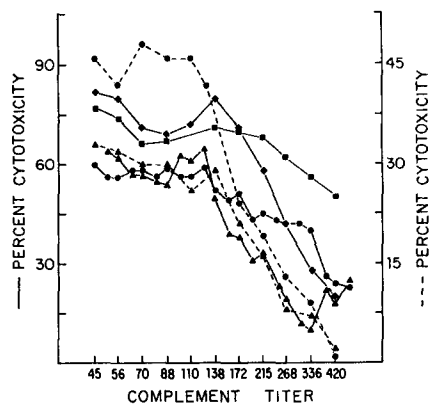


FIGURE 6. Sensitivity of cloned CTLs to complement-mediated lysis. The standard CTL target cell EL-4 (◆) was tested for sensitivity to complement lysis, along with the following CTL clones: (—▲—) L3; (—●—) AB.1; (—■—) AB.2; (—◆—) 83.4; (---▲---) KB1.24. After labeling with  $^{51}\text{Cr}$ , the target cells were incubated 30 min on ice with an IgM mAb specific for Thy-1. The cells were washed in PBS, and then incubated 60 min at  $37^\circ\text{C}$  with varying dilutions of guinea pig complement, after which  $^{51}\text{Cr}$  release was measured.

### Discussion

In direct, class I antigen-specific cytolysis, we find cloned CTLs used as target cells to be completely refractory to lysis by other cloned CTLs at E/T ratios and for assay periods sufficient for complete destruction of lysis-susceptible target cells normally used to measure cytotoxicity. We have tested 11 CTL clones generated in our and other laboratories, and have found no exception to this rule. Cloned CTL targets are also completely resistant to lysis by cloned CTL effector cells in both Con A- and PHA-mediated LDCC, and by cloned CTL induced to kill in a promiscuous fashion by PMA.

Unlike cloned CTLs as effectors, we find that primary CTLs are capable of lysing cloned CTL targets, although only to a low degree. With primary CTL effectors, we find that on average cloned CTL targets are 5% or less as sensitive to lysis as are susceptible target cells, that is, it requires on average at least 20 times more effector cells to achieve comparable levels of lysis of cloned CTL targets, compared to susceptible targets. Inasmuch as the limited lysis of cloned CTL by primary CTL populations that we do observe is class I dependent (Fig. 3), we conclude that the lytic activity is indeed expressed by CTL, and not by other effector cells such as NK cells. We find the same low degree of sensitivity to lysis in LDCC, and in promiscuous killing by TCGF-induced spleen cells. Why cloned CTL targets should be absolutely refractory to lysis by cloned CTL effectors, yet slightly sensitive to lysis by primary CTLs, is unclear. Cloned CTLs are extremely potent effectors toward normal target cells, and may be presumed at least potentially capable of killing cloned CTL target cells.

Luciani et al. (27) have recently reported that a given CTL clone is refractory to lysis by itself in LDCC, but is not refractory to lysis in LDCC by unrelated CTL clones. While we also find that cloned CTLs do not lyse themselves in LDCC (Table II), we find in every case we have examined, using either Con A or PHA, that cloned CTLs are completely resistant to LDCC by all CTL clones. At present, we cannot explain the differences in our results.

Several laboratories (6-8) have demonstrated that cytotoxic T cell lines maintained in long-term, IL-2-driven culture *in vitro* possess cytotoxic granules, the contents of which are highly toxic for a wide range of target cells. A lytic PFP has recently been partially enriched from these granules (18, 19, 28, 29) that

appears to share structural/functional/immunological homologies with the terminal components of the membrane attack complex of complement (30–33). We thus tested our CTL clones for sensitivity to cytotoxic granules. We found that cloned CTLs but not other non-CTL targets are highly resistant to lysis by granules, reflecting closely the extraordinary resistance of cloned CTLs to lysis by other CTLs. This would be consistent with, although it does not prove, a role for cytotoxic granules in target cell killing by cloned CTLs. The resistance of cloned CTLs to the granule/PFP-mediated cytolytic pathway is consistent with a protective mechanism that would spare CTLs from lysis by their own granule contents during the cytolytic reaction. The possibility that a membrane polypeptide of CTLs could play this protective role, by complexing rapidly with PFP in the plane of the bilayer, thereby avoiding its further aggregation and lytic transmembrane pore formation, is currently being assessed in our laboratories. A similar protective membrane polypeptide (termed C8-binding protein or homologous restriction factor) has already been described for the complement system that presumably avoids channel formation at the C8/C9 stage (34, 35). However, this polypeptide only seems to protect erythrocyte targets against homologous complement. Thus, the finding that cloned CTLs are susceptible to lysis by heterologous complement does not rule out that the resistance mechanisms/polypeptides that protect cells from CTL- and complement-mediated killing may be similar. Experiments are presently being carried out in our laboratories to address this issue.

Since primary CTLs are able to kill cloned CTLs to some extent, it is possible that primary CTLs and long-term, IL-2-driven CTL clones kill target cells through at least partially different mechanisms. Indeed, in preliminary experiments, we have not succeeded in staining granules of murine primary effector spleen cells with antiserum derived against isolated PFP (our unpublished observations). Two other groups have also recently failed to demonstrate a role for cytotoxic granules and their contents in killing by primary CTL (36, 37).

While this manuscript was in preparation, Kranz and Eisen (38) reported on similar findings of resistance of several CTL clones to lysis by a clone of CTL. Unlike our use here of various effectors and targets in different combinations of antigen specificity, these authors circumvented variations in target recognition by attaching to all the targets a monoclonal antibody to the antigen-specific receptor of a cloned CTL cell line (clone 2C) and using the 2C cell line as the only effector cell type. Here we have demonstrated that cloned CTLs are not only resistant to lysis by whole effector CTLs but also to lysis by granule proteins. It remains to be seen whether the resistance of cloned CTLs to killing is also a property of primary cytotoxic effector cells. Although the resistance to lysis exhibited by cloned CTLs may be related to their long-term culture in the presence of high levels of IL-2, this type of resistance may be physiologically relevant. Prolonged exposure to IL-2 causes a number of changes in CTL, including conversion to a broad specificity, promiscuous lytic activity (20–25). This would in effect recruit any CTL, or potential CTL, in the vicinity of a stimulus eliciting high IL-2 production (e.g., a massive viral infection, a tumor focus, etc.), to begin displaying immediate and vigorous lytic activity. It is even possible, perhaps likely, that the utilization of a lytic pathway involving cytotoxic

granules and their contents is itself qualitatively or quantitatively affected by long-term culture with IL-2. If CTL and NK cells did convert to a promiscuous mode of killing, perhaps involving a secreted mediator, it would make sense for these cells to acquire a defense against damage by such a mechanism. In general, it would make sense for any cytolytic cell type to acquire some form of defense against damage that might be inflicted by its own lytic mediators.

### Summary

Cloned CTLs show an unusually high resistance to lysis by effector CTLs. Several cloned CTL lines in our laboratories are absolutely refractory to lysis by other cloned CTLs, either (a) directly, (b) in the presence of lectin, or (c) by PMA-induced CTLs. They can be lysed to some extent by primary CTL, although they are <5% as sensitive as target cells normally used to assay primary CTL lytic activity. Lysis of cloned CTLs by primary CTL effector cells is not enhanced by the presence of lectin, and cloned T cells are also highly resistant to lysis by primary lymphokine-activated killer cells. Cloned CTLs are highly resistant to lysis by isolated CTL granules that contain the membranolytic pore-forming protein (PFP or perforin), while non-CTL targets are highly susceptible to granule-mediated killing, indicating that cloned CTLs resist lysis not only at the intact effector cell level but also when soluble effector proteins are used. This resistance mechanism may explain how CTLs kill but spare themselves from being killed during the cytolytic event.

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