Target Cell Death Triggered by Cytotoxic T Lymphocytes: a Target Cell Mutant Distinguishes Passive Pore Formation and Active Cell Suicide Mechanisms

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The role of the target cell in its own death mediated by cytotoxic T lymphocytes (CTL) has been controversial. The ability of the pore-forming granule components of CTL to induce target cell death directly has been taken to suggest an essentially passive role for the target. This view of CTL-mediated killing ascribes to the target the single role of providing an antigenic stimulus to the CTL; this signal results in the vectoral degranulation and secretion of pore-forming elements onto the target. On the other hand, by a number of criteria, target cell death triggered by CTL appears fundamentally different from death resulting from membrane damage and osmotic lysis. CTL-triggered target cell death involves primary internal lesions of the target cell that reflect a physiological cell death process. Orderly nuclear disintegration, including lamin phosphorylation and solubilization, chromatin condensation, and genome digestion, are among the earliest events, preceding the loss of plasma membrane integrity. We have tested directly the involvement of the target cell in its own death by examining whether we could isolate mutants of target cells that have retained the ability to be recognized by and provide an antigenic stimulus to CTL while having lost the capacity to respond by dying. Here, we describe one such mutant, BW87. We have used this CTL-resistant mutant to analyze the mechanisms of CTL-triggered target cell death under a variety of conditions. The identification of a mutable target cell element essential for the cell death response to CTL provides genetic evidence that target cell death reflects an active cell suicide process similar to other physiological cell deaths.

The mechanism by which cytotoxic T lymphocytes (CTL) trigger the death of their target cells has remained controversial. Although target cell death is not cell autonomous and does not require target cell macromolecular synthesis (48, 59; but see references 25 and 73), the metabolic activity of the targeted cell appears to be required (30), and in many morphological and biochemical respects, the cell death process is indistinguishable from cell autonomous physiological cell deaths (64). Indeed, the concept of internal disintegration, with prelytic nuclear events, including genome digestion, preceding the loss of plasma membrane integrity, comes from studies of CTL-induced target cell death (48). We recently described intracellular structural changes, including mitotic-like phosphorylation and solubilization of nuclear lamins, as among the earliest events detectable in the CTL-triggered death of target cells (67).

The nearly ubiquitous presence in effector cells of pore-forming cytolytic components sequestered in secretory granules (5, 6, 12, 27, 28, 33) has been taken to suggest that target cells die when they trigger the exocytosis of these lytic granules from CTL (18). The granule exocytosis model of CTL action (54) proposes that the target, in providing an antigenic stimulus to the CTL, triggers a degranulation response from the CTL, resulting in the vectoral deposition of pore-forming elements onto the target. Perforin, the pore-forming protein of effector cell granules (also termed cytolsin), can kill nucleated target cells in a calcium-dependent manner, but the cell lysis that results is not a physiological cell death (9, 10, 14, 67).

Together with perforin, cytolytic granules contain a variety of molecules that may be involved in cell death, including a family of serine proteases (for example, granzyme A and granzyme B/fragmentin 2 [31, 36, 42, 52, 53]) and an RNA-binding protein (TIA-1 [61]). These granule components, under certain circumstances and in purified form, have been shown to induce genome digestion or even physiological cell death (15, 52, 53, 61), giving rise to the proposal that target cell death actually results from the action of these non-pore-forming components following their transfer from the effector cell to the target cell (via a polyperforin pore).

The validity of these models remains unresolved. Genetic manipulations to date have yielded only equivocal results. Antisense ablation of expression of perforin (1) exerted partial loss of cytolytic effector function under conditions of redirected lysis (see below); the effect on antigen-specific CTL activity was not tested. Similarly, ablation of granzyme A expression did not correlate consistently with loss of cytolytic activity among the antisense transfectant clones and populations examined (60). The expression of perforin following gene transfer into exocytic but noncytotoxic cells conferred potent lytic activity against erythrocytes but only weak cytolytic activity against nucleated target cells (54). The weak cytolytic activity against nucleated targets generated by the expression of both perforin and granzyme A was associated additionally with genome digestion (55); whether this DNA breakdown occurred prelytically and represents an authentic physiological cell death has not been determined.

A more fundamental reservation regarding the role of cytolytic granule components in CTL activity concerns the lack of correlation of expression of these presumptive lytic elements with the ability of effector cells to trigger target cell death. Among CTL populations with roughly comparable

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effector cell activities, levels of expression of perforin and granzymes vary widely. While some long-term in vitro CTL clones display substantial levels of perforin and granzyme activity, primary CTL express low levels of perforin and granzyme mRNA, and some CTL generated in vivo have undetectable levels of expression (5, 6, 17, 19). On the other hand, abundant expression of granule components has been observed consistently in natural killer and lymphokine-activated killer cells (19, 45, 52, 53, 56, 73). It may be that cytolytic granule molecules are more relevant to the action of these effector populations than to the action of antigen-specific CTL. The lack of correlation between cytolytic granule activity and CTL-triggered target cell death is evidenced strikingly with calcium-independent target cells. CTL can trigger the death of these targets equally in the presence of calcium, associated with typical CTL degranulation, and in the absence of calcium, when CTL degranulation and granzyme release are undetectable (39, 62). Whether this reflects the absolute absence of degranulation or only the limits of the sensitivity of the assay is not easily resolved.

We have explored the mechanism of CTL-triggered target cell death by characterizing genetically the involvement of the target cell in its own death. Our approach is motivated by the hypothesis that mutable elements of the target cell which reveal response elements necessary for the cell death process induced by CTL can be identified. Recently, we reported one target cell mutant that fails to digest its genome during cell death, allowing us to identify the enzyme responsible for genome digestion as DNase I and to conclude that genome digestion is a dispensable consequence of CTL-triggered target cell death (67). Here we describe another somatic cell mutant that is recognized by CTL and triggers CTL degranulation but that fails to die in response. We have used this mutant to probe the mechanisms by which target cells are triggered to die by CTL under a variety of circumstances. The behavior of the CTL-resistant mutant demonstrates that normal, antigen-specific CTL-triggered target cell death is a physiological cell death process, involving the active participation of the target cell in its own death.

MATERIALS AND METHODS

Cell culture. All cells were grown in RPMI 1640 medium (Whittaker Bioproducts, Walkersville, Md.) supplemented with glutamine (2 mM), 2-mercaptoethanol (50 μ M), and heat-inactivated fetal bovine serum (10% [vol/vol]; Tissue Culture Biologicals, Tulare, Calif.). The cell lines used included the AKR-derived (*H-2^k*) thymoma BW5147 (see below), the C57BL/6-derived (*H-2^b*) thymoma EL4, and the DBA/2-derived (*H-2^d*) mastocytoma P815.

CTL effector populations were harvested on day 5 from primary mixed lymphocyte cultures (MLC): 7×10^6 spleen cell responders and 3×10^6 irradiated (3,000 R) allogeneic spleen cell stimulators per 2 ml of culture. CTL clones Nancy (specific for *H-2D^k* [66]), Jennifer (specific for *H-2^a* [67]), and CTL 3 (a heteroclitic clone specific for *H-2K^k* [49]) were stimulated on a 7-day cycle with appropriate targets and interleukin-2 (IL-2) (2.5×10^5 CTL and 5×10^6 irradiated spleen cells from AKR, DBA/1, and BALB/c mice, respectively, per 2 ml of culture containing 10 U of IL-2 per ml). CTL clone 36 (CTL 36), specific for the peptide corresponding to residues 365 to 380 of the A/PR/8/34 influenza virus nucleoprotein (NP) sequence presented by *H-2D^b*, was similarly maintained with peptide-pulsed C57BL/6 spleen cells as described previously (21).

Mutant isolation. BW1083, a freshly subcloned glucocorticoid-resistant derivative of the AKR-derived (*H-2^k*), thymidine kinase-negative thymoma BW5147, was treated with *H-2^k*-specific CTL (from a C57BL/6 anti-AKR MLC). From two rounds of treatment (coculture of 10^7 nonmutagenized target cells with 5×10^7 CTL for 3 days, followed by recovery and outgrowth of surviving target cells for 1 week), a CTL-resistant population was obtained. The CTL-resistant BW87 clone was derived by plating this population at limiting dilution. In all of the experiments reported here, we used a subclone of the BW87 mutant, BW87.G7. The doubling time of the BW87 mutant (approximately 16 h) is indistinguishable from that of its parent.

Somatic cell fusion. IL-2-independent, hypoxanthine-aminopterin-thymidine-resistant hybrids from the fusion of the C57BL/6 (*H-2^b*)-derived Mls^a-reactive, IL-2-dependent helper T-cell clone L2 (13) (kindly provided by Andy Glasebrook) with BW87 or BW1083 were prepared as described previously (35). Hybrids were tested for Mls^a reactivity by the production IL-2 in response to irradiated CBA/J (*Mls^a*) spleen cells (10^6 per microwell) (65). Positive cells were subcloned at limiting dilution.

Transfection. Cells were transfected by a modification of the spheroplast fusion technique of Sandri-Goldin et al. (51). Bacterial spheroplasts were prepared essentially as described elsewhere (51). T cells (10^7) were washed once in serum-free medium, collected by centrifugation (5 min at $500 \times g$, 25°C), and resuspended by gentle pipetting in the spheroplast suspension (approximately 2×10^9 spheroplasts in 5 ml). The mixture was transferred to a 60-mm-diameter dish and centrifuged at $1,500 \times g$ for 7 min. After gentle aspiration of the supernatant, 1.5 ml of polyethylene glycol solution (70% [wt/vol] PEG-4000 [Roth] and 15% [vol/vol] dimethyl sulfoxide in phosphate-buffered saline [PBS]) was added to the film of cells and spheroplasts left on the surface of the plate. The dish was spun at $750 \times g$ for 60 s. Cells were collected by gently pipetting prewarmed serum-free medium across the plate. Cells were resuspended in growth medium containing kanamycin (100 μ g/ml) and plated in 96-well dishes at 2×10^4 cells per well. After 24 h, G418-containing medium (1.2 mg/ml) was added.

Cytotoxicity assays. Cells were labeled with 5'-[¹²⁵I]iodo-2'-deoxyuridine ([¹²⁵I]IUdR; 5 Ci/mg; Amersham, Arlington Heights, Ill.) and/or sodium [⁵¹Cr]chromate (400 to 1,200 mCi/mg; New England Nuclear, Boston, Mass.) as described previously (50). Cells (5×10^6) were incubated in 0.5 ml with 20 μ Ci of [¹²⁵I]IUdR and/or 100 μ Ci of [⁵¹Cr]chromate for 90 min at 37°C, washed, and incubated for another 60 min at 37°C without label. For redirected lysis, cells were derivatized with 3 mM trinitrobenzene sulfonate in PBS for 15 min at 37°C after labeling as described previously (41). Cells were tested for susceptibility to CTL-triggered cell death essentially as described previously (67). Reactions (200- μ l volume) were performed in V-bottom 96-well plates, included 10^4 labeled targets and effector CTL at the indicated effector-to-target cell (E:T) ratios, and were incubated at 37°C for 4 h or as indicated. Plates were centrifuged (5 min at $500 \times g$, 25°C), and 100 μ l of each supernatant was collected for determination of ⁵¹Cr and/or Triton-independent [¹²⁵I]IUdR release. Then 100 μ l of 0.4% Triton X-100 in PBS was added to the wells, and another 100 μ l of each supernatant was collected after recentrifugation for determination of Triton-dependent [¹²⁵I]IUdR release. Specific ⁵¹Cr release was calculated according to the following formula: [(experimental release - spontaneous release) $\times$ 100]/(maximal release - spontaneous release). Spontaneous release

was generally less than 10%. Maximal release was determined by lysing targets with 0.25% sodium dodecyl sulfate. Specific [125 I]IUdR release was calculated similarly; total [125 I]IUdR release included both Triton-dependent and Triton-independent release (49).

Lysis by cytolytic granules and ATP-mediated cell death were assessed similarly. Serial dilutions of ATP were incubated with ^{51}Cr -labeled target cells in medium additionally buffered as described previously (4). Cytolytic granules purified from a rat large granular lymphocyte tumor (32) (graciously provided by Pierre Henkart) were diluted serially in calcium-free PBS and incubated with the labeled target cells in complete medium for 1 h.

BLT serine esterase release. *N* $^{\alpha}$ -benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) serine esterase activity in supernatants collected after 3 h of incubation was determined by the ability to cleave the chromogenic BLT substrate as described previously (67). Briefly, 20 μl of each culture supernatant was added to 180 μl of reaction buffer [2×10^{-4} M BLT ester and 1.1×10^{-4} M 5,5'-dithio-bis-(2-nitrobenzoic acid) in PBS, pH 7.4], and the reaction mixtures were incubated at 25°C for 60 min. Optical densities were measured at 405 nm in an automated enzyme immunoassay plate reader (EL309; Bio-Tek Instruments, Burlington, Vt.).

Cytofluorimetric analyses. Cells were stained for expression of total H-2^k class I molecules with M1.42 antibody (rat antibody specific for mouse major histocompatibility complex class I [58]) and for specific expression of H-2D^b with 28-14-8S antibody (mouse antibody specific for H-2D^b [40]). Cells (10^6) were incubated with 100 μl of culture supernatant of the primary antibody at 4°C for 45 min. The washed cells were resuspended in 100 μl of buffer (Hanks' balanced salt solution with 0.2% bovine serum albumin) containing 10 μl of fluorescein isothiocyanate-conjugated secondary antibody (goat anti-mouse or mouse anti-rat immunoglobulin; Fisher Biotech, Pittsburgh, Pa.) and incubated at 4°C for another 45 min. Cells were analyzed cytofluorimetrically on a FACScan analyzer (Becton Dickinson, San Jose, Calif.).

Conjugates of CTL and target cells were analyzed by a method previously described (26). CTL effectors were labeled with fluorescein diacetate, mixed with target cells at an E:T ratio of 2 (so as to favor the formation of targets conjugated with a single effector; 4×10^5 labeled effectors and 8×10^5 unlabeled targets in a 200- μl reaction), and briefly centrifuged (3 min at $500 \times g$, 25°C). Pelleted cells were gently resuspended and diluted into 500 μl of buffer for cytofluorimetric analysis after approximately 10 min. Target cells exhibited greater light scatter properties than did effectors. Conjugates exhibiting both CTL-like fluorescein labeling and target cell-like high light scatter properties were identified.

Reagents and chemicals. ATP and kanamycin were purchased from Sigma Chemical Co. (St. Louis, Mo.). G418 (G418 sulfate; Geneticin) was purchased from GIBCO (Grand Island, N.Y.). Concanavalin A (Sigma) was resuspended and dialyzed in tissue culture medium without serum at 1 mg/ml. The synthetic glucocorticoid dexamethasone (Sigma) was prepared as a stock solution (10^{-3} M) in 95% ethanol. Ionomycin and valinomycin (Calbiochem, La Jolla, Calif.) were resuspended in dimethyl sulfoxide at 1.0 mM.

RESULTS

Recovery of a CTL-resistant mutant. That a defect in DNase I expression in a target cell results in the failure of that cell to digest its genome without altering its ability to die

in response to CTL indicates that genome digestion is a dispensable target cell function associated with CTL-triggered cell death (67). To identify target cell functions that are essential to the CTL-induced cell death process, we have selected for target cell mutants that are resistant to CTL-triggered death while retaining the ability to be recognized by CTL. BW87 is one such antigenic and resistant clone.

BW87 was isolated from BW1083, a glucocorticoid-resistant subclone of the AKR-derived (*H*-2^k) thymoma BW5147 (see Materials and Methods). BW87 was selected for resistance to alloantigen-specific CTL. As demonstrated in a simple ^{51}Cr release assay (Fig. 1), BW1083, like its parent BW5147, expresses H-2^k determinants (Fig. 2) and is sensitive to cell death triggered by H-2^k-specific CTL effectors. Independently, activation of CTL specific for an irrelevant *H*-2 haplotype and agglutination with target cells by the lectin concanavalin A will induce the death of the CTL-sensitive cells (Fig. 1). In contrast, CTL can induce neither antigen-specific nor lectin-dependent death of the CTL-resistant BW87 mutant (Fig. 1).

How much less sensitive to CTL-triggered cell death is BW87 than its parent? We have quantified relative CTL susceptibility in terms of the number of effector cells per target (Effector dose) required to trigger 50% specific ^{51}Cr release (ED_{50}). The data in Fig. 1, for primary MLC-derived CTL effectors, yield an observed ED_{50} for BW1083 of 11 in the absence and 2 in the presence of concanavalin A. In contrast, the calculated ED_{50} for BW87 with the same effectors is greater than 1,000 with or without lectin. Data compiled from 20 experiments with MLC-derived effectors and long-term CTL clones indicate that BW87 cells are at least 400 times less sensitive to CTL-triggered death than are BW1083 cells (Table 1).

These ^{51}Cr release experiments are consistent with other measures of CTL-triggered death. The plating efficiency of BW1083 cells is reduced to less than 1% of the control value following treatment with antigen-specific or lectin-dependent CTL (at an E:T ratio of 20); in contrast, BW87 cells retain 80% of colony-forming ability following CTL treatment (data not shown). The release of free fatty acids from target cells is another indication of CTL-triggered death (46). While BW1083 cells release substantial levels of free fatty acids when subjected to antigen-specific or lectin-dependent CTL, no free fatty acid release is induced from BW87 cells (22).

BW87 resistance is not due to a failure in recognition by CTL. The failure of BW87 to die in response to CTL is not due to an inability to be recognized by CTL. BW87 cells continue to express H-2^k determinants, although at levels reduced to about 10% of that of the parental cells (based on mean immunofluorescence staining intensities; Fig. 2). This reduced H-2^k expression is sufficient, nonetheless, to allow BW87 cells to be recognized and form antigen-specific conjugates effectively with H-2^k-specific CTL (Fig. 3).

Still, to obviate the possibility that the failure of BW87 to die in response to CTL is due to this quantitative or other, subtle alteration in the expression of endogenous H-2^k molecules, we introduced a distinct *H*-2 gene into BW87 cells and tested whether expression of the new recognition molecule conferred sensitivity to CTL-triggered death. BW87 cells were transfected with an *H*-2D^b construct driven by a Moloney murine leukemia virus long terminal repeat promoter and bearing a G418 resistance gene (3). Transfected clones, selected for G418 resistance, were screened for functional expression of the transfected H-2 determinant by virtue of the ability to present an H-2D^b-restricted viral peptide antigen to a CTL clone. Presentation was assessed

FIG. 1. The BW87 mutant is resistant to antigen-specific and lectin-dependent CTL-triggered cell death. ^{51}Cr -labeled target cells were tested for susceptibility to death mediated by H-2^k-specific CTL (from a C57BL/6 anti-AKR MLC; ●) and by H-2^b-specific CTL (from an AKR anti-C57BL/6 MLC) in the absence (□) and presence (Δ) of 5 μg of concanavalin A per ml. Specific ^{51}Cr release was determined after 4 h at the indicated E:T ratios. EL4 cells (H-2^b) and wild-type BW5147 cells (H-2^k) serve as specificity controls. The BW87/D^b clones are H-2D^b-expressing transfectants of the BW87 mutant (see Figs. 2 and 4).

as the ability to trigger CTL degranulation with release of serine protease activity (granzyme A [31]). Figure 4 presents data for three positive transfectant clones. The BW87/D^b transfectant clone B6, for example, expresses H-2D^b at 50% of the level of an authentic H-2^b-expressing CTL-sensitive

target, EL4 (Fig. 2). This clone forms conjugates with and competes for recognition by H-2^b-specific CTL as effectively as EL4 does (Figs. 3 and 5). Although H-2D^b expression was able to confer effective antigen-specific CTL recognition on the BW87 mutant, it did not confer susceptibility to CTL-triggered cell death (Fig. 1 and 4). Thus, the CTL resistance of BW87 cannot be accounted for by a failure of recognition.

The phenotype of CTL resistance is recessive. In a complementary set of experiments, we tested whether the BW87 H-2^k molecules could confer recognition and susceptibility to CTL-triggered death on somatic cell hybrids derived from BW87. We generated hybrid clones between the CTL-sensitive, nontransformed (and IL-2-dependent) helper T-cell clone L2 (expressing H-2^b determinants [13]) and BW87 or its parent BW1083.

In all cases, the hybrids were found to be susceptible to cell death triggered by CTL specific for either H-2^b or H-2^k (Fig. 6). The BW87 hybrids express a lower density of surface H-2^k molecules than do BW1083 hybrids (comparable to the BW87 parent; data not shown), accounting for the greater extent of death induced with H-2^b-specific effectors. Apparently, the phenotype of reduced H-2^k expression on BW87 cells relative to parental BW1083 expression persists in their hybrids. Still, this reduced level of H-2^k expression is sufficient to allow CTL recognition and CTL-triggered cell death.

Significantly, the phenotype of resistance to CTL-triggered death is extinguished in a hybrid between the BW87 mutant and a wild-type cell. The BW87 mutation conferring CTL resistance therefore behaves as a recessive lesion. This implies that the resistance to CTL-triggered death of the BW87 mutant represents a loss of target cell function essential for cell death. Interestingly, the BW1083 phenotype of glucocorticoid resistance also is extinguished in the hybrids (16). As with this and other cases of cell autonomous physiological cell death (35), target cells are involved actively in their own death triggered by CTL.

TABLE 1. Relative target cell susceptibility to cytolytic activity

Effector function	CTL	ED ₅₀ ratio (relative to BW1083) ^a	
		BW87	CTL ^b
Antigen-specific CTL ^c	MLC	4.2×10^2	ND
	Cloned	1.3×10^3	1.8×10^3
Lectin-dependent CTL ^d	MLC	1.5×10^3	ND
	Cloned	6.2×10^2	1.8×10^3
Redirected lysis ^e	MLC	7.7×10^0	ND
	Cloned	5.9×10^0	ND
Bystander killing ^f	Cloned	1.0×10^0	ND
Granules ^g		1.3×10^0	1.5×10^5

^a The ED₅₀ was determined by linear regression as described in the text. The ratios of ED₅₀ values for the BW87 mutant and the BW1083 parent, or for a CTL clone and BW1083, were calculated from data from a single experiment as a measure of relative susceptibility to effector function. Each value represents the mean of ED₅₀ ratios from multiple (*n*) experiments. For experiments in which resistant cells failed to exhibit significant specific ^{51}Cr release, calculated ED₅₀ values greater than 10^3 were arbitrarily set equal to 10^3 . ND, not determined.

^b The CTL clones used as target cells for cytolytic granules and for lectin-dependent CTL activity were Jennifer, Nancy, and CTL 3. Jennifer (expressing H-2^b) was used exclusively as a target for H-2^k-specific CTL activity.

^c H-2^k-specific (cognate) CTL-triggered death was assayed as in Fig. 1 with MLC-derived effectors (*n* = 10) or the H-2^k-specific clones Nancy and CTL 3 (*n* = 10).

^d Determined in the presence of 5 μg of concanavalin A per ml, as in Fig. 1, with H-2^k-specific MLC-derived CTL (*n* = 10) or with the CTL clone Jennifer (*n* = 5).

^e Assessed as in Fig. 7 (*n* = 4).

^f Bystander killing of the H-2D^b-negative targets by CTL 36 was monitored following stimulation with EL4 cells pulsed with NP peptide as in Fig. 4 (*n* = 4).

^g Lysis by cytolytic granules was quantified as in Fig. 8 (*n* = 3).

FIG. 2. Cell surface expression of H-2^k class I molecules on target cells. Cells were stained for expression of H-2^k class I molecules with M1.42 antibody (a rat antibody specific for mouse major histocompatibility complex class I that does not recognize H-2D^b [58]; A to C) and for specific expression of H-2D^b with 28-14-8S antibody (mouse antibody specific for H-2D^b [40]; D to F). Specific staining is displayed with solid lines; dotted lines represent background staining with secondary antibody alone. (A) BW5147; (B) BW1083; and (C and D) BW87; (E) BW87/D^b transfectant clone B6; (F) EL4.

BW87 retains sensitivity to extreme forms of CTL killing. In addition to antigen-specific and lectin-dependent triggering of target cell death, CTL can kill target cells directly when they are activated and linked to the targets by antibody

FIG. 3. CTL form antigen-specific conjugates with BW87 cells. Target cells were tested for the ability to form antigen-specific conjugates with MLC-derived CTL. Fluorescein-labeled CTL were mixed with the indicated targets (the BW87/D^b transfectant was clone B6) at an E:T ratio of 2 and analyzed cytofluorimetrically following brief centrifugation and incubation. Conjugates exhibiting both CTL-like fluorescein labeling and target cell-like high light scatter properties were identified. Results are expressed as the fraction of CTL that were in conjugates. Conjugates with H-2^k-specific CTL are displayed as open bars; conjugates with H-2D^b-specific CTL are shown as hatched bars. Conjugates between fluorescein-labeled and unlabeled cells also were visualized microscopically; microscopic enumeration and cytofluorimetric analysis gave similar results.

FIG. 4. Functional expression of transfected H-2D^b on CTL-resistant target cell clones does not confer susceptibility to CTL-triggered death. Functional expression of H-2D^b on target cells was assessed as the ability to present the H-2D^b-restricted viral peptide corresponding to residues 365 to 380 of the A/PR/8/34 influenza virus NP sequence to CTL 36, a clone specific for that peptide presented by H-2D^b (21). Presentation was quantified as the specific release of serine protease activity (granzyme A [31]) from CTL 36 incubated for 3 h with target cells (E:T = 0.5) that had been pulsed with NP peptide (hatched bars) or not pulsed (open bars). In parallel, target cell death triggered by CTL 36 (E:T = 1.7) was assessed by specific ⁵¹Cr release as in Fig. 1. BW87/D^b clones B6, C6, and H9 are three independent clones of the BW87 mutant, selected for G418 resistance following transfection with an H-2D^b construct driven by a Moloney murine leukemia virus long terminal repeat promoter and bearing a G418 resistance gene (3).

FIG. 5. CTL recognize BW87 cells without triggering their death. The ability of unlabeled target cells to compete with ^{51}Cr -labeled EL4 targets for recognition by H-2^b -specific CTL was determined in a specific ^{51}Cr release assay as in Fig. 1. Each well contained 2×10^4 ^{51}Cr -labeled targets and 6×10^4 CTL (i.e., the effector-to-labeled target cell ratio equaled 3:1). Unlabeled target cells (the CTL-sensitive, H-2^k parent BW1083 [○], CTL-resistant BW87 [△], BW87/ D^b transfectant clone B6 [▽], and H-2^b -expressing EL4 [□]) were included with labeled targets at the indicated ratios. Specific ^{51}Cr release from targets in the absence of unlabeled competitors is also presented (filled symbols).

bridging (redirected lysis [24, 41, 57]) or indirectly when strongly activated (bystander killing [70]). In contrast to resistance to antigen-specific and lectin-dependent CTL-triggered death, BW87 cells exhibit virtually wild-type sensitivity to redirected and bystander killing (Fig. 7; Table 1).

BW87 cells also are fully sensitive to lysis effected by cytolytic granules (Fig. 8) and by purified perforin (data not shown). CTL clones and populations are resistant to CTL attack and to lysis by cytolytic granule components (29, 34,

FIG. 6. The BW87 phenotype of resistance to CTL-triggered cell death is recessive. ^{51}Cr -labeled target cells were tested for susceptibility to death mediated by H-2^k -specific CTL (from a C57BL/6 anti-AKR MLC; ●) and by H-2^b -specific CTL (from an AKR anti-C57BL/6 MLC; □) as in Fig. 1. EL4 cells (H-2^b) and wild-type BW5147 cells (H-2^k) serve as specificity controls. Hybrids were prepared by fusing the H-2^b T-cell clone L2 to the CTL-resistant BW87 mutant or to its CTL-sensitive parent BW1083. Data for two representative hybrids from each group are presented.

FIG. 7. The BW87 mutant is sensitive to redirected lysis by primary and cloned CTL. ^{51}Cr -labeled and trinitrophenol-modified target cells were tested for susceptibility to death mediated by primary CTL (from an AKR anti-BALB/c MLC; ○) or the H-2^b -specific clone Jennifer (□) in the presence of heteroconjugated antibody (50 ng/ml) with specificities for CD3 and dinitrophenol (41). Specific ^{51}Cr release was determined as in Fig. 1 after 90 min.

69) (Table 1). The sensitivity of BW87 to lysis by pore formation (a nonphysiological cell death [64; also see references 9, 10, 14, and 67]) distinguishes the pattern of resistance of the mutant from the resistance to CTL of CTL themselves.

Extracellular ATP is another cytotoxic agent that has been proposed to mediate CTL-triggered target cell death (11, 72). However, the CTL-resistant BW87 mutant exhibits sensitivity to cell death induced by ATP indistinguishable from that of BW1083, its CTL-sensitive parent (Fig. 8). Similarly, the sensitivities of the BW87 mutant and its parent to the calcium-specific ionophore ionomycin (Fig. 8) and to the potassium-specific ionophore valinomycin (data not shown) are not different.

CTL can induce target cell death occurring by two mechanisms. We wondered whether conditions of CTL-mediated lysis to which BW87 retained sensitivity occurred by the same process that is associated with antigen-specific and lectin-dependent CTL activity. Typical CTL-triggered target cell death is a physiological cell death that occurs by a process of internal disintegration (48, 67). Dissolution of nuclear elements, including nuclear envelope lamin solubilization and digestion of chromosomal DNA, precedes plasma membrane breakdown: in a physiological cell death, the release of cytoplasmic markers (^{51}Cr labeled) through a disintegrating plasma membrane follows kinetically the solubilization of DNA fragments (labeled with [^{125}I]IUdR). Prelytic genome digestion is an unambiguous and convenient marker (albeit a dispensable consequence) of physiological cell death (64, 67).

We examined the kinetics of [^{125}I]IUdR-labeled DNA solubilization and ^{51}Cr release in CTL-sensitive target cells dying in response to CTL under a variety of triggering conditions. Data from one representative target cell are presented in Fig. 9. Only antigen-specific and lectin-dependent (not shown) CTL-triggered cell death resulted in unambiguous prelytic genome digestion (including the generation of a typical ladder of oligonucleosomal DNA fragments; data not shown). Redirected lysis clearly was associated with

FIG. 8. The BW87 mutant retains susceptibility to death induced by cytolytic granules, extracellular ATP, and calcium and potassium ionophores. ⁵¹Cr-labeled BW1083 (○) and BW87 (Δ) cells were tested for susceptibility to lysis by cytolytic granules (A) in a 60-min ⁵¹Cr release assay and to death mediated by extracellular ATP (B) and calcium ionophore (C) in a 4-h assay.

rapid plasma membrane rupture and little genome digestion; bystander killing appeared to involve both early membrane damage and genome digestion (Fig. 9). BW87 cells behaved similarly in cases of bystander killing and redirected lysis.

These results indicate that under certain circumstances, CTL are able to induce nonphysiological (pathological) target cell death. These data also underscore the important kinetic aspect of genome digestion as a marker of cell death: only prelytic DNA solubilization is diagnostic of the physiological cell death process. Strikingly, the biochemical determination of the type of cell death induced by CTL is completely concordant with the relative susceptibility of the

BW87 mutant (Table 1): BW87 cells are sensitive to pathological cell death and are resistant to physiological cell death triggered by CTL. The BW87 mutant distinguishes different mechanisms of CTL action.

DISCUSSION

Cytolytic granules and CTL action. BW87 is resistant to antigen-specific and lectin-dependent CTL action but is as effective at inducing CTL degranulation as a CTL-sensitive target and is fully sensitive to lysis by cytolytic granules and by purified perforin. These results suggest that granule-generated membrane lesions are not the immediate cause of CTL-triggered cell death and that perforin itself cannot be the lethal mediator of normal CTL action. On the other hand, they do not exclude the possibility that, for example, a transient pore created by perforin could be healed after permitting the entry of an inducer of the target cell suicide process from the CTL.

In this context, it is significant that nucleated cells are able to reverse potentially cytolytic membrane damage inflicted by small amounts of perforin (20). Unlike erythrocytes, which can be lysed by a single complement channel, the osmotic lysis of nucleated cells requires the formation of several channels (23). Under certain circumstances (for example, redirected lysis; Fig. 9), CTL are able to induce nonphysiological target cell death. Observations that redirected lysis is much more efficient against erythrocytes than against nucleated cells (41, 44, 54, 55) and appears to be dependent on perforin expression (54, 55) support the view that this type of killing results directly from pore formation mediated by CTL-derived granules. We propose that extreme forms of CTL action (redirected lysis and bystander killing) employ this lethal pore formation mechanism.

CTL can effect two types of target cell death. CTL can trigger target cell death by two mechanisms: a physiological cell death process that involves typical internal disintegration, genome digestion, and nuclear disintegration, and under more extreme circumstances, an essentially passive lytic process associated with osmotic lysis due to membrane damage, resulting in a pathological cell death. The BW87 mutant reveals and distinguishes these two mechanisms. The

FIG. 9. The kinetics of release of cytoplasmic and nuclear markers indicate different mechanisms of CTL-mediated target cell death. Specific release of ⁵¹Cr (○) and [¹²⁵I]IUdR (□, ■) from double-labeled P815 (H-2^d) target cells was determined kinetically following treatment with CTL under conditions of antigen-specific action with H-2^d-specific primary CTL (from an AKR anti-BALB/c MLC; E:T = 1.7) (A), bystander killing, in which the labeled P815 cells (E:T = 0.6) and unlabeled NP-pulsed EL4 targets were incubated with CTL 36 (see Fig. 4; E:T = 0.6) (B), and redirected lysis of trinitrophenol-modified P815 targets with CTL from an AKR anti-DBA/1 MLC (see Fig. 7; E:T = 5) (C). Both total [¹²⁵I]IUdR release (■) and Triton-independent release (□) are presented.

ability of CTL to induce different types of target cell death has been suggested previously (38, 73) and is confirmed by differences in the patterns of release of nuclear and cytoplasmic markers during target cell death. Normal CTL activity, triggered in an antigen-specific or lectin-dependent manner, employs the physiological process.

Target cells are involved actively in their death. That the BW87 phenotype of resistance to physiological cell death triggered by CTL is recessive indicates that the failure of the target cell to die results from the loss of a function or activity necessary for the cell death response. This is not a trivial deficit, for example, the inability of the target to be recognized or the failure of the mutant to trigger a degranulation response from the CTL. Instead, the defect of the mutant reveals an essential functional contribution that the target cell makes normally to its own death. In this sense, even death triggered by a CTL effector is an induced cell suicide process.

CTL-triggered physiological target cell death is unusual among physiological cell deaths in a number of respects. Most obviously, it is not a cell autonomous process. The rapidity of the process (within minutes; Fig. 9) reflects the absence of a requirement for macromolecular synthesis (48, 59). We hypothesize that the effector cell is able to provide a molecule or signal introduced into the target (for example, via a polyperforin lesion) which obviates earlier steps of the physiological cell death process that include *de novo* gene expression (as well as rigorous cell cycle control and sensitivity to BCL-2 [64, 68]). Nonetheless, target cell metabolic activity appears to be necessary for cell death (30), consistent with the notion that the target cell plays an active role in its own death.

The results presented here for the BW87 mutant independently demonstrate that the target cell actively contributes an essential function to the cell death process. The loss of that function prevents target cells from responding to CTL by dying. Because the CTL-triggered process can occur in the absence of macromolecular synthesis, the function absent in BW87 is not one that must be induced in susceptible cells for cell death to occur; further, the absence of this function in the BW87 mutant is not deleterious for cell survival. We conclude that an activity constitutively present in the target cell is essential for CTL-triggered cell death.

BW87 resistance and physiological cell death. Simplistically, the resistance to CTL action of the BW87 mutant could result either from an inability of the mutant to receive an inductive signal from the CTL or from the mutant's failure to respond to that signal. BW87 is not pleiotropically resistant to the induction of physiological cell death. For example, the CTL-resistant mutant retains susceptibility to ATP-triggered cell death (Fig. 8), to death resulting from elevated intracellular calcium levels (Fig. 8), and to death induced by low-dose irradiation and by elevated intracellular cyclic AMP levels (16). Thus, the physiological cell death response in BW87 cells appears generally to be intact. This implies either that these cells are defective specifically in one of the afferent signaling steps between the lethal hit delivered by the CTL and distal elements of the cell death process or that the CTL-triggered cell death process is genetically distinct from that of other physiological cell deaths.

We have argued that all physiological cell deaths likely share common components (64; also see reference 8). However, this hypothesis, particularly relating to CTL-triggered cell death, remains unproven. Previously, we reported that another target cell mutant, 4R.D, appeared to be resistant to cell death induced both by glucocorticoids and by CTL as a

result of a single genetic lesion, suggesting that a common mutable element governs the ability of a cell to die (63). Subsequent work (7) has shown that the 4R.D mutant cannot be considered generally resistant to CTL, however. Therefore, no genetic linkage among CTL-triggered death and other cell suicide processes yet has been established.

Mediators of CTL-triggered physiological cell death. A number of potential mediators of CTL-triggered target cell death have been proposed, including extracellular ATP, granule-derived molecules, and elevated intracellular ion fluxes (2, 11, 15, 43, 52, 53, 61, 72). Our observation that the CTL resistance of BW87 does not confer resistance to ATP-mediated cell death complements another recent somatic cell genetic study of ATP sensitivity. Avery et al. (4) found that target cell mutants resistant to ATP-induced death were not resistant to CTL-triggered death. Together, these results demonstrate that the induction of cell death by CTL and that by ATP occur by genetically distinct mechanisms.

While the role of calcium in cell death in general is controversial, it is clear that elevated free calcium within the target cell (2, 43) is not sufficient to account for CTL-triggered target cell death (64). For example, the induction of physiological cell death in T cells by calcium ionophores is dependent on macromolecular synthesis (71). The undiminished sensitivity of the BW87 mutant to elevated intracellular calcium levels caused by calcium ionophore further demonstrates that a calcium flux likely is not involved in mediating CTL-triggered cell death. The potassium ionophore valinomycin, in contrast, has been shown to mimic CTL-triggered cell death kinetically and with respect to independence from macromolecular synthesis requirements (2, 37). The observation that CTL-resistant BW87 cells exhibit unaltered sensitivity to cell death induced by valinomycin argues, however, that cell deaths triggered by CTL and by presumptive intracellular potassium changes also are distinct.

It also is worth noting that cells derived from the BW5147 thymoma are not susceptible to CTL action in the absence of calcium. Consequently, the Fas antigen, which has been proposed to serve in triggering target cell death under calcium-independent conditions of CTL action (47), likely is not involved here.

The involvement of non-pore-forming granule components such as granzyme A, granzyme B/fragmentin 2, and TIA-1 (15, 52, 53, 61) have not been tested with the BW87 mutant. It may be revealing to examine whether the BW87 mutant is susceptible to these molecules when the molecules are introduced with low doses of perforin. It also will be interesting to explore the sensitivity of this mutant to other forms of cell-mediated target cell death, including helper T-cell cytotoxicity (59). CTL-resistant target cell mutants such as BW87 will permit the role of these and other potential mediators to be tested rigorously.

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REFERENCES

- Acha-Orbea, H., L. Scarpellino, S. Hertig, M. Dupuis, and J. Tschopp. 1990. Inhibition of lymphocyte mediated cytotoxicity by perforin antisense oligonucleotides. *EMBO J.* 9:3815-3819.
- Allbritton, N. L., C. R. Verret, R. C. Wolley, and H. N. Eisen. 1988. Calcium ion concentration and DNA fragmentation in target cell destruction by murine cloned cytotoxic T lymphocytes. *J. Exp. Med.* 167:514-527.
- Allen, H., J. Fraser, D. Flyer, S. Calvin, and R. Flavell. 1986. $\beta 2$ -Microglobulin is not required for cell surface expression of the murine class I histocompatibility antigen H-2D^b or of a truncated H-2D^b. *Proc. Natl. Acad. Sci. USA* 83:7447-7451.
- Avery, R. K., K. J. Bleier, and M. S. Pasternack. 1992. Differences between ATP-mediated cytotoxicity and cell-mediated cytotoxicity. *J. Immunol.* 149:1265-1270.
- 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.* 141:1429-1436.
- Dennert, G., C. G. Anderson, and G. Prochazka. 1987. High activity of N⁶-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* 84:5004-5008.
- Dennert, G., C. Landon, and M. Nowicki. 1988. Cell-mediated and glucocorticoid-mediated target cell lysis do not appear to share common pathways. *J. Immunol.* 141:785-791, 2187.
- Dowd, D. R., and R. L. Miesfeld. 1992. Evidence that glucocorticoid- and cyclic AMP-induced apoptotic pathways in lymphocytes share distal events. *Mol. Cell. Biol.* 12:3600-3608.
- Duke, R. C., P. M. Persechini, S. Chang, C.-C. Liu, J. J. Cohen, and J. D.-E. Young. 1989. Purified perforin induces target cell lysis but not DNA fragmentation. *J. Exp. Med.* 170:1451-1456.
- Duke, R. C., K. S. Sellins, and J. J. Cohen. 1988. Cytotoxic lymphocyte-derived lytic granules do not induce DNA fragmentation in target cells. *J. Immunol.* 141:2191-2194.
- Filippini, A., R. E. Taffs, and M. V. Sitkovsky. 1990. Extracellular ATP in T-lymphocyte activation: possible role in effector functions. *Proc. Natl. Acad. Sci. USA* 87:8267-8271; 88:6899.
- Garcia-Sanz, J. A., G. Plaetinck, F. Velotti, D. Masson, J. Tschopp, H. R. MacDonald, and M. Nabholz. 1987. Perforin is present only in normal activated Ly2⁺ T lymphocytes and not in L3T4⁺ cells, but the serine protease granzyme A is made by both subsets. *EMBO J.* 6:933-938.
- Glasebrook, A. L., M. Sarmiento, M. R. Loken, D. P. Dialynas, J. Quintans, L. Eisenberg, C. T. Lutz, D. Wilde, and F. W. Fitch. 1981. Murine T lymphocyte clones with distinct immunological functions. *Immunol. Rev.* 54:225-266.
- Gromkowski, S. H., T. C. Brown, D. Masson, and J. Tschopp. 1988. Lack of DNA degradation in target cells lysed by granules derived from cytolytic T lymphocytes. *J. Immunol.* 141:774-778.
- Hayes, M. P., G. A. Berrebi, and P. A. Henkart. 1989. Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. *J. Exp. Med.* 170:933-946.
- Hebshi, L. D., and D. S. Ucker. Unpublished data.
- Helgason, C. D., J. A. Prendergast, G. Berke, and R. C. Bleackley. 1992. Peritoneal exudate lymphocyte and mixed lymphocyte culture hybridomas are cytolytic in the absence of cytotoxic cell proteinases and perforin. *Eur. J. Immunol.* 22:3187-3190.
- Henkart, M. P., and P. A. Henkart. 1981. Lymphocyte mediated cytotoxicity as a secretory phenomenon, p. 227-247. *In* W. R. Clark and P. Golstein (ed.), *Mechanisms of cell-mediated cytotoxicity*. Plenum Press, New York.
- Joag, S. V., C.-C. Liu, B. S. Kwon, W. R. Clark, and J. D.-E. Young. 1990. Expression of mRNAs for pore-forming protein and two serine esterases in murine primary and cloned effector lymphocytes. *J. Cell. Biochem.* 43:81-88.
- Jones, J., M. B. Hallett, and B. P. Morgan. 1990. Reversible cell damage by T-cell perforins: calcium influx and propidium iodide uptake into K562 cells in the absence of lysis. *Biochem. J.* 267:303-307.
- Kane, K. P., A. Vitiello, L. A. Sherman, and M. F. Mescher. 1989. Cytolytic T-lymphocyte response to isolated class I H-2 proteins and influenza peptides. *Nature (London)* 340:157-159.
- Kleinfeld, A. M. Personal communication.
- Koski, C. L., L. E. Ramm, C. H. Hammer, M. M. Mayer, and M. L. Shin. 1983. Cytolysis of nucleated cells by complement: cell death displays multi-hit characteristics. *Proc. Natl. Acad. Sci. USA* 80:3816-3820.
- Kranz, D. M., S. Tonegawa, and H. N. Eisen. 1984. Attachment of an anti-receptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 81:7922-7926.
- Landon, C., M. Nowicki, S. Sugawara, and G. Dennert. 1990. Differential effects of protein synthesis inhibition on CTL and targets in cell-mediated cytotoxicity. *Cell. Immunol.* 128:412-426.
- Lebow, L. T., and B. Bonavida. 1990. Purification and characterization of cytolytic and noncytolytic human natural killer cell subsets. *Proc. Natl. Acad. Sci. USA* 87:6063-6067.
- Liu, C.-C., S. Rafii, A. Granelli-Piperano, J. A. Trapani, and J. D.-E. Young. 1989. Perforin and serine esterase gene expression in stimulated human T cells: kinetics, mitogen requirements and effects of cyclosporin A. *J. Exp. Med.* 170:2105-2118.
- Lu, P., J. A. Garcia-Sanz, M. G. Lichtenheld, and E. R. Podack. 1992. Perforin expression in human peripheral blood mononuclear cells: definition of an IL-2-independent pathway of perforin induction in CD8⁺ T cells. *J. Immunol.* 148:3354-3360.
- Luciani, M.-F., J.-F. Brunet, M. Suzan, F. Denizot, and P. Golstein. 1986. Self-sparing of long-term *in vitro*-cloned or uncloned cytotoxic T lymphocytes. *J. Exp. Med.* 164:962-967.
- MacDonald, H. R., and C. J. Koch. 1977. Energy metabolism and T-cell-mediated cytotoxicity I. Synergism between inhibitors of respiration and glycolysis. *J. Exp. Med.* 146:698-709.
- Masson, D., and J. Tschopp. 1987. A family of serine esterases in lytic granules of cytolytic T lymphocytes. *Cell* 49:679-685.
- Millard, P. J., M. P. Henkart, C. W. Reynolds, and P. A. Henkart. 1984. Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. *J. Immunol.* 132:3197-3204.
- Nagler-Anderson, C., M. Lichtenheld, H. N. Eisen, and E. R. Podack. 1989. Perforin mRNA in primary peritoneal exudate cytotoxic T lymphocytes. *J. Immunol.* 143:3440-3443.
- 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.* 141:3299-3305.
- Nickas, G., J. Meyers, L. D. Hebshi, J. D. Ashwell, D. P. Gold, B. Sydora, and D. S. Ucker. 1992. Susceptibility to cell death is a dominant phenotype: triggering of activation-driven T-cell death independent of the T-cell antigen receptor complex. *Mol. Cell. Biol.* 12:379-385.
- Ojcus, D. M., L. M. Zheng, E. C. Sphicas, A. Zychlinsky, and J. D.-E. Young. 1991. Subcellular localization of perforin and serine esterase in lymphokine-activated killer cells and cytotoxic T cells by immunogold labeling. *J. Immunol.* 146:4427-4432.
- Ojcus, D. M., A. Zychlinsky, L. M. Zheng, and J. D.-E. Young. 1991. Ionophore-induced apoptosis: role of DNA fragmentation and calcium fluxes. *Exp. Cell Res.* 197:43-49.
- Ostergaard, H. L., and W. R. Clark. 1989. Evidence for multiple lytic pathways used by cytotoxic T lymphocytes. *J. Immunol.* 143:2120-2126.
- Ostergaard, H. L., K. P. Kane, M. F. Mescher, and W. R. Clark. 1987. Cytotoxic T lymphocyte mediated lysis without release of serine esterase. *Nature (London)* 330:71-72.
- Ozato, K., T. H. Hansen, and D. H. Sachs. 1980. Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the H-2L^d antigen, the products of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol.* 125:2473-2477.
- Perez, P., R. W. Hoffman, S. Shaw, J. A. Bluestone, and D. M.

- Segal. 1985. Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. *Nature (London)* **316**:354–356.
42. Peters, P. J., J. Borst, V. Oorschot, M. Fukuda, O. Krahenbuhl, J. Tschopp, J. W. Slot, and H. J. Geuze. 1991. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J. Exp. Med.* **173**:1099–1109.
 43. Poenie, M., R. Y. Tsien, and A.-M. Schmitt-Verhulst. 1987. Sequential activation and lethal hit measured by $[Ca^{2+}]_i$ in individual cytolytic T cells and targets. *EMBO J.* **6**:2223–2232.
 44. Redegeld, F. A., S. Chatterjee, N. A. Berger, and M. V. Sitkovsky. 1992. Poly-(ADP-ribose) polymerase partially contributes to target cell death triggered by cytolytic T lymphocytes. *J. Immunol.* **149**:3509–3516.
 45. Reynolds, C. W., D. Reichardt, M. Henkart, P. Millard, and P. Henkart. 1987. Inhibition of NK and ADCC activity by antibodies against purified cytoplasmic granules from rat LGL tumors. *J. Leukocyte Biol.* **42**:642–652.
 46. Richieri, G. V., and A. M. Kleinfeld. 1991. Free fatty acids are produced in and secreted from target cells very early in cytotoxic T lymphocyte-mediated killing. *J. Immunol.* **147**:2809–2815.
 47. Rouvier, E., M.-F. Luciani, and P. Golstein. 1993. Fas involvement in Ca^{2+} -independent T cell-mediated cytotoxicity. *J. Exp. Med.* **177**:195–200.
 48. Russell, J. H. 1983. Internal disintegration model of cytotoxic lymphocyte-induced target damage. *Immunol. Rev.* **72**:97–118.
 49. Russell, J. H., and C. B. Dobos. 1983. Characterization of a “heteroclitic” cytotoxic lymphocyte clone: heterogeneity of receptors or signals? *J. Immunol.* **130**:538–541.
 50. Russell, J. H., V. R. Masakowski, and C. B. Dobos. 1980. Mechanisms of immune lysis. I. Physiological distinction between target cell death mediated by cytotoxic T lymphocytes and antibody plus complement. *J. Immunol.* **124**:1100–1105.
 51. Sandri-Goldin, R. M., A. L. Goldin, M. Levine, and J. C. Glorioso. 1981. High-frequency transfer of cloned herpes simplex virus type I sequences to mammalian cells by protoplast fusion. *Mol. Cell. Biol.* **1**:743–752.
 52. Shi, L., C. M. Kam, J. C. Powers, R. Aebersold, and A. H. Greenberg. 1992. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. *J. Exp. Med.* **176**:1521–1529.
 53. Shi, L., R. P. Kraut, R. Aebersold, and A. H. Greenberg. 1992. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *J. Exp. Med.* **175**:553–566.
 54. Shiver, J. W., and P. A. Henkart. 1991. A noncytotoxic mast cell tumor line exhibits potent IgE-dependent cytotoxicity after transfection with the cytolysin/perforin gene. *Cell* **64**:1175–1181.
 55. Shiver, J. W., L. Su, and P. A. Henkart. 1992. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. *Cell* **71**:315–322.
 56. Smyth, M. J., Y. Norishisa, and J. R. Ortaldo. 1992. Multiple cytolytic mechanisms displayed by activated human peripheral blood T cell subsets. *J. Immunol.* **148**:55–62.
 57. Staerz, U. D., O. Kanagawa, and M. J. Bevan. 1985. Hybrid antibodies can target sites for attack by T cells. *Nature (London)* **314**:628–633.
 58. Stallcup, K. C., T. A. Springer, and M. F. Mescher. 1981. Characterization of an anti-H-2 monoclonal antibody and its use in large-scale antigen purification. *J. Immunol.* **127**:923–930.
 59. Strack, P., C. Martin, S. Saito, R. H. Dekuyff, and S.-T. Ju. 1990. Metabolic inhibitors distinguish cytolytic activity of CD4 and CD8 clones. *Eur. J. Immunol.* **20**:179–184.
 60. Talento, A., M. Nguyen, S. Law, J. K. Wu, M. Poe, J. T. Blake, M. Patel, T.-J. Wu, C. L. Manyak, M. Silberklang, G. Mark, M. Springer, N. H. Sigal, I. L. Weissman, R. C. Bleackley, E. R. Podack, M. L. Tykocinski, and G. C. Koo. 1992. Transfection of mouse cytotoxic T lymphocyte with an antisense granzyme A vector reduces lytic activity. *J. Immunol.* **149**:4009–4015.
 61. Tian, Q., M. Streuli, H. Saito, S. F. Schlossman, and P. Anderson. 1991. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. *Cell* **67**:629–639.
 62. Trenn, G., H. Takayama, and M. V. Sitkovsky. 1987. Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes. *Nature (London)* **330**:72–74.
 63. Ucker, D. S. 1987. Cytotoxic T lymphocytes and glucocorticoids activate an endogenous suicide process in target cells. *Nature (London)* **327**:62–64.
 64. Ucker, D. S. 1991. Death by suicide: one way to go in mammalian cellular development? *New Biol.* **3**:103–109.
 65. Ucker, D. S., J. D. Ashwell, and G. Nickas. 1989. Activation-driven T cell death I. Requirements for de novo transcription and translation and association with genome fragmentation. *J. Immunol.* **143**:3461–3469.
 66. Ucker, D. S., J. Meyers, and P. S. Obermiller. 1992. Activation-driven T cell death. II. Quantitative differences alone distinguish stimuli triggering nontransformed T cell proliferation or death. *J. Immunol.* **149**:1583–1592.
 67. Ucker, D. S., P. S. Obermiller, W. Eckhart, J. R. Appgar, N. A. Berger, and J. Meyers. 1992. Genome digestion is a dispensable consequence of physiological cell death mediated by cytotoxic T lymphocytes. *Mol. Cell. Biol.* **12**:3060–3069.
 68. Vaux, D. L., H. L. Aguila, and I. L. Weissman. 1992. *Bcl-2* prevents death of factor-deprived cells but fails to prevent apoptosis in targets of cell mediated killing. *Int. Immunol.* **4**:821–824.
 69. 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**:1536–1546.
 70. Vitiello, A., W. R. Heath, and L. A. Sherman. 1989. Self-presentation of peptide antigen by CTL. *J. Immunol.* **143**:1512–1517.
 71. Wyllie, A. H., R. G. Morris, A. L. Smith, and D. Dunlop. 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.* **142**:67–77.
 72. Zanovello, P., V. Bronte, A. Rosato, P. Pizzo, and F. Di Virgilio. 1990. Responses of mouse lymphocytes to extracellular ATP. II. Extracellular ATP causes cell type-dependent lysis and DNA fragmentation. *J. Immunol.* **145**:1545–1550.
 73. Zychlinsky, A., L. M. Zheng, C.-C. Liu, and J. D.-E. Young. 1991. Cytolytic lymphocytes induce both apoptosis and necrosis in target cells. *J. Immunol.* **146**:393–400.