Brief Definitive Report

A CALCIUM- AND PERFORIN-INDEPENDENT PATHWAY OF KILLING MEDIATED BY MURINE CYTOLYTIC LYMPHOCYTES

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It has long been thought that CTL lyse cells by a calcium-dependent mechanism (1–3). Calcium is not required at the initial stage of CTL-target conjugation but is thought to be absolutely necessary for subsequent delivery of the lethal hit, during which stage the target cell is "programmed" for cell death. These initial observations were corroborated by recent studies suggesting a role for CTL granules in mediating target membrane damage (4–6). A granule pore-forming protein (PFP, perforin, or cytolysin) was postulated to be deposited on the target cell surface by exocytotic degranulation and to assemble into functional channels, a process known to require Ca^{2+}. In other studies, the contact between the CTL and target cell has recently been shown to elicit an abrupt rise in cytosolic calcium levels within the CTL (7–9), and within the target cell immediately before lysis (9). Moreover, engagement of the T cell antigen receptor complex is known to be accompanied by increases in cytoplasmic-free calcium levels in T cells, including CTLs (reviewed in reference 10). Finally, calcium is also thought to be required for the reorientation of certain cytoskeletal elements of CTL towards the target after CTL-target conjugation (11). Together, these studies have implied that both the signaling and the effector mechanisms of CTLs are all strictly calcium-dependent.

Here, we have reexamined the cytotoxic properties of a number of murine CTL lines (CTLL). We report that CTLL cultured in vitro are capable of lysing cells in a calcium- and PFP/perforin-independent manner.

Materials and Methods

Cell Lines and Subcellular Fractionation. The origin, phenotype, and maintenance of CTLL AB.2, KB1.24, 83.4, L3, A2, A11, and R8 were described in detail elsewhere (12, 13). CTLL-1 was obtained from Dr. K. A. Smith, Dartmouth Medical School, Hanover, NH. YAC-1, P815, EL-4, and WEHI-164 cells were maintained in RPMI 1640/FCS (13). Fractionation of cells and protein and enzyme microassays were done exactly as described (12). Proteoglycans were assayed using dimethylene blue (14).

J. D.-E. Young is a Lucille P. Markey Scholar. This work was supported in part by grants from the Cancer Research Institute/Francis L. & Edwin L. Cummings Memorial Fund Investigators Award, the Lucille P. Markey Charitable Trust, and by grants AI-14747, CA-30198, and AI-07012 from the National Institutes of Health.

TABLE I

Efficiency of Target-Cell Lysis Mediated by CTLL in the Presence and Absence of Extracellular Calcium

Effector CTLL	Target cell	Calcium	Net percent ^{51}Cr release at E/T ratio of:	
			5:1	1:1
R8	YAC-1	+	99	85
		−	97	88
	P815	+	98	90
		−	99	85
A2	P815	+	91	73
		−	98	63
A11	P815	+	85	53
		−	99	75
AB.2	EL-4	+	90	81
		−	93	73
83.4	P815	+	71	45
		−	17	9
L3	P815	+	67	39
		−	39	21
1	P815	+	49	27
		−	18	11
KB1.24	EL-4	+	71	37
		−	65	31

Effector and target cells were mixed together at the given ratios and incubated for 3 h, either in medium containing 1 mM $CaCl_2$ (with calcium) or in the absence of calcium and with 2 mM EGTA (without calcium). Spontaneous release values were <10% and were subtracted from the final readings. Data represent averages of triplicates.

Cytotoxicity Assays. Cytotoxicity against targets was quantitated in 4-h ^{51}Cr-release assays (13). Ca^{2+}-free, RPMI 1640 containing 1 or 2 mM EGTA was used when the effect of Ca^{2+} depletion was tested. This medium was supplemented with 5% FCS that was previously dialyzed extensively against PBS. For Figs. 2 and 3, the MTT dye reduction microassay (15) was performed exactly as described (14). Percoll was removed from fractions by high-speed centrifugation before use (14). To deplete fractions of perforin activity, these were extracted twice with diethyl ether (14). The samples were resuspended to original volumes with PBS. 6×10^4 targets/200 μl/well were tested in triplicates with cytotoxic reagents added in volumes ≤20 μl/well. Plates were incubated for different intervals before addition of MTT (14), followed by additional incubation for 30 min. Plates containing nonadherent cells (YAC-1) were centrifuged (200 *g*, 10 min) before aspiration of MTT supernatant.

Planar Bilayer System. Bilayers were formed from soybean lipids (16). The membrane bathing buffer consisted of 0.1 M NaCl, 1 mM $CaCl_2$, 3 mM NaN_3, and 5 mM Pipes, pH 7. PFP channels were incorporated into bilayers through addition of PFP into the aqueous phase.

Results and Discussion

All eight CTLL tested lysed their respective targets to a variable extent in the absence of any extracellular calcium, with lytic efficiencies ranging from 25 to 150% when compared with the extent of lysis obtained in the presence of calcium (Table I). The time course of lysis was also comparable to that observed in the presence of 1 mM Ca^{2+} in the medium (Fig. 1). Since assembly of PFP is thought to be Ca^{2+}-dependent, we analyzed each of these CTLL for PFP content. CTL were grown to at least 10^8 cells and their subcellular fractions were obtained by Percoll gradient centrifugation and assessed by using various granule markers.

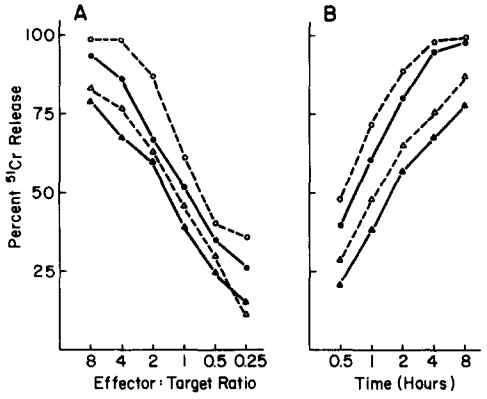

FIGURE 1. Lysis of target cells by CTL in the absence of calcium: CTLL A11 (b anti-d) against P815 (H-2^d) (○, ●) and CTLL AB.2 (d anti-b) against EL-4 (H-2^b) (△, ▲). ^{51}Cr-release assays were performed in medium containing 1 mM $CaCl_2$ (●, ▲), or were done in the absence of Ca^{2+} (medium containing 2 mM EGTA) (○, △). (*A*) 3-h assays were done in triplicates. (*B*) Time-course of killing, with incubation periods as indicated. Spontaneous release values for P815 and EL-4 targets were <10% in all assays done within 4 h of incubation and ranged 8–17% in assays done at the 8-h point.

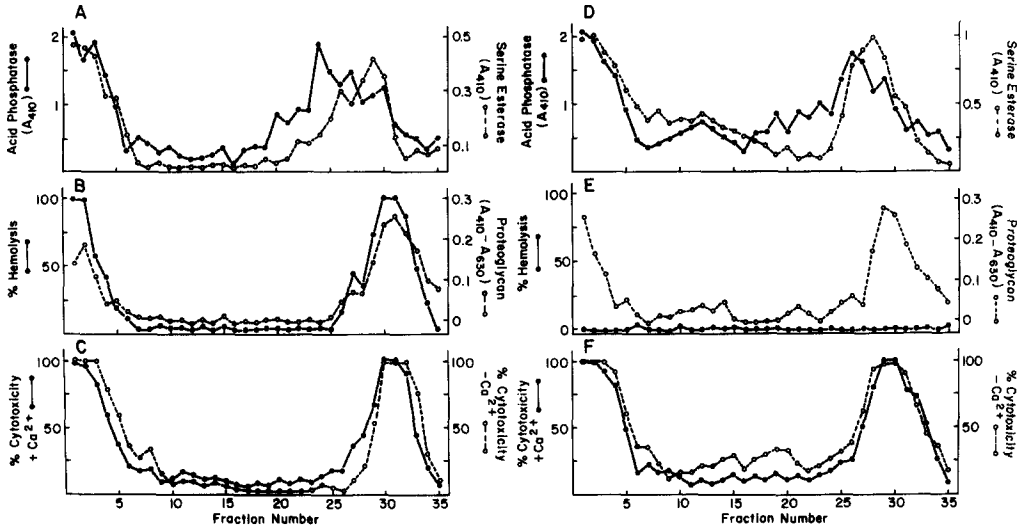

FIGURE 2. Subcellular fractionation of CTLL R8 (*A–C*) and CTLL AB.2 (*D–F*). Percoll fractions were collected from top (*left to right*). Hemolytic and serine esterase activities and proteoglycan were used as granule markers. Cytotoxicity of Percoll fractions to YAC-1 (*C*) and WEHI-164 (*F*) cells was assayed by MTT reduction assay (see Materials and Methods). An incubation time of 12 h was used. Spontaneous cytotoxicity measured in response to added control PBS was subtracted from each point. Calcium conditions for *C* and *F* are exactly the same as those of Fig. 1.

Granules of all CTLL were enriched for proteoglycan, serine esterase, and acid phosphatase (small peak), and in the case of CTLL R8 (Fig. 2, *A–C*), A2, A11, and L3, for hemolytic activity. Granule markers were associated with two separate peaks of the Percoll gradient: one at the bottom of the gradient and the other one on top, possibly corresponding to material that did not migrate into the gradient. The relative distribution of granule markers between these two peaks varied in each experiment. The dense, lytic fractions represented granule populations, as ascertained by ultrastructural analysis of four cell lines (CTLL R8, A11, 83.4, and 1; micrographs not shown). It appeared that at least part of the top fractions could have been derived from limited granule breakdown during

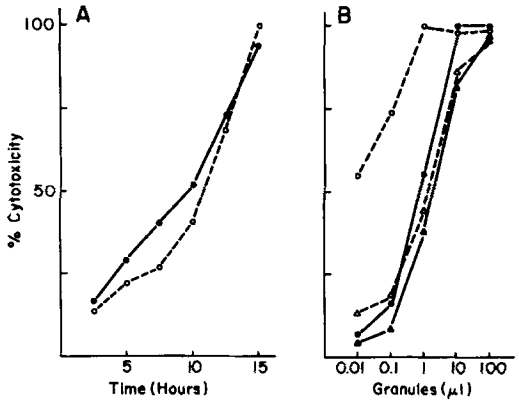

FIGURE 3. (*A*) Time-course of killing mediated by granule fractions from CTLL AB.2. Granule fractions from 2 × 10^4 cell equivalents that had previously been depleted of perforin-activity (see Materials and Methods) were added to each well in triplicates. The MTT assay was used. (●) 1 mM $CaCl_2$ in the medium; (○) 0 $CaCl_2$, 1 mM EGTA. In the absence of calcium, spontaneous cytotoxicity was 23% by 15 h, 17% at the 12-h point, and <10% at time points before 10 h. Spontaneous cytotoxicity was <5% when 1 mM $CaCl_2$ was present in the medium. (*B*) Granule fractions from CTLL R8 (○, ●) and CTLL AB.2 (△, ▲) were incubated with WEHI-164 targets for 15 h, in the presence of 1 mM $CaCl_2$ in the medium. Granules were obtained after pelleting Percoll and used directly (○, △) or after depletion of perforin activity (●, ▲). 1 μl of granules corresponded to material from 10^6 cells. Data points represent averages of triplicates.

nitrogen cavitation of cells. In those CTLL (AB.2, KB1.24, 83.4, and 1) lacking hemolytic activity, serine esterase and proteoglycan were, however, abundantly present in the cells, as shown in Fig. 2, *D–F*, for CTLL AB.2. Subcellular fractions from these CTLL that were frozen and thawed 3× and extracted with a high-phosphate buffer, which would have ruptured granules, remained nonhemolytic. The lack of pore-forming activity in these CTLL was further ascertained by testing subcellular fractions in planar lipid bilayers that would have allowed us to detect 0.1–0.5 pmol of PFP (data not shown).

These results made it unlikely that PFP could have been involved in the cytolysis mediated by at least some CTLL. Notably, subcellular fractions derived from all eight CTLL, including those lacking PFP, were cytolytic to tumor targets. Fig. 2 illustrates that regardless of whether or not PFP was present in the CTLL, dense granule fractions were highly cytotoxic to nucleated targets. A second peak of lytic activity was associated with light Percoll fractions. These results imply that a PFP-independent mechanism could be involved in the killing of tumor targets. This notion was strengthened by the finding that subcellular fractions tested in the absence of calcium remained cytotoxic (Fig. 2). In addition to lysing YAC-1 cells (Fig. 2*C*), for example, subcellular fractions from CTLL-R8 lysed P815, WEHI-164, and EL-4 cells in the absence of calcium (data not shown).

The time course of killing mediated by calcium-independent cytotoxic factor(s) partially enriched in granule fractions was relatively slow, as shown in Fig. 3. In contrast to PFP, which lyses targets within minutes in the presence of calcium (4–6), subcellular fractions of CTLL required hours to lyse targets in the absence of calcium, and up to 15–18 h for lysis to be completed (Fig. 3*A*, shown here only for CTLL AB.2). Notably, granules obtained from CTLL AB.2 (PFP^-) also required several hours to lyse targets, even in the presence of millimolar amounts of calcium (Fig. 3*A*). Granules of CTLL R8 and AB.2 clearly lysed WEHI-164 cells in a dose-dependent manner in the presence or absence of calcium (Fig. 3*B*). In the presence of calcium, granule material from 10^4 R8 cell and 10^6 AB.2

cell equivalents were capable of lysing 3×10^4 cells in a 200-μl volume after a 10-h incubation. Depletion of calcium significantly reduced the lytic efficiency of CTLL R8 granules (perforin-positive), but not of CTLL AB.2 granules (perforin-negative), when compared on an equivalent granule protein basis. These results suggest that the calcium-dependent and -independent pathways may both contribute to target cell lysis mediated by CTLL that contain PFP. At this time, we cannot explain the apparent discrepancy observed between the slow kinetics of killing mediated by subcellular fractions and the much faster killing time course seen with intact effector CTL. It is possible that other unidentified factors may be involved that could result in the rapid delivery of the lethal hit only in a whole cell context.

Our results suggest that in addition to PFP, IL-2-driven murine CTLL also contain other effector mechanisms of killing that are not restricted by calcium requirements. A Ca^{2+}-independent pathway appears to be present in all CTLL tested here. In marked contrast, the Ca^{2+}-dependent pathway involving perforin is present only in some but not all murine CTLL. Whether the Ca^{2+}-independent lytic pathway is expressed against all targets or is restricted to a selected panel of tumor cells remains to be tested. Tirosh and Berke (17) have suggested earlier that calcium requirements are dictated solely by the targets and not by the effector CTL being tested. It is not clear whether the Ca^{2+}-independent pathway involves only one mediator, or perhaps multiple mediators that may be differentially distributed between the granules and the cytosol of CTL. Initial studies (14) indicate that one such lytic factor found in all murine CTLL tested so far is antigenically related but not identical to tumor necrosis factor/cachectin, a cytokine that has been associated in the past with monocyte-mediated cytotoxicity (18, 19). Taken together, these results suggest that killing by CTL may involve a combination of multiple mechanisms and/or mediators that appear not to be mutually exclusive.

Summary

Cytotoxic T lymphocytes have been thought to lyse cellular targets in the past by a calcium-dependent pathway. This notion was recently supported by the identification and purification of a pore-forming protein (perforin) from the granules of these cell types. Here, we show that perforin is absent from a number of cell lines that nevertheless display vigorous cytolytic activity toward target cells. The cytotoxic activity of eight murine CTL lines is completely or partially retained in the absence of calcium. The calcium-independent lytic activity is associated with two subcellular fraction peaks isolated by Percoll gradient centrifugation, e.g., a heavy density band migrating with granule markers and a lighter band corresponding to free cytosolic material. These results suggest a complex picture of lymphocyte-mediated killing involving probably multiple mechanisms and mediators that may operate in concert or independently in the delivery of the lethal hit.

We thank A. Blakely, L. G. Leong, A. Damiano, and G. Butler for excellent technical assistance. We also wish to acknowledge initial collaborative studies done with D. Campanelli and Dr. C. F. Nathan.

Received for publication 16 September 1987.

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