T Cell–T Cell Killing Is Induced by Specific Epitopes: Evidence for an Apoptotic Mechanism

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Summary

Epstein-Barr virus–specific cytotoxic T lymphocyte clones were shown to be an effective target for their own lysis when incubated in the presence of their specific epitopes but not in the presence of irrelevant epitopes. The mode of cell killing appeared to be by apoptosis and was prevented by previously described inhibitors of the process. Degranulation, as measured by serine esterase activity, was involved in this form of T cell–T cell killing. This is the first report of T cell–T cell killing by apoptosis and is only observed in the presence of a specific epitope. This result may be of significance in the use of peptide-based vaccines.

It now seems likely that cytotoxic T lymphocytes kill their targets by a complex process of multiple mechanisms of cell lysis involving apoptosis. The best described processes involve either cytotoxic granules that are directed towards the cleft between CTL-target pairs (1–3) or by non–pore-forming contact-induced stimulated disintegration (4). Cytotoxic granules contain a pore-forming protein (perforin or cytolysin) that forms transmembrane channels in the target cell, as well as serine esterases that are frequently used as a marker for the abundance of secretory activity (5–7). Evidence for the existence of mechanisms of cell killing other than that involving pore formation has recently been presented. Loss of target cell membrane permeability was found to follow prelytic events such as DNA fragmentation and elevation of Ca^{2+} in target cells (8). In vivo primed, peritoneal exudate CTLs do not form complement-like pores during lysis and lytic granules were not detected (9). However, failure to detect hemolytic activity in primary CTL is not in itself evidence for the presence of a perforin-independent pathway since these cells have been shown to contain significant levels of mRNA (10).

It has been demonstrated previously that cloned CTLs are highly resistant to lysis by CTLs (11–13). Murine CTLs have been shown to be a poor target for lysis and are also resistant to killing by purified perforin-rich granules (14–17). Functionally, the concept of resistance of CTLs to lysis is to be expected since it provides a mechanism of avoiding self destruction by their own cytolytic mediators.

However, we have recently made several observations that suggest that CTLs are an effective target for lysis when the target antigen is presented on CTLs themselves in the form of a specific CTL epitope. Consistent with this observation, a recent report has demonstrated that peptide-specific $CD4^+$ CTLs are able to lyse themselves by a mechanism as yet undefined (18). The system under investigation uses EBV CTL clones that recognize defined CTL epitopes in association with either HLA B8 or B44. In this study, we demonstrate T cell–T cell killing in the presence of the relevant epitope resulting in DNA fragmentation and the appearance of morphological changes characteristic of apoptosis.

Materials and Methods

Generation and Description of CTL Clones. Briefly, lymphocytes from three EB virus seropositive donors were stimulated (100:1, responder to stimulator ratio) with irradiated (80 Gy) autologous lymphoblastoid cell lines (LCLs) and cloned in agar as previously described (19). CTLs were propagated in medium containing RPMI 1640, 20% FCS, 25% IL-2-containing supernatant from MLA 144 T cell cultures, and highly purified human rIL-2 from *Escherichia coli* (30 U/ml) (20, 21). The $CD8^+$ CTL clones from the three seropositive donors involved in this study have been previously described: HLA B8-restricted clone 13 from donor LC (HLA A1, B8, B18), which recognizes the epitope TETAQAWNAGFLRGRAYGIDLLRTE (referred to as TETA) (22); HLA B44-restricted clone 3 from donor DM (HLA AW24, 29; B44, 47), which recognizes the epitope EENLLDFVRFMGVMSSCNNP (referred to as EENL) (23); and HLA A2-restricted clone 6 from donor JS (HLA A1,2; B8,51) (24).

Target Cells. The CTLs described above were used in this study not only as effector cells but were also used as target cells. Two other categories of target cells were used. First, B lymphocytes from donors LC and DM were transformed with EBV derived from the cell line IARC-BL74 (25) (designated LC\BL74 and DM\BL74). Second, PHA blast cell lines from these two donors were established as follows: PHA was added to lymphocytes for 3 d, and growth medium containing MLA 144 supernatant and rIL-2 was added. Cultures were propagated with bi-weekly replacement of IL-2 and MLA supernatant. PHA blasts were maintained for up to 6 wk using this procedure (cell lines designated LC\PHA and DM\PHA).

Cytotoxicity Assay. Two different experimental protocols were used. In the first (as in experiment illustrated in Fig. 1), target cells (LCLs and PHA blasts) were preincubated with peptide (40 μg/ml)

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for 1 h, washed, labeled with ^{51}Cr, and incubated with effector cells (E/T ratio of 10:1) in U-shaped microtiter wells at 37°C for 5 h. In the second protocol, in which CTLs acted as both effector cells and target cells (as in experiment illustrated in Fig. 2), CTL clones were labeled with ^{51}Cr, washed three times, and plated out in either U- or flat-bottomed microtiter wells in medium containing IL-2 at 5×10^4 U/well (180 μl). Peptide was added to the well (20 μl, 200 μg/ml) and incubated for 1 h at 37°C before being centrifuged at 200 *g* and incubated at 37°C for 5 h. In some experiments, either 5 mM EGTA, 5 mM $ZnSO_4$, or cycloheximide (50 μg/ml) was included in the reaction mixture.

DNA Extraction and Electrophoresis. In experiments to determine DNA fragmentation, clone 13 was incubated for 4 h (5×10^5 cells/U-bottomed-well) in the presence of TETA or EENL (20 μg/ml) with and without 5 mM EGTA or 5 mM $ZnSO_4$. DNA was extracted from 10^7 cells according to the salting-out method of Miller et al. (24). After digestion with RNAase, ~10 μg of DNA was loaded into each lane of a 1.4% agarose gel, subjected to electrophoresis for 15 h at 15 mA, stained with ethidium bromide and visualized using a UV transilluminator. The degree of fragmentation was determined by densitometry.

Light and Electron Microscopy. To detect the morphological features characteristic of apoptosis, clone 13 was incubated (5×10^5 cells/U-bottomed well) in the presence of TETA or EENL (20 μg/ml) for 4 h and examined both by electron and light microscopy as previously described (26).

BLT-Esterase Activity. Serine esterase activity was determined in CTL culture supernatants (27), after incubations (4 h) of clone 13 (5×10^5 cells/U-bottomed well) in the presence of TETA or EENL (20 μg/ml) with and without 5 mM EGTA or 5 mM $ZnSO_4$.

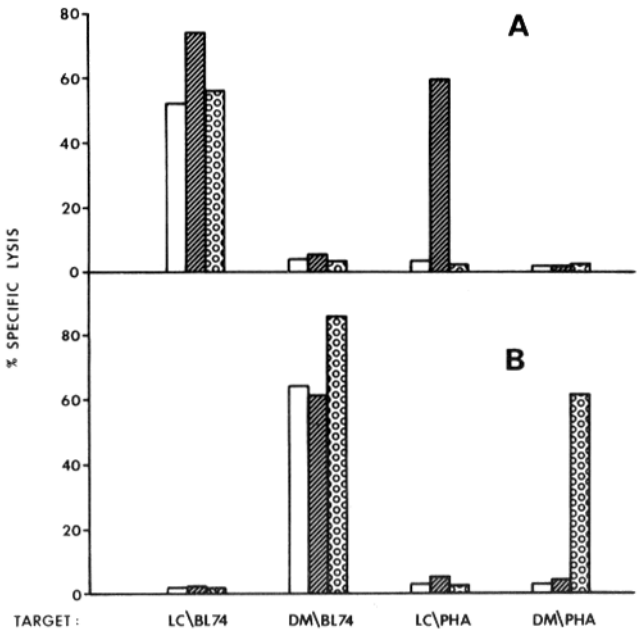

Figure 1. (*A*) Lysis by clone 13 from donor LC of autologous (LC\BL74 and LC\PHA) and allogeneic (DM\BL74 and DM\PHA) cell lines either in the absence of added peptide (□) or in the presence of TETA (▨) or EENL (▩). (*B*) Lysis by clone 3 from donor DM of autologous (DM\BL74 and DM\PHA) and allogeneic (LC\BL74 and LC\PHA) cell lines either in the absence of added peptide (□) or in the presence of TETA (▨) or EENL (▩).

Results and Discussion

The reactivity of the CTL clones LC 13 and DM 3 towards autologous and allogeneic cells in the presence and absence of relevant epitopes (TETA for clone 13 and EENL for clone 3) is illustrated in Fig. 1. Each clone lyses the autologous but not allogeneic LCL, and the extent of lysis was greater in the presence of relevant peptide. A dramatic increase in lysis by the CTL clones of the autologous PHA blast cell line exposed to the relevant epitope was also observed. It is thus quite clear that each clone specifically recognizes a peptide epitope in association with its restricting allele.

An overwhelming body of evidence has suggested that CTLs are resistant to self lysis. To determine whether CTLs are a target for self lysis when antigen is presented in the form of a specific CTL epitope, we have incubated ^{51}Cr-labeled clone 13 and 3 in the presence of their specific epitope. Fig. 2 illustrates the effect of incubating these clones with TETA and EENL respectively. Incubation of the TETA peptide with clone 13 results in lysis, while incubation of this epitope with the CTL clone 3 results in little or no lysis. Similarly, incubation of clone 3 with EENL results in specific lysis of this clone. Exposure of another EBV-specific CTL clone (clone 6) to TETA or EENL failed to elicit any cytotoxic response. It should be noted that the level of peptide-induced lysis of CTLs and LCLs was comparable, indicating that both cell types were equally efficient targets. Overall, these experiments demonstrate that CTL epitopes can specifically induce lysis of CTLs. Toxicity can be excluded as the mediator of lysis since peptides have been used at five times the concentration (100 μg/ml) used in these experiments with little or no increase in spontaneous release of ^{51}Cr from either LCLs or CTLs (not specific for the peptide). Recent characterization of the third EBV CTL epitope (manuscript in preparation) recognized by clone 6 from donor JS has revealed that T cell–T cell killing also occurs after exposure to the specific epitope (results not shown).

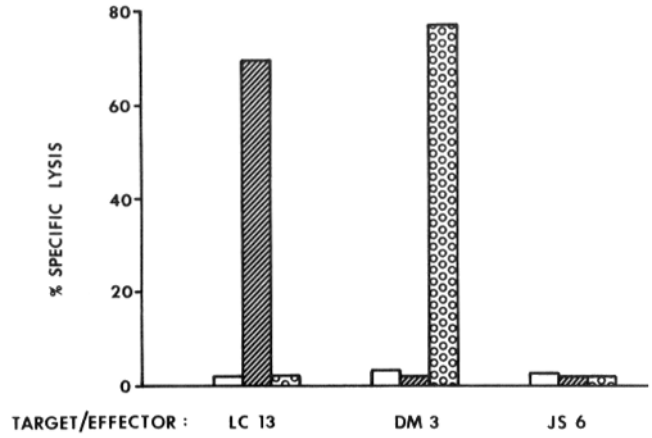

Figure 2. Lysis of ^{51}Cr-labeled CTL clone 13 from donor LC, clone 3 from DM, and clone 6 from JS incubated either without peptide (□) or in the presence of TETA (▨) or EENL (▩).

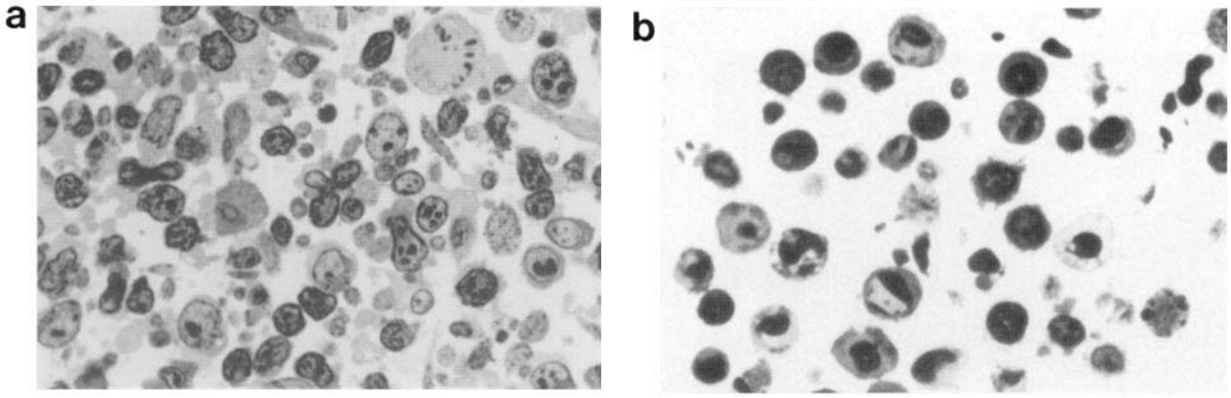

Figure 3. Morphological characteristics of CTL clone 13 (light micrograph, ×1,000). (*a*) Clone 13 incubated for 4 h with the irrelevant epitope EENL. (*b*) Clone 13 incubated for 4 h with the specific epitope TETA.

Microscopy and DNA fragmentation analysis were used to examine the mode of cell death of clone 13 after exposure to the peptide epitopes. Light microscopic examination showed that 40% of cells were undergoing programmed cell death or apoptosis in the presence of the specific epitope TETA (Fig. 3 *b*). When this clone was incubated with the irrelevant epitope EENL, no apoptosis was evident (Fig. 3 *a*). More detailed examination by electron microscopy revealed loss of microvilli, and cytoplasmic and nuclear condensation was apparent, as previously described, in cells undergoing apoptosis (Fig. 4 *B*). This evidence of apoptosis was further enhanced by demonstrating DNA fragmentation in clone 13 after exposure to TETA (Fig. 5, lane *2*). Quantitation of the extent of fragmentation by densitometry revealed that ~30% of the fragmentation ladder was represented by DNA varying from mononucleosome to octanucleosome size fragments. Zn^{2+}

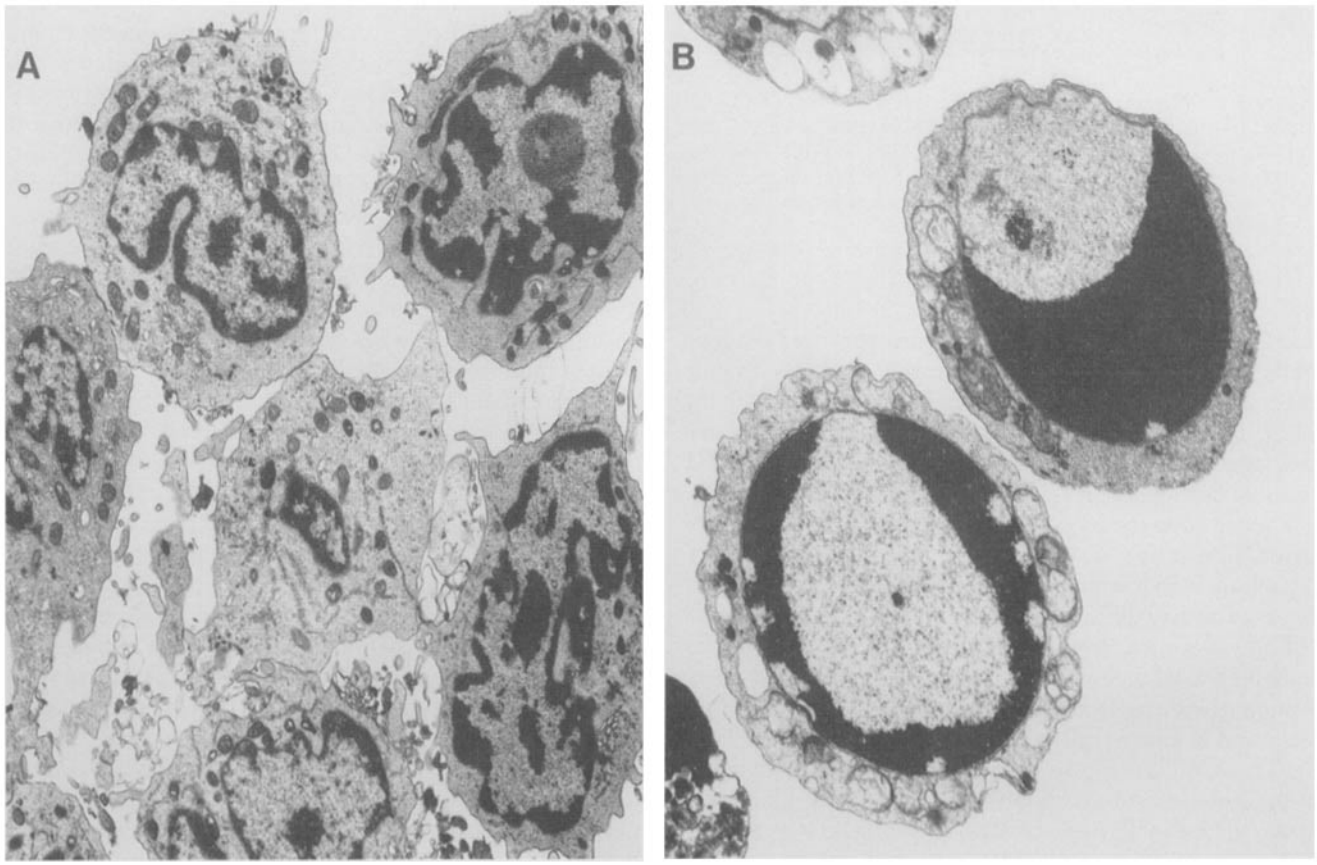

Figure 4. Morphological characteristics of CTL clone 13 (electron micrograph). (*A*) Cells of normal CTL morphology incubated with the irrelevant epitope EENL. (*B*) A cluster of cells at various stages of apoptosis after 4-h incubation in the presence of TETA.

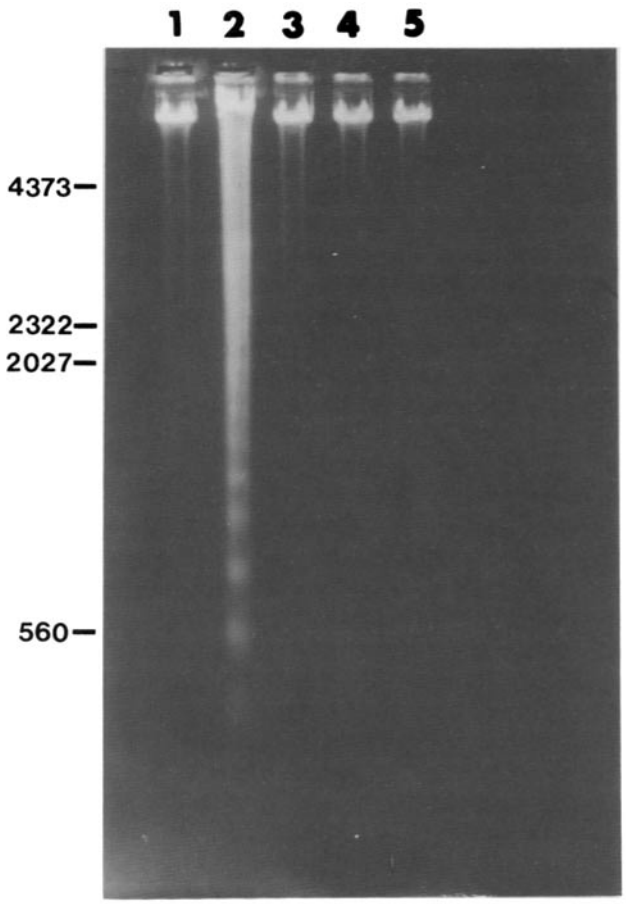

Figure 5. DNA fragmentation in CTL clone 13. (Lane *1*) CTLs incubated for 4 h; (lane *2*) CTLs incubated in the presence of TETA; (lane *3*) CTLs incubated in the presence of EENL; (lane *4*) CTLs incubated in the presence of TETA and 5 mM EGTA; (lane *5*) CTLs incubated in the presence of TETA and 5 mM $ZnSO_4$. Size markers (bp) are fragments generated from λDNA by HindIII digestion.

and EGTA inhibited both DNA fragmentation and cell lysis in this clone. These compounds have been shown to inhibit CTL-mediated killing and apoptosis in other systems. To investigate in more detail the mechanism of self killing, inhibitors were used to determine the effects on release of ^{51}Cr and whether degranulation as measured by serine esterase activity was involved in the process. The results in Table 1 illustrate that CTLs incubated with the specific epitope (TETA) for 4 h release high levels of serine esterase into the supernatant. No esterase activity was detected when an irrelevant peptide (EENL) was used. EGTA and Zn^{2+}, which are known to inhibit CTL killing, were shown to markedly reduce degranulation (shown in Table 1 by inhibition of serine esterase release) and to prevent cell lysis as judged by ^{51}Cr release (Table 1). These inhibitors may prevent apoptosis by functioning at the level of exocytosis for which evidence has been presented above, or by inhibiting the Ca^{2+}/Mg^{2+} endonuclease and thus preventing chromatin cleavage, as observed in several other cell systems (26, 28–30). The protein synthesis inhibitor, cycloheximide, did not prevent DNA fragmentation in

Table 1. *Serine Esterase Activity and Percent Specific Lysis of CTL Clone 13 Cells after Treatment*

Treatment	BLT-esterase activity (OD 412 nm/ 10^6 cells/15 min)	Percent specific lysis
CTL cells only	0.1	3.0
CTL + TETA	3.1	62.0
CTL + EENL	0.1	3.0
CTL + TETA + ZN^{2+}	0.3	0.5
CTL + TETA + EGTA	0.4	2.0

this system in agreement with previously observed results for CTL-mediated cell killing (29, 31). Indeed in this system, cycloheximide enhanced fragmentation in cells exposed to TETA and caused fragmentation when added alone, as observed previously in other human cells (26).

Apoptosis is a widely distributed form of cell death occurring during normal tissue turnover and in embryogenesis (32). It has been reported in lymphocytes exposed to γ radiation (30): after removal of IL-2 from dependent T cell lines (33); after isolation of lymphocytes from blood of leukemic patients (26); and in target cells exposed to CTLs (29). It is now evident that target cell lysis involves more than one pathway and is mediated by a series of specific recognition events between effector and target (31). In the present study, CTLs appear to undergo this process when cultured in the presence of the specific epitope? The observed cytotoxicity could arise due to either binding of the epitope to an individual cell resulting in self recognition and self lysis or, more likely, due to lysis mediated by other members of the CTL population that treat the CTL presenting the epitope as a target. The latter proposal is supported by results that demonstrate that lysis of clone 13 exposed to the specific epitope was prevented when the incubation was carried out in flat-bottomed microtiter wells (when tested at 5×10^3 cells/well) in contrast to the high level of cytotoxicity in U-shaped wells. Presumably, the geometry of the U-shaped well provides for greater contact between the CTLs and suggests that the observed lysis involves cell-to-cell contact.

The results presented here are at variance with a number of reports that have demonstrated that CTLs are resistant to killing by intact effector cells (11). While this report was being considered for publication, three other groups have reported the phenomenon of T cell–T cell killing in murine cells (34–36). Pemberton et al. (34) showed that murine cytotoxic T cell clones underwent self lysis when incubated with specific influenza peptide. In that case, the cells had not undergone irreversible damage and had not been programmed for lysis. On the other hand, Walden and Eisen (36) have demonstrated that cloned murine CTLs undergo self destruction by exposure to their cognate peptides in a concentration-dependent manner. In contrast, the evidence provide in the present study suggests that cell-to-cell contact is important in this process rather than cell suicide. Self lysis of $CD4^+$ CTLs in the pres-

ence of specific peptide has also been demonstrated for human cells (18). In all of the above studies, while T cell–T cell killing was reported the mechanism of cell death was not investigated.

It is clear that CTL lysis is a complex process involving several different mechanisms as well as multiple mediators (37). The model described here makes it a useful approach to study the molecular events involved in CTL cytotoxicity. In this model, small defined peptides epitopes bound to HLA B8 and A24 alleles induce T cell–T cell killing in a CTL clone. The simplicity of this system may assist in the identification of new functional molecules involved in the CTL response and in defining the mechanism of apoptosis. Furthermore, these findings have important implications in the use of peptide epitopes for vaccination since these may bind to and be recognized by specific CTLs in vivo.

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