INDUCTION OF TARGET CELL DNA RELEASE BY THE CYTOTOXIC T LYMPHOCYTE GRANULE PROTEASE GRANZYME A

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The mechanism(s) by which CTL kill their targets remains controversial (1). Two major hypotheses have been advanced as models for target cell cytolysis. The granule exocytosis model, for which we (2) and others (3, 4) have provided evidence, proposes that preformed lytic mediators that exist in secretory granules are released by the cytotoxic lymphocyte upon stimulus of an effector cell receptor by the target cell. One important granule-associated lytic mediator that has been well characterized is cytolysin or perforin, a protein that induces lysis by formation of pores in the target cell membrane. An alternative model that has been proposed to account for lymphocyte cytotoxicity is the internal disintegration model (5), in which the CTL induces an autolytic mechanism in the target; i.e., the ultimate lytic mediators are derived from the target cell. This model derives from the observation that target cell DNA is released from the nucleus before lysis during lymphocyte-mediated cytotoxicity (5, 6). This phenomenon occurs during programmed cell death (7–11) as well as during cytotoxicity by both CTL and NK cells (5, 6, 12), but has not been observed when cells are killed by osmotic lysis or with complement. Recent reports have indicated the possibility that the disruption of ion gradients in the plasma membrane of some target cells, using valinomycin (13), staphylococcal α-toxin, or purified perforin (14), is sufficient for the induction of DNA release.

The discovery that CTL secretory granules contain large amounts of several unique serine proteases (15–19) has raised questions as to their function. While inhibition of cytotoxicity by serine esterase inhibitors has been interpreted as evidence that these enzymes are a necessary component of the cytolytic mechanism (20–23), the natural substrates for these enzymes remain unknown. By sequence analysis, CTL granule serine proteases appear to belong to a family of granule enzymes (granzymes) (19) that also include those found in granules of mast cells and neutrophils (24). There appears to be no firmly established physiological function for the granule proteases in any of these cells. We have recently shown that inhibition of granzyme A activity in CTL granules does not significantly reduce the lytic activity of these cells (25).

We have recently considered the possibility that several lymphocyte granule components can contribute to target cell damage, particularly DNA fragmentation and

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release (26). We have shown that granules, isolated from large granular lymphocytes (LGL)[1] and CTL, can mediate target cell DNA release from ^{125}I-UdR-labeled target cells. Purified cytolysin induces ^{51}Cr release, but not DNA release, from double-labeled target cells. While granules with inactivated cytolysin could induce neither ^{51}Cr nor ^{125}I-DNA release, addition of purified cytolysin to such granules restored the release of both ^{51}Cr and DNA (26). These data suggested that a mediator(s) of DNA release was present in the granules, but was active only if provided access to the nucleus of the target cell. We present evidence here that the prominent CTL granule protease granzyme A (alternatively referred to as Hannukah factor, T cell–specific protease 1, SE-1, or BLT-esterase) is a potent mediator of DNA breakdown in permeabilized target cells.

Materials and Methods

Cell Lines. The mouse CTL clone BM10.37 (H-2K^{bm10} anti-H-2K^{b}) has been described (27). Cloned CTL were maintained by weekly stimulation with irradiated B10 spleen cells and cultured in RPMI supplemented with 10% FCS and with 2% rat T cell Polyclone (Collaborative Research Inc., Bedford, Mass.) and 20 U/ml rIL-2. EL-4 lymphoma cells were cultured in RPMI/10% FCS and passed in fresh medium 1 d before use as target cells.

Inactivation of Granule Proteases in Intact CTL. Cloned CTL were harvested, washed in HBSS, and divided into six equal aliquots. Two groups each were initially treated with medium alone, 10 mM NH_4Cl, or 3 μg/ml monensin at 37°C for 30 min. PMSF (1 mM) or DMSO (solvent control) was then added to one of each of the three treatment groups for an additional 30 min at 37°C. The CTL were washed three times and added to wells with double-labeled EL-4 lymphoma target cells (^{51}Cr and ^{125}I-UdR; Amersham Corp., Arlington Heights, IL) at the indicated E/T ratios. Release of each of the isotopes was determined after 4 h at 37°C. ^{125}I-DNA release was measured after termination of the assay with Triton X-100 (0.3%).

Subcellular Fractionation of CTL. BM10.37 CTL were harvested, washed in HBSS, suspended in disruption buffer (0.25 M sucrose, 4 mM EGTA, 10 mM Hepes, pH 7.4), and subjected to nitrogen cavitation as previously described (28). Nuclei were removed by centrifugation at 500 *g* for 10 min. The cavitate was then layered onto a 52% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradient and subjected to centrifugation at 29,000 rpm for 26 min using a Beckman 50.2Ti rotor in a Beckman L5-75B ultra-centrifuge (Beckman Instruments, Fullerton, CA). Fractions (0.8 ml) were collected from the bottom of the tube and analyzed for cytolysin, BLT-esterase, and nuclear DNA-releases (NDR) activities (see below). Active fractions were pooled and frozen until further use.

Cytolysin, BLT-Esterase, and NDR Assays. Cytolysin activity was measured as previously described (29), using sheep erythrocytes as targets. 1 U/ml is defined as that concentration required to cause 50% lysis of sheep erythrocyte targets at 37°C in 10 min. BLT-esterase activity was measured using a modification (24) of the method of Pasternak and Eisen (15). 1 U/ml BLT-esterase activity is that concentration that produces an OD of 1.0 at 414 nm in 30 min at 25°C. NDR activity was measured using Triton X-100–permeabilized EL-4 target cells labeled with ^{125}I-UdR (26). Briefly, 5 × 10^6 EL-4 cells were harvested in logarithmic growth and incubated with 50 μCi of ^{125}I-UdR in a volume of 0.5 ml for 2 h at 37°C. Labeled cells were then washed and incubated for an additional hour at 37°C before use. Granules or column fractions were diluted in PBS containing 0.6% Triton X-100. An equal volume of target cells in HBSS containing 1 mg/ml BSA was then added to the diluted samples in a 96-well plate. The plates were incubated for 2 h at 37°C, after which they were centrifuged at 400 *g* to pellet the nuclei. The upper 100 μl of supernatant fluid was then harvested for counting in a Micromedic ME Plus gamma spectrometer (Micromedic Systems, Inc., Horsham, PA). 1 U/ml is defined as that concentration that causes 50% of maximal release of ^{125}I-DNA under these conditions.

[1] *Abbreviations used in this paper:* LGL, large granular lymphocytes; NDR, nuclear DNA-releasing.

Immunoabsorption Experiments. Antibodies were produced by immunization of rabbits with granzyme A that was purified by sequential gel filtration and heparin-agarose chromatography from LGL dense granules. This preparation migrated as a single band under reducing and nonreducing conditions by silver stain of SDS polyacrylamide gels. For immunoabsorption, 100 μl packed protein A-Sepharose was incubated for 1 h at room temperature with 100 μl of rabbit antisera. The beads were washed with PBS and resuspended with a soluble extract of CTL light granules for 1 h at room temperature. The beads were centrifuged and the resulting supernatant fluid was tested for NDR and BLT-esterase activities.

Purification of Cytolysin. Cytolysin was purified from pooled dense granules derived from Percol gradients of rat RNK tumor cell homogenates. Briefly, granules were solubilized by suspension in 2 M NaCl followed by centrifugation at 100,000 *g* for 90 min. The soluble extract was fractionated by gel filtration on an AcA54 column (Pharmacia Fine Chemicals) equilibrated in phosphate-buffered 2 M NaCl, 0.5 mM EGTA, 0.2% polyethylene glycol (PEG) pH 7.4, dialyzed exhaustively into PBS, and further purified by heparin-agarose chromatography. The latter column was equilibrated in PBS, 0.2% PEG, and the cytolysin activity was eluted with 0.45 M NaCl. This preparation was homogeneous by silver stain of SDS polyacrylamide gels and contained no detectable BLT-esterase activity.

Purification of BLT-Esterase. BLT-esterase and NDR activities were solubilized from pooled active fractions of Percoll gradients of CTL nitrogen cavitates by the addition of solid NaCl to a final concentration of 2 M. The mixture was rapidly freezed-thawed twice and subjected to centrifugation at 100,000 *g* for 60–90 min to remove insoluble material. This extract was loaded onto a 2.5 × 100 cm AcA54 column equilibrated with 10 mM Hepes, pH 7.0, 1 M NaCl, 0.2% PEG. 5-ml fractions were collected and assayed for BLT-esterase and NDR activities. Peak fractions were pooled and loaded directly onto an aprotinin-Sepharose column (1.0 × 18 cm), equilibrated in the same buffer with 0.2 M NaCl. aprotinin-Sepharose was prepared by coupling aprotinin (Trasylol; Boehringer Mannheim Biochemicals, Indianapolis, IN) to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals) according to the manufacturer's instructions. The aprotinin column was washed with equilibration buffer and activity was eluted with 0.1 M acetate buffer, pH 3.0, containing 0.2 M NaCl and 0.2% PEG. Eluted fractions were immediately neutralized with 1 M Tris-HCl, pH 7.5, assayed for activity, pooled, and dialyzed exhaustively against the original equilibration buffer. Protein concentrations were determined by the bicinchoninic acid procedure (BCA assay; Pierce Chemical Co., Rockford, IL). Purified material was stored at −70°C until further use.

Results

Inhibition of Target Cell DNA Release by Pretreatment of CTL with PMSF and Agents That Raise the pH of Intracellular Compartments. Previous studies have shown that treatment of CTL with the serine esterase inhibitor PMSF reduced their BLT-esterase activity significantly; this effect was markedly enhanced when PMSF was added in the presence of agents that are known to raise the pH of acidic intracellular compartments by different mechanisms, ammonium chloride (NH_4Cl) and monensin (24). Since PMSF is known to react optimally with serine esterases at neutral pH, the interpretation of this result is that the BLT-esterase activity in CTL resides in an acidic intracellular compartment. The ability of CTL to lyse targets was affected only slightly by pretreatment of the effectors with these reagents, consistent with a lack of involvement of granule serine proteases in lytic activity. We have extended these studies to ask if target cell DNA release is affected by the same treatments. CTL were pretreated with combinations of PMSF and NH_4Cl or monensin, washed, and incubated with EL-4 lymphoma target cells that had been labeled with ^{51}Cr and ^{125}I-UdR (Fig. 1). As previously found (25), none of the treatments had a substantial effect on the capacity of these effectors to lyse target cells, as measured by ^{51}Cr release (Fig. 1, *top*). However, pretreatment with the combination of PMSF with either NH_4Cl or monensin resulted in a significant decrease in the capacity

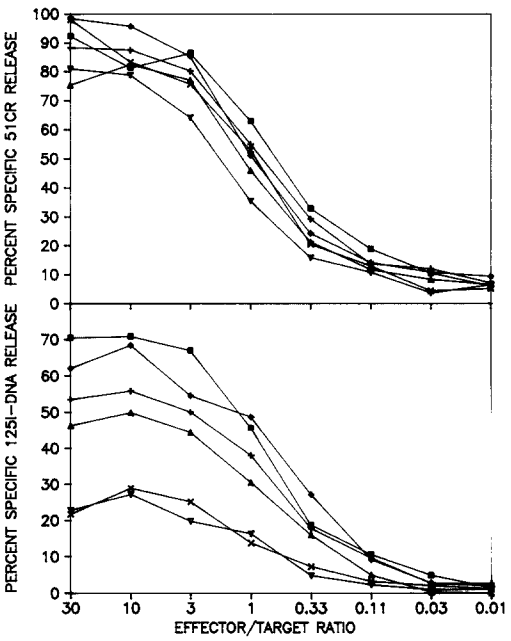

FIGURE 1. Inactivation of granule proteases of intact CTL inhibits target cell ^{125}I-DNA release without affecting ^{51}Cr release. CTL (clone 37) were pretreated as described in Materials and Methods and incubated with double-labeled EL-4 lymphoma target cells. After 4 h, ^{51}Cr release (*top*) and ^{125}I-DNA release (*bottom*) were determined. ^{125}I-DNA release was measured by terminating the assay with Triton X-100 (final concentration, 0.3%) to release DNA trapped in any membranous compartment. Treatments of CTL were as follows: control (*squares*), NH_4Cl (*diamonds*), monensin (*triangles*), PMSF (+), NH_4Cl and PMSF (x), monensin and PMSF (inverted triangles).

of the CTL to induce target cell DNA release (Fig. 1, *bottom*). These results imply that a serine protease residing in an acidic intracellular compartment of the CTL is involved in target cell DNA release. Therefore, further studies were designed to identify potential mediators of this activity, and to assess the potential role of granule proteases in DNA release.

Subcellular Compartmentalization of BLT-Esterase and NDR Activities. We have reported that both LGL and CTL contain an activity, which has been termed nuclear DNA-releasing activity, that is present in distinct subcellular compartments after fractionation of effector cell homogenates over Percoll gradients (26). This NDR activity is defined by the induction of release of ^{125}I-DNA from Triton X-100-permeabilized target cells (see Materials and Methods). Homogenates of cloned CTL were examined to identify compartments within the cells that contained the nuclear DNA-releasing activity and to assess its association with BLT-esterase activity. After a low speed centrifugation to remove nuclei, the homogenates were fractionated by density over a 52% self-forming Percoll gradient, as described in Materials and Methods. In Fig. 2, profiles from representative gradients are shown for NDR and BLT-esterase activities. The majority of both activities are present in "light granule" fractions, 19–26, which also contained most of the membranous material in these gradients, with a much smaller peak associated with previously characterized dense granules (fractions 1–5, which contain cytolysin activity) (28, 29). These activities always comigrated in Percoll gradients, although the ratio of dense to light granular material varied among preparations.

Effects on Activities by Esterase Inhibitors. Previous experiments in our laboratory had demonstrated that LGL dense granule-derived NDR activity was inhibited by

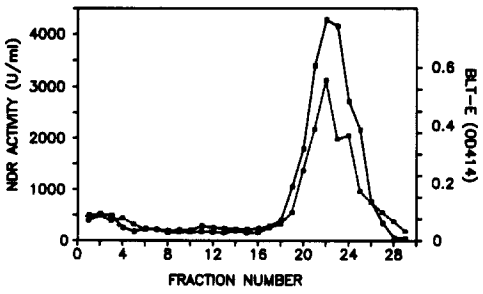

FIGURE 2. Subcellular fractionation of NDR and BLT-esterase activities in CTL. Clone 37 (bm10 anti-B10) CTL were subjected to nitrogen cavitation and the cavitate was fractionated over a 52% Percoll gradient as described in Materials and Methods. Each fraction (0.8 ml) was analyzed for NDR (*solid line*) and BLT-esterase (*dashed line*) activities after freeze-thawing. Active fractions were pooled and stored for further purification.

serine esterase-reactive agents such as diisopropyl fluorophosphate (DFP) and PMSF (26). NDR activity from the light granules of cloned CTL is also sensitive to micromolar concentrations of PMSF (data not shown). Inhibition profiles of CTL light granule-derived NDR and BLT-esterase activities were determined using two additional esterase inhibitors, benzamidine and aprotinin. Virtually identical profiles were derived with benzamidine, with 50% inhibition occuring at 0.79 and 0.71 mM for BLT-esterase and NDR activities, respectively (Fig. 3, *top*). However, when the macromolecular inhibitor aprotinin was used, 50% inhibition occurred at 0.6 μg/ml for NDR activity and at 7.2 μg/ml for BLT-esterase activity (Fig. 3, *bottom*).

Immunoabsorption of NDR Activity by Anti-BLT-Esterase Antibodies. To detect any immunological relationship between NDR and BLT-esterase activities, rabbit antisera raised against BLT-esterase purified from rat LGL tumor dense granules were used

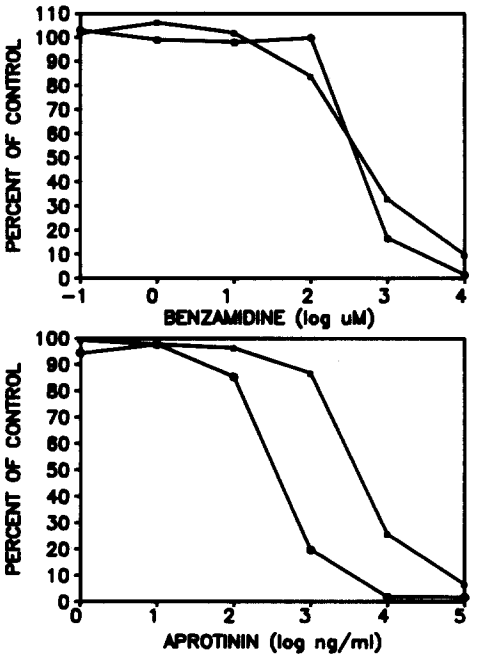

FIGURE 3. Effect of esterase inhibitors on NDR and BLT-esterase activities. Pooled light membrane fractions (see Fig. 2) were incubated with benzamidine (*top*) or aprotinin (*bottom*) at the indicated concentrations and assayed for NDR (*circles*) and BLT-esterase (*squares*) activities. The control value for NDR activity was release of 17,433 ± 386 cpm of ^{125}I-DNA. The control value for BLT-esterase activity was an OD_{414} of 0.930 ± 0.023.

in immunoabsorption experiments. Protein A-Sepharose beads were incubated with rabbit antisera against BLT-esterase or KLH, washed, and incubated with soluble extracts from CTL light granules. The supernatant, after centrifugation of the beads, was then assayed for NDR and BLT-esterase activities. Both activities were completely absorbed by the anti-BLT-esterase but not by the control antibody (Table I).

Purification of NDR/BLT-Esterase Activities. Since the above results suggested that BLT-esterase and NDR activity could be mediated by the same enzyme, the activities were purified. As a first step, soluble extracts of CTL light granules were fractionated over an AcA54 gel filtration column. The activities comigrated on this column in a single peak at ~60,000 daltons (Fig. 4). The peak fractions from this column were pooled and immediately applied to an aprotinin-Sepharose column in buffer containing 1 M NaCl. The column was washed in 0.2 M NaCl and eluted with acetate buffer at pH 3. Again, the activities comigrated and eluted in a single peak with the acid wash (Fig. 5). The effective purification of these activities is summarized in Table II, which shows little change in the ratio of the two activities throughout the purification. The affinity-purified material was analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 6). Under the latter, a single prominent band migrating at ~60,000 daltons was revealed by Coomassie blue staining. Upon reduction, the band shifted to 30,000 daltons. These properties are identical to those previously reported for granzyme A (19, 30); such dimeric structure is unique among known serine proteases. Further evidence that NDR activity was mediated by the granzyme A BLT-esterase and not by an undetected contaminant was provided by the demonstration that NDR activity remains associated with BLT-esterase during reduction and alkylation. The purified esterase was reduced and alkylated (31) and migration of the two activities was measured after gel filtration (Fig. 7). The control (treated as the experimental without the inclusion of DTT) esterase/NDR activities migrated as before as a single peak at 60,000 daltons. The esterase/NDR activities remained associated after reduction and alkylation, but at the expected monomer position of 30,000 daltons.

Release of DNA from Target Cells Treated with Purified Cytolysin and BLT-Esterase. It was critical to assess the activity of purified BLT-esterase on target cells permeabi-

TABLE I

Immunoabsorption of NDR Activity by Anti-BLT-Esterase Antibodies

	Remaining activity	
	NDR (% release)	BLT-E
		OD_{414}
Control AB	75.3	0.553
Anti-granzyme A	0.6	0.003

A soluble extract prepared by suspension of CTL light granules in 2 M NaCl was incubated with protein A-Sepharose beads coated with rabbit antisera made against granzyme A or a control antiserum against keyhole limpet hemocyanin. The beads were centrifuged and the resulting supernatant fluid was tested for NDR and BLT-esterase activities. NDR activity is expressed as percent specific ^{125}I-DNA release after 2 h at 37°C, and BLT-esterase activity is expressed as optical density at 414 nm.

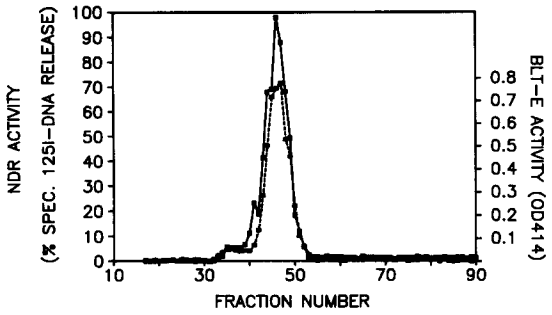

FIGURE 4. Gel filtration of solubilized light membranous material. Pooled light membrane fractions were solubilized by the addition of 2 M NaCl and centrifugation at 100,000 *g* for 90 min. The soluble material was applied to a 2.5 × 100 cm AcA54 gel filtration column equilibrated with 10 mM Hepes, pH 7.0, 1 M NaCl, and 0.2% PEG (see Materials and Methods). 5-ml fractions were collected and assayed for NDR (*solid line*) and BLT-esterase (*dashed line*) activities. The column was calibrated with gel filtration standards BSA, ovalbumin, chymotrypsinogen A, and ribonuclease A (Pharmacia Fine Chemicals).

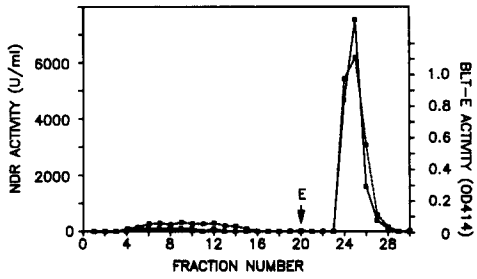

FIGURE 5. Aprotinin-Sepharose chromatography of AcA54 peak fractions. Pooled active fractions from an AcA54 preparative run were added directly to a 1 × 18 cm aprotinin-Sepharose column, which was washed with 10 mM Hepes, 0.2 M NaCl, and 0.2% PEG before elution (at fraction 20) with 0.1 M acetic acid, pH 3.0. Eluted fractions were immediately neutralized with 1 M Tris-HCl, pH 7.5, and tested for NDR (*solid line*) and BLT-esterase (*dashed line*) activities.

TABLE II

Purification of NDR/BLT-Esterase Activities from CTL

Step*	Specific activity		
	NDR‡	BLT-E§	NDR/BLT-E‖
Light granules	684 (1.0)	744 (1.0)	0.919
1 M NaCl extract	626 (0.9)	837 (1.1)	0.748
AcA54 peak pool	1,563 (2.3)	2,731 (3.7)	0.572
Aprotinin-Sepharose eluate	13,871 (20.3)	20,694 (27.8)	0.670

* See Materials and Methods for purification steps.

‡ NDR activity in units per milligram; numbers in parentheses are -fold purification.

§ BLT-esterase activity in units per milligram.

‖ Ratio of NDR to BLT-esterase activity at each step.

lized with the pore-forming granule protein cytolysin instead of detergent. EL-4 target cells were labeled with ^{51}Cr and ^{125}I-UdR, washed and incubated in the presence of purified cytolysin (from LGL granules, reference 29) and/or purified BLT-esterase. In Fig. 8, EL-4 lymphoma target cells were treated for 4 h with dilutions of purified cytolysin alone, and specific release of ^{51}Cr and ^{125}I-DNA was assessed. ^{125}I-DNA

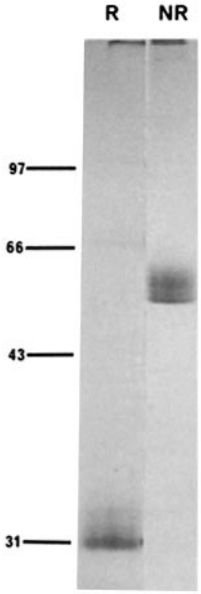

FIGURE 6. SDS-PAGE of aprotinin-Sepharose-purified NDR and BLT-esterase activity. 5 Mg (BCA assay) of affinity-purified protein was resolved on a 10% polyacrylamide gel under reducing (*R*) and nonreducing (*NR*) conditions and the gel was stained with Coomassie blue R-250. Molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were as follows: phosphorylase b (97,400), BSA (66,200), ovalbumin (42,699), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

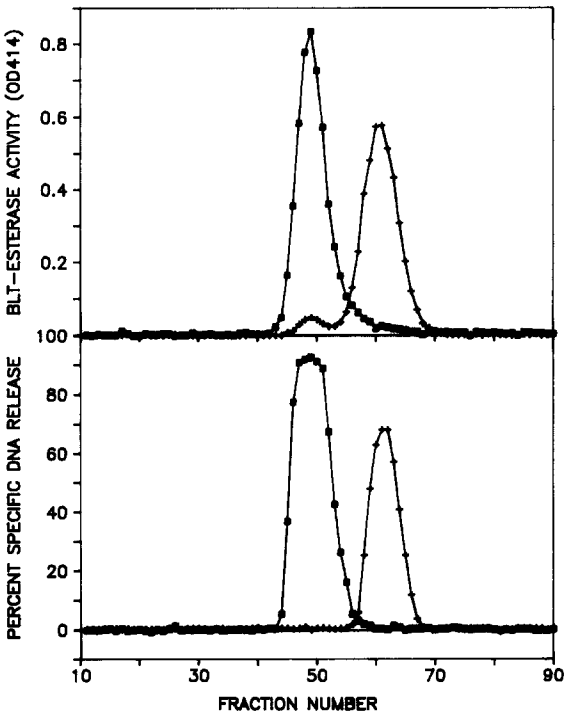

FIGURE 7. Reduction and alkylation of esterase/NDR activities. 60 μg (1 ml) of purified esterase was incubated in the presence of 5 mM benzamidine (to protect the active site) and 1 mM dithiothreitol for 1 h at 37°C (*crosses*). A control sample was treated with benzamidine only (*squares*). Both samples were then reacted with 4 mM iodoacetamide for 1.5 h at 37°C, and then dialyzed exhaustively against AcA54 running buffer (see Fig. 4). Residual esterase activity in the reduced sample was ~80% of the control. The samples were fractionated over an AcA54 gel filtration column as in Fig. 4, and assayed for BLT-esterase (*top*) and NDR (*bottom*) activities.

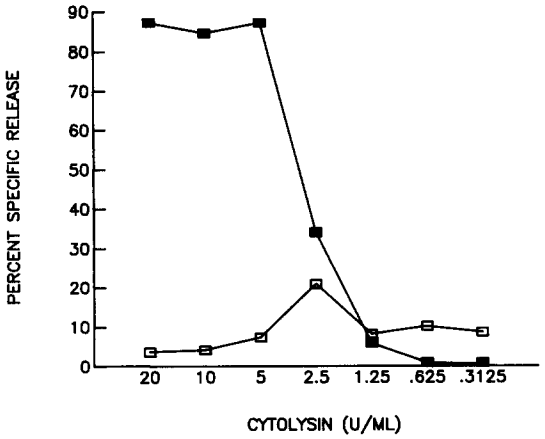

FIGURE 8. Purified cytolysin alone induces minimal DNA release. EL-4 cells were labeled with ^{51}Cr or ^{125}I-UdR and exposed to dilutions of purified LGL granule cytolysin (U/ml as assayed on RBC) for 4 h at 37°C. At harvest, the cells were pelleted and the supernatant fluid was taken directly for determination of ^{51}Cr release (*filled squares*). For determination of ^{125}I-DNA release (*open squares*), Triton X-100 was added to a final concentration of 0.3% immediately before centrifugation and harvest.

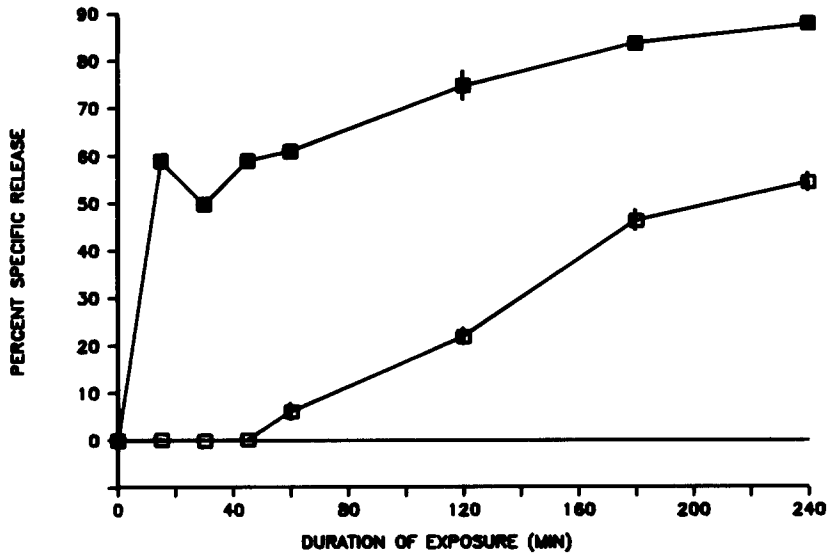

FIGURE 9. Cytotoxicity mediated by purified cytolysin and BLT-esterase. EL-4 target cells were labeled with ^{51}Cr or ^{125}I-UdR and exposed to BLT-esterase (310 ng/ml) in the presence of purified cytolysin (20 U/ml as assayed on RBC) for the indicated periods of time. Specific release of each isotope (^{51}Cr, *filled squares*; ^{125}I-DNA, *open squares*) was measured as in Fig. 8.

release was minimal at fully lytic concentrations of cytolysin, but a detectable amount (20%) was released at marginally lytic concentrations. When purified granzyme A was added to target cells in the presence of purified cytolysin at a concentration where no DNA release was observed with cytolysin alone (1/200), significant release of ^{125}I-DNA occurred after 1 h and rose gradually over 4 h (Fig. 9). However, DNA release required addition of detergent at harvest, indicating that it was still trapped in some membranous compartment in the target cells. ^{51}Cr release occurred within the first 15 min of incubation, and increased gradually over 4 h under the same conditions.

Discussion

The studies described here provide clear evidence for a role for the CTL granule protease granzyme A in target cell nuclear DNA breakdown. Experiments with PMSF treatment of CTL indicate that their capacity to cause target cell DNA release is dependent on a serine protease activity that resides in a low pH subcellular compartment (such as a secretory granule). In support of this functional result, we have shown that the nuclear DNA-releasing activity present in CTL is mediated by the T cell–specific granule protease that has previously been identified as granzyme A (19, 30). These results are significant both because they identify a potential mechanism through which CTL can mediate target cell DNA release by way of a granule exocytosis pathway, and because they indicate the existence of functionally important substrates for granzyme A.

The functional data presented in Fig. 1 suggest that a granule serine protease(s) is involved in the release of target cell DNA during CTL-mediated cytotoxicity. We have previously shown that PMSF treatment of CTL substantially inhibits BLT-esterase activity; this inhibition is virtually complete when PMSF is added in the presence of NH_4Cl or monensin, agents, which raise the pH of intracellular compartments by independent mechanisms (24). As seen in Fig. 1 (*top*), the release of ^{51}Cr from target cells is only marginally affected, suggesting that BLT-esterase activity is apparently not required for the lytic activity of these effectors. However, the ability of these same effector cells to induce target cell DNA release was significantly reduced upon treatment with these agents. The synergistic effect of PMSF and NH_4Cl or monensin on target cell DNA release was substantial, implying that the functionally relevant molecule(s) affected resides in an acidic intracellular compartment. These results, combined with the fact that CTL-derived NDR activity was also inhibited by serine esterase–reactive agents, led us to investigate the relationship between BTL-esterase activity and NDR activity present in CTL.

The molecular identity of NDR and BLT-esterase activities was demonstrated by physicochemical and immunological results. First, the two activities reside in the same subcellular compartments, as shown by density fractionation over Percoll gradients of CTL homogenates (Fig. 2). The secretory capacity of these compartments is difficult to demonstrate directly. However, BLT-esterase has been widely used as a secretory marker for many CTL studies. Second, NDR activity and BLT-esterase comigrate in single peaks upon gel filtration and an affinity column (aprotinin-Sepharose). Third, the affinity-purified material is composed predominantly of a single protein that migrates at ~60,000 daltons on SDS-PAGE and, upon reduction, at 30,000 daltons. This behavior has already been described for purified granzyme A, and distinguishes this enzyme from all of the other described granule proteases (19, 30). Fourth, the two activities show similar inhibition profiles with benzamidine and aprotinin. Aprotinin was an order of magnitude more effective at inhibition of NDR than of BLT-esterase activity. One likely explanation for this is that the aprotinin peptide does not compete as effectively for the BLT substrate as for the unknown NDR substrate. The common inhibitor susceptibilities, however, strengthen the likelihood that the two activities are mediated by an identical enzyme. An additional compelling argument for this relationship is the immunological crossreactivity of these activities. Both are specifically absorbed by a rabbit antiserum made against granzyme A purified by a different procedure from rat LGL

granules. Finally, when the esterase was reduced and alkylated, the activities continued to be associated when refractionated over a gel filtration column. Taken together, these data indicate the identity of NDR and BLT-esterase activities, and imply a role for granzyme A in target cell DNA release.

Others have reported that DNA release or fragmentation can be induced by isolated CTL granules (32–34). However, in two recent reports, the authors have argued that there is no DNA-releasing activity in CTL granules (35, 36). In both of these studies, however, the granules were used at concentrations at which DNA release was not likely to be observed. We have observed elsewhere (Munger, W. E., C. W. Reynolds, and P. A. Henkart, manuscript in preparation) that, with LGL-derived dense granules, DNA release from detergent-permeabilized nuclei is detected only at concentrations greater than that required for maximal ^{51}Cr release. We would predict, based on the data presented here, that the extent of DNA release induced by granules would depend upon (*a*) the susceptibility of the target cell to DNA degradation induced by ionic leakage, and (*b*) the ratio of cytolysin to granzyme A in the secretory granules. Nevertheless, we would argue that DNA release observed when granule extracts are added to cell suspensions is inefficient with respect to ^{51}Cr release compared with its delivery by intact CTL. Our reasoning is that, when added in solution, the cytolysin, due to its amphiphilic nature in the presence of calcium, should partition efficiently into the target cell plasma membrane. Granzyme A, however, will not be subject to such influences on partitioning to its relevant substrate(s) and suffers a dilutional effect when added in solution. During killing by intact CTL, this inefficiency is overcome by the polarized secretory mechanism that delivers a high local concentration of the active mediator(s) to the appropriate target sites (e.g., the nucleus) within the cell.

Recent reports have indicated that DNA release can occur from various target cells treated with agents that disrupt membrane ion gradients, including valinomycin (13), staphylococcal α-toxin, and murine perforin (14). We observed small but detectable amounts of DNA release in EL-4 targets treated with purified cytolysin, but only at minimally lytic concentrations (Fig. 8). At higher, fully lytic concentrations, no DNA release was observed. The discrepancy over cytolysin/perforin-induced DNA release may be due to differences in target cells or assay conditions; the latter should take into consideration the active concentration of the pore-forming protein. Further experiments should clarify the role of cytolysin/perforin in target cell DNA release.

Since the premise of our approach was that detergent permeabilization of target nuclei could be used to mimic that provided by cytolysin released from CTL granules, it was important to demonstrate that granzyme A can mediate DNA release from cells permeabilized with cytolysin. Fig. 9 clearly demonstrates that granzyme A has access to its substrate when cytolysin is present. However, the DNA release was not detectable unless the cells were further solublized by terminating the assay with Triton X-100, in a manner analogous to that of Russell and Dobos (37). This suggests that, although granzyme A can enter cytolysin-treated cells, DNA fragments remain trapped in some membrane-bound compartment after DNA breakdown has occurred.

The rate of target nuclear DNA release by granzyme A (Fig. 9) is considerably slower than the rate of ^{51}Cr release by cytolysin or by CTL. It can thus be argued that we have not reproduced the findings of DNA breakdown preceding lysis which

gave rise to the "internal disintegration" model (5). Current studies in our laboratory, aimed at dissecting the molecular pathway of nuclear DNA breakdown by granzyme A, show that the rate of granzyme A induced nuclear DNA breakdown can be increased by altering the assay conditions. We speculate that with the high local granzyme A concentrations produced during granule exocytosis, target DNA breakdown may occur rapidly. In any case, target cell DNA breakdown induced by cytolysin and granzyme A from CTL, and involving existing target cell components, would not require de novo gene expression. This model would explain the resistance of CTL-induced target cell DNA breakdown to inhibitors of RNA and protein synthesis (12), which is in strong contrast to the results in systems undergoing programmed cell death (7–11). Such target RNA and protein synthesis is clearly not required in our experimental system, where lysis by detergent or cytolysin occurs very rapidly.

The mechanism by which granzyme A mediates nuclear DNA release is still unclear. There is no reason to expect that granzyme A has any DNase activity. Two models can be proposed as possible means by which DNA release is mediated. (*a*) Granzyme A acts upon nuclear matrix proteins to ultimately allow the accessibility of cytosolic nucleases to the DNA and/or simply allow its release from the nucleus; and (*b*) granzyme A acts upon a cytosolic substrate and activates this substrate in a zymogen-like manner to induce DNA fragmentation. Experiments in progress to distinguish between these possibilities favor the former model.

Summary

The rapid breakdown of target cell DNA during CTL-mediated lysis has been difficult to explain by the granule exocytosis model of cytotoxicity. The involvement of CTL granule proteases in this process was strongly suggested by experiments in which CTL were pretreated with the serine protease inhibitor PMSF, in combination with agents that raise the pH of acidic intracellular compartments. While PMSF pretreatment alone had little effect on target lysis or DNA breakdown, the combination of PMSF and NH_4Cl or monensin profoundly reduced target cell DNA release, while little effect was observed on target lysis, as measured by ^{51}Cr release. CTL granule extracts cause release of ^{125}I-DNA from detergent-permeabilized cells. This nuclear DNA-releasing (NDR) activity is inhibited by serine esterase inhibitors that also inhibit the granule BLT-esterase activity, and is specifically immunoabsorbed by antibodies to the CTL granule protease granzyme A. The NDR activity comigrates with BLT-esterase activity during subcellular fractionation, solubilization, gel filtration, and aprotinin-Sepharose affinity chromatography. SDS-PAGE analysis of the affinity-purified product indicates a molecular mass of 60,000 daltons under nonreducing conditions, which moves to 30,000 daltons upon reduction, consistent with previously reported behavior of granzyme A. When the purified material was reduced and alkylated, both esterase and NDR activities comigrated at 30,000 daltons upon gel filtration. Although fully lytic concentrations of purified LGL granule cytolysin alone failed to induce target cell DNA release, a combination of purified granzyme A and the cytolysin induces substantial DNA release.

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