Synergistic Roles of Granzymes A and B in Mediating Target Cell Death by Rat Basophilic Leukemia Mast Cell Tumors Also Expressing Cytolysin/Perforin

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Summary

We have studied the cytotoxic activity of rat basophilic leukemia (RBL) cells transfected with cDNAs for the cytotoxic T lymphocyte (CTL) granule components, cytolysin (perforin), granzyme A, and granzyme B. With red cell targets, cytolysin expression conferred potent hemolytic activity, which was not influenced by coexpression of granzymes. With tumor targets, RBL cells expressing cytolysin alone were weakly cytotoxic, but both cytolytic and nucleolytic activity were enhanced by coexpression of granzyme B. RBL cells expressing all three CTL granule components showed still higher cytotoxic activities, with apoptotic target death. Analysis of the cytotoxic activity of individual transfectant clones showed that cytolytic and nucleolytic activity correlated with granzyme expression but was independent of cytolysin expression within the range examined. A synergism between granzymes A and B was apparent when the triple transfectant was compared with RBL cells expressing cytolysin and one granzyme. These data implicate granzymes as the major mediators of tumor target damage by cytotoxic lymphocytes.

The granule exocytosis model for lymphocyte cytotoxicity postulates that target cell recognition triggers a secretory process in which preformed cytoplasmic granules fuse with the effector cell plasma membrane in the vicinity of the bound target cell, releasing cytotoxic molecules into a junctional region created by cell–cell adhesion (1). The initial cytotoxic mediator identified in cytotoxic lymphocyte granules was cytolysin or perforin, a 70-kD protein that undergoes a calcium-dependent conformational change and inserts into membranes, forming large functional pores with corresponding structures visible by negative stain electron microscopy (2). There is considerable evidence that this pathway is the major effector mechanism for cytotoxic lymphocytes, including the major defects in antiviral and allogeneic CTL and NK activity in mice whose perforin/cytolysin gene was rendered nonfunctional (3).

The granule exocytosis pathway with cytolysin as the sole lethal mediator is deficient in two significant respects. First, membrane damage does not account for the observed average delay of 1–2 h required for target death after delivery of the lethal hit (4). Second, it does not account for the apoptotic morphology of most dying target cells and the DNA breakdown ("nucleolytic activity"), which typically accompanies apoptotic morphology (5). We have sought other granule-delivered mediators that could cause secondary lethal damage after entry into target cells whose membranes were permeabilized (6), focusing on the serine proteases termed granzymes.

Although seven granzymes are expressed by cloned murine CTL lines maintained in IL-2 (7), only granzymes A and B are expressed by the potent in vivo PEL CTL (8). These two enzymes have markedly different enzymatic specificities, with granzyme A being a "tryptase," that is, cleaving at Arg and Lys residues, while granzyme B prefers Asp at its cleavage site. Granules from NK cells and human CTL also express multiple granzymes, including two with nominally similar specificities to granzymes A and B (9, 10).

Several lines of evidence argue that granzymes mediate target cell damage during lymphocyte-mediated cytotoxicity. Both granzyme A– and B–like proteases have the ability to trigger apoptotic nuclear damage and cell death when introduced into cells by detergents or cytolysin (11). More directly, mice whose granzyme B gene was inactivated produced CTL with defective cytolytic and nucleolytic activity (12). Another approach to testing the functional role of granzymes in cytotoxicity has been their controlled expression along with cytolysin in the rat basophilic leukemia (RBL)[1] cell line, which has a well-defined regulated secretory pathway controlled by the IgE Fc receptor. When transfected with the cDNA for cytolysin, RBL expressed the protein in secretory granules, and these RBL-cy acquired a potent IgE–anti-

[1] *Abbreviations used in this paper:* BAAD-pNA, Boc-Ala-Ala-Asp-pNA; BLT, Cbz-Lys-SBz; RBL, rat basophilic leukemia.

hapten–dependent lytic activity against haptenated red blood cells (13). However, these cells had only a modest cytolytic ability on tumor targets, without accompanying apoptotic nuclear damage (14). When granzyme A was coexpresssed along with cytolysin in RBL, these double transfectants killed tumor cells considerably more efficiently with apoptotic nuclear damage, but there was no additional lytic potency against red cell targets (15).

The above-mentioned studies provide strong evidence that granzymes can trigger an "internal disintegration" pathway of target death after their entry into the cell. In our experiments, we have tested the abilities of both granzymes A and B to contribute to cytotoxic activity on tumor targets when expressed in RBL cells along with cytolysin. We find that expression of granzyme B along with cytolysin enhances cytotoxic activity compared to RBL cells expressing cytolysin only (as was found for granzyme A), and RBL cells expressing both granzymes as well as cytolysin display a potent cytotoxic activity comparable with cloned CTL. Granzymes A and B appear to interact synergistically to provide enhanced cytolytic and nucleolytic activity. Analysis of individual transfectant clones shows that granzyme expression controls their cytotoxic activity.

Materials and Methods

Cells and Chemicals. RBL-2H3 cells (a gift of Dr. Reuben Siriganian, National Institutes of Health [NIH], Bethesda, MD) were maintained in DME containing 15% FCS, 4 mM L-glutamine, and 100 μg/ml each of penicillin and streptomycin, as described previously (13). Cloned murine CTL line TIM448, specific for H-$2K^b$ (provided by Terri Munitz and Alfred Singer, NIH) was used as a positive control for cytotoxic activity and granule protein expression. Affinity-purified anti-DNP IgE (16) was a gift from Juan Rivera (NIH), and covalently cross-linked heteroconjugate antibody 2C11xα-DNP (17) was kindly provided by Dr. David Segal (NIH). The granzyme B substrate Boc-Ala-Ala-Asp-pNA was purchased from Bachem (King of Prussia, PA).

RBL Transfection and Selection. For RBL transfection with granzyme B cDNA, a granzyme B expression vector was constructed by use of the mouse granzyme B (CTLA1) cDNA in PUC9 (a gift from Dr. Pierre Golstein, Centre d'Immunologie Marseille-Luminy, Marseille, France). The granzyme B cDNA was excised with BamHI and SmaI digestions and recloned into the pSVL expression vector (Pharmacia Inc., Piscataway, NJ) via blunt and BamHI ligations. Cytolysin and granzyme A cDNAs were cloned into the pCDL/SRα expression vector as described (15). Transfection was by electroporation (500 mV, 260 μF) by use of a genepulser (Bio-Rad Laboratories, Richmond, CA) as described (14). For a single gene transfection with 10^7 RBL cells, 30 μg of expression vector was mixed with 1 μg of pSV3-neo, while double transfectants with cytolysin and one granzyme were made with 15 μg of each expression vector and 1 μg pSV3-neo. Selection with geneticin (GIBCO BRL, Gaithersburg, MD) was begun 2 d after transfection, and the antibiotic resistant clones that grew out were tested for expression. For these screening purposes, cytolysin expression was assayed by cytotoxicity with red cell targets, granzyme A expression by Cbz-Lys-SBz (BLT)–esterase assay, and granzyme B by reverse transcription PCR (18) (sense primer, 5'GATCCTCCTGCTACTGCTGACCTTG3', and antisense primer, 5'GGCCTTACTCTTCAGCTTTAGCAGC3').

RBL-cy-gza-gzb were produced by secondary transfection of the RBL-cy-gzb clone 501 as described above, with 1 μg of the selection vector pSV2/bsr (Funakoshi, Tokyo, Japan) (19). This provides resistance to the antibiotic blasticidin S (ICN, Aurora, Ohio), which was used at 10 μg/ml for selection after 3 d.

Expression of Transfected Granule Components. Granzyme A protein expression was assessed using the BLT–esterase assay (20) on cell extracts in 0.1% Triton X-100. Similarly, granzyme B protein expression was measured on an extract of 2 $\times$ 10^6 cells by following hydrolysis of Boc-Ala-Ala-Asp-pNA (21) after a 24-h incubation.

Granule protein mRNA expression was monitored by Northern blot analysis of the transfectant clones. Total RNA was extracted via the guanidinium thiocyanate-phenol-chloroform procedure (22). For Northern blot analysis, 20 μg of total cellular RNA from each sample was electrophoresed on a 1% formaldehyde gel and transferred to a membrane (Nytran; Schleicher & Schuell, Keene, NH). Full length mouse cDNAs of cytolysin, granzyme A, granzyme B, and β-actin (Clonetech, Palo Alto, CA) were cut out from each expression vector and labeled with α-[^{32}P]dCTP (Dupont, Boston, MA) by use of a random hexamer oligonucleotide priming kit (Pharmacia Inc.). Labeled probe was hybridized with the filters at 42°C in formamide-containing hybridization solution and washed with 2× SSC, 0.1% SDS, at 60°C before analysis. After analysis, bound probe was removed by washing with 5 mM Tris, pH 8.0, 0.2 mM EDTA, 0.05% pyrophosphate, 0.1× Denhart's for 2 h at 64°C, and the blots were reexposed to other probes. For quantitation, the membrane was exposed to phosphor screens, and specific radioactivity was quantitated by phosphorimager (Molecular Dynamics, Sunnyvale, CA). Granule component mRNA levels were normalized to those of the housekeeping gene β-actin.

To allow for easy comparison, expression of each of the granule proteins and mRNAs in the murine CTL line was measured, and the values in the RBL transfectants were reported as a percentage of this CTL level.

Cytotoxicity Experiments. For target cells, we used fresh human RBC, mouse thymoma line EL4, mouse mastocytoma line P815, human acute T cell leukemia line Jurkat, and mouse fibroblast line L929. Target cell labeling by ^{51}Cr and ^{125}I-UDR for DNA release assays were performed as described in detail previously (13). In all cases, target cells were TNP modified by use of trinitrobenzene sulfonate, CTL recognition was via the antihapten $\times$ anti-CD3 heteroconjugate, and RBL recognition was via IgE anti-DNP, as previously described (13). Experiments were carried out in 200 μl vol in 96-well round-bottom microtiter plates with 10^4 target cells and varying numbers of effector cells per well. After 4 h of incubation, 100 μl of supernatant was harvested from each well, followed by addition of 100 μl of 0.2% Triton X-100 in PBS. After mixing and centrifugation, a second 100 μl of supernatant was harvested from each well. Triplicate data points were used and the released ^{125}I-DNA combined from each harvest for each well. Each marker release was calculated by the following formula, which includes a correction for spontaneous release from target cells alone: Corrected percent marker release = $(cpm_{sample} - cpm_{spontaneous})/(cpm_{total} - cpm_{spontaneous})$.

Data relating cytotoxicity and granule component expression were entered into CricketGraph (Computer Associates, San Diego, CA) and analyzed by its linear regression analysis program.

Microscopic Analysis for Apoptotic Nuclei in Target Cells. To identify apoptotic nuclear morphology in target cells incubated with RBL transfectants, 1–2 $\times$ 10^6 target cells were prelabeled with the lipid probe $DiIC_{16}$ (Molecular Probes, Inc., Eugene, OR) by incubation with a 10 μM solution for 20 min at 37°C, followed by

washing. Target cells were then incubated with effectors under the standard conditions in microtiter plates for four hours. Triplicate wells were combined at harvest, pelleted cells were labeled with 8 μg/ml of the DNA stain HOECHST 33342 (Sigma Chemical Co., St. Louis, MO) for 15 min, propidium iodide was added (10 μg/ml final concentration), and the cells were examined in a fluorescence microscope with a HOECHST filter set. Target cells were identified by their red membrane stain, and their nuclear morphology was assessed as normal or apoptotic.

Results

Characterization of mRNA and Protein Expression in RBL-cy-gzb Transfectant Clones. Following a generally similar strategy to that previously used with granzyme A, RBL cells were transfected by use of expression vectors with granzyme B cDNA, or with both granzyme B and cytolysin cDNAs, and individual transfectant clones were isolated and characterized. Fig. 1 *A* shows Northern blots measuring expression of the CTL granule mRNAs cytolysin, granzyme A, granzyme B, as well as the housekeeping gene β-actin. All seven RBL-cy-gzb transfectant clones express cytolysin and granzyme B mRNA, but no granzyme A mRNA, as seen by a comparison with the cloned CTL control. Fig. 1 *B* presents quantitative data derived from these blots, as well as the expression of granzyme B enzymatic activity (hydrolysis of its enzymatic substrate Boc-Ala-Ala-Asp-pNA (BAAD-pNA) measured on extracts of these clones. The parental RBL line expresses a negligible level of cytolysin or either granzyme. Five of the seven RBL-cy-gzb clones studied in detail express higher levels of cytolysin mRNA than the reference cloned CTL line used for comparison, while the total range of expression levels of this mRNA was approximately 10-fold. In contrast, however, granzyme B mRNA as well as enzymatic activity in all seven clones was lower than that of the CTL clone. Expression levels of cytolysin and granzyme B mRNAs were not correlated in these RBL-cy-gzb transfectant clones, although as expected, granzyme B mRNA levels correlated well with its enzymatic expression. The clones with the highest granzyme B expression had 40–50% of the levels expressed in the reference CTL clone.

Cytotoxic Activity of RBL-cy-gzb Transfectant Clones. The cytotoxic activity of RBL-cy-gzb, RBL-cy, and RBL-gzb clones was assessed on both red cell and tumor targets by use of standard 4-h assays. Fig. 2 shows cytotoxicity results with several of the most active clones of each type tested on two tumor targets. In agreement with our previous results (15), RBL-cy showed significant but weak cytolytic activity against such targets, as seen by ^{51}Cr release, and negligible nucleolytic activity, as measured by ^{125}I-DNA release. Generally similar results were obtained with EL4 thymoma and P815 mastocytoma targets. As shown by the horizontal distance between titration curves in Fig. 2, the most active RBL-cy-gzb clones were 3–4-fold more active than the most active RBL-cy clones in mediating the lysis (^{51}Cr release) of EL4 and P815 targets, and were <10-fold more active in their ability to cause ^{125}I-DNA release. These cytolytic activities were roughly 30–70-fold less than cloned CTL, while the nucleolytic activity was >100-fold less than CTL. In all cases, cytotoxic activity was negligible in the absence of IgE αTNP antibody (data not shown), as previously shown for RBL-cy and RBL-cy-gza (14). In contrast to the RBL transfected with cytolysin, RBL-gzb clones were in no case active against tumor targets.

When red cell targets were tested, a potent hemolytic activity was obtained as a result of cytolysin expression as de-

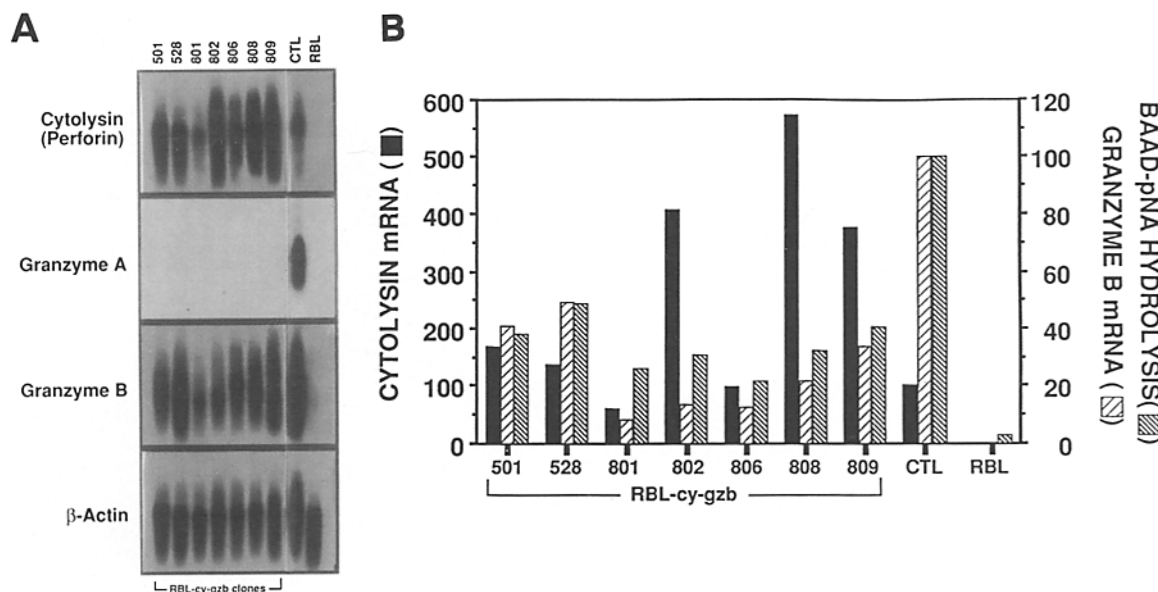

Figure 1. Expression of cytolysin and granzyme B in RBL transfectant clones. (A) A Northern blot hybridized with probes for cytolysin, granzyme A, granzyme B, and β-actin of RNA of seven RBL-cy-gzb clones and the CTL and RBL controls. (B) The quantitative expression of these mRNAs normalized to actin and expressed as a percentage, relative to the CTL control. In addition, the expression of granzyme B protein was assessed by assaying a detergent lysate of each cell type for hydrolysis of the granzyme B substrate BAAD-pNA. This activity is also plotted relative to the CTL control.

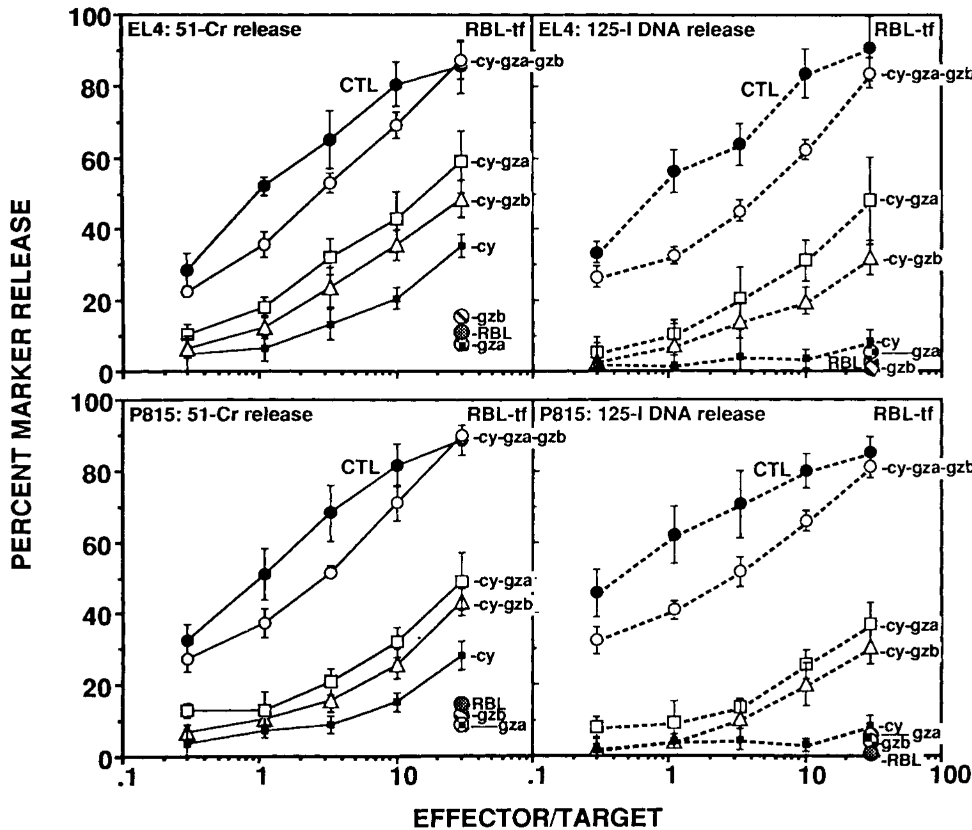

Figure 2. Cytotoxic activity of the most active of each type of RBL transfectant on tumor targets. Several clones of each type were included, and the mean data for several experiments with each transfectant type were averaged and are shown with the SEM. The following were included: RBL (three experiments); RBL-gza (two clones, two experiments each); RBL-gzb (two clones, two experiments each); RBL-cy (three clones, three experiments each); RBL-cy-gza (three clones, four experiments each); RBL-cy-gzb (clones 501, 528, and 809, four experiments each); RBL-cy-gza-gzb (clones 5011, 5014, 5015, and 50110, three experiments each). The cytotoxic activity of a single cloned CTL line, redirected by use of aCD3xaTNP heteroconjugate, is shown for comparison. The top panels show the results with EL4 lymphoma targets, and the bottom panels show the results with P815 mastocytoma targets. The left panels show the release of ^{51}Cr, while the right panels show the release of ^{125}I-DNA after detergent treatment. The assay incubation time was 4 h.

scribed previously (14), but there was no enhancement of this hemolytic activity attributable to granzyme B expression (Fig. 3). This result is similar to that obtained previously with granzyme A (15). Fig. 3 displays the results with the same clones as were shown in Fig. 2, and the contrast with tumor targets is clear.

Although the results shown in Fig. 2 represent the most cytotoxic of the clones studied, there was considerable variation within each group. To assess whether the expression levels of one or the other of these genes might limit the cytotoxic activity of the RBL-cy-gzb transfectants, the cytotoxic activities of each of these clones on tumor targets were plotted against their level of expression of cytolysin and granzyme B (Fig. 4). It can be seen that while neither cytolytic nor nucleolytic activities were significantly correlated with cytolysin mRNA expression (*A*), both activities on both targets are significantly correlated with the level of granzyme B enzymatic activity (p <0.0025 for all four independent variables, *B*). As expected from Figs. 4 and 1 *B*, a similar plot against granzyme B mRNA expression also shows a significant correlation (p <0.0025 for the activities on EL4, p <0.025 for the activities on P815; data not shown). When cytolytic activity against RBC targets was similarly tested for correlation with expression levels, this activity showed no significant correlation with either granzyme B level, but did show a significant positive correlation with cytolysin mRNA levels (p <0.01; data not shown).

mRNA and Protein Expression in RBL-cy-gza-gzb Transfectant Clones. To examine the cytotoxic properties of RBL transfectants expressing both granzyme A and B as well as cytolysin, a most potent RBL-cy-gzb clone (#501) was secondarily transfected with granzyme A cDNA as previously described (14), this time by use of the blasticidin selection marker. Again, individual clones were isolated and their expression levels and cytotoxic activities studied. Fig. 5 *A* shows Northern

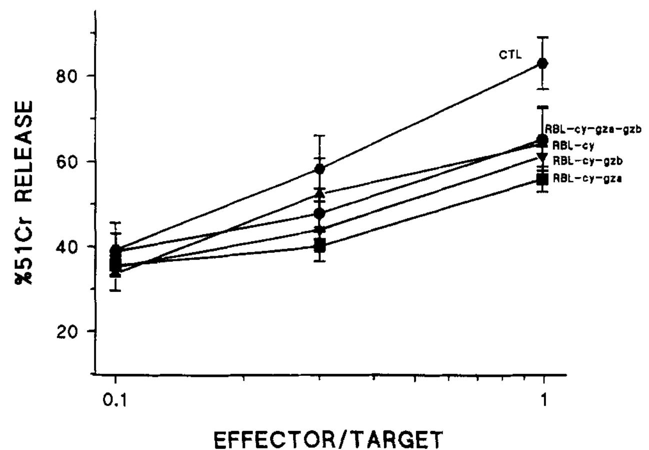

Figure 3. Cytotoxic activity of the most active of each type of RBL transfectant on red cell targets. The same clones as were shown in Fig. 2 are shown in experiments with human red cells as targets. Again, CTL were redirected by use of aCD3xaTNP heteroconjugate.

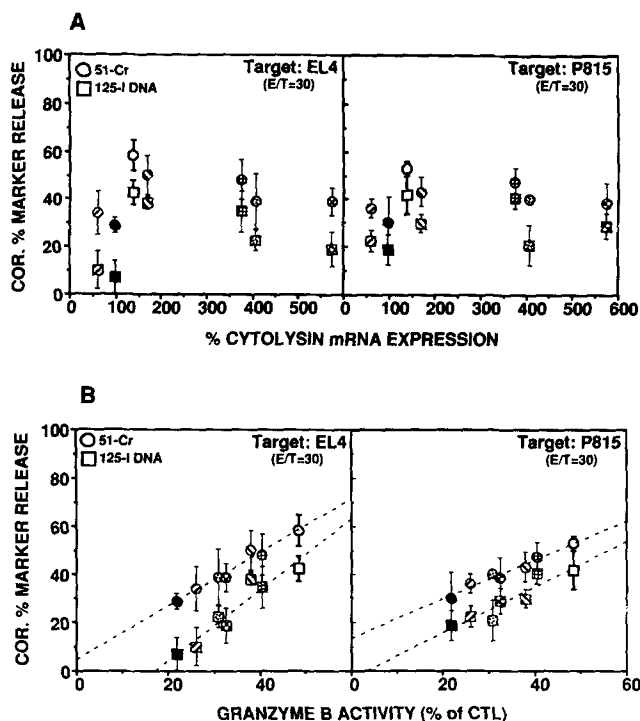

Figure 4. Cytotoxic activity of individual RBL-cy-gzb clones as a function of their expression of cytolysin and granzyme B. The mean marker release values at E/T ratio = 30 are plotted versus cytolysin mRNA (*A*) and granzyme B enzymatic activity in lysates (*B*) for EL-4 targets (*left*) and P815 targets (*right*). In each case, the mean values of experiments are shown. Circles indicate ^{51}Cr release and squares ^{125}I-DNA release. The clones (in increasing order of cytolysin expression) are 801, 806, 528, 501, 809, 802, and 808.

blots of the expression of the three granule protein genes as well as β-actin for 11 of these clones, and Fig. 5 *B* shows the quantitation of these mRNA levels. Not surprisingly, there is less variability between the levels of cytolysin and granzyme B in these clones. The granzyme B mRNA expression levels of these clones were 25–46% of the CTL level, as opposed to 40% of the parental RBL-cy-gzb clone, and the cytolysin expression levels were 77–229% of the CTL level. Expression levels of the newly acquired granzyme A mRNA ranged from 59 to 614% of the CTL level, and its enzyme activity ranged from 22 to 91% of the CTL level. In this case, for unknown reasons, the enzymatic and mRNA levels did not correlate well with each other.

Cytotoxic Activity of RBL-cy-gza-gzb Clones. As shown in Fig. 2, the most active of the 11 RBL-cy-gza-gzb clones studied gave potent cytolytic and nucleolytic activities on both tumor targets examined. These activities were roughly 3 and 5 times less, respectively, than the cloned CTL line used as a comparison, while they were 8–100 times more potent than the most potent of the RBL-cy-gza and RBL-cy-gzb clones. Fig. 6 shows the cytotoxic activity of several of the most active RBL transfectant clones of each type plotted in terms of lytic units, which is a linear measurement of relative cytotoxic activity. These data show clearly that both the cytolytic (*A*) and nucleolytic (*B*) activity of the triple transfectants is considerably greater than the sum of the two types of double transfectant, implying a synergistic interaction between the two granzymes in their ability to cause cytolysis and DNA damage. The granzyme expressions for the transfectant clones used in this comparison are shown in Fig. 6, which argues that the enhanced cytotoxic activity of the triple transfectants is not due to higher expression of granzymes than the double transfectants used for comparison.

As was the case with RBL-cy-gzb clones, there was considerable variation in cytotoxic activity among the individual RBL-cy-gza-gzb clones. Fig. 7 shows the cytolytic and nucleolytic activity of each of the 11 clones on four different tumor lines plotted as a function of their granzyme A enzymatic activity. The cytolytic activity is significantly correlated ($p < 0.001$) with granzyme A activity in three of four of these target cells (with Jurkat the exception), while the nucleolytic activity is significantly correlated ($p < 0.0005$) with expression of this protease in all four cases. Activity correlations with granzyme A mRNA levels were generally significant, but the fits were much better with the enzymatic activity data. Because these triple transfectant clones were derived from a single RBL-cy-gzb clone, they did not show a large range of expression of cytolysin and granzyme B (Fig. 5). Although their cytotoxic activities also correlated with granzyme B expression, this was less significant because of the smaller range. No correlation with cytolysin expression was found (data not shown).

In contrast to the results with tumor target cells, the most active RBL-cy-gza-gzb clones showed no greater activity on RBC targets than did the other cytolysin expressing RBL transfectants (Fig. 3). In analyzing the activity of individual clones on RBC targets, a highly significant ($p < 0.0005$) positive correlation was found with cytolysin mRNA expression. However, the roughly threefold range of cytolysin mRNA expression of these clones gave only a small variation in lytic activity. Granzyme expression levels of these triple transfectant clones showed no significant correlation with cytotoxic activity on RBC targets.

Induction of Apoptotic Morphology in Target Nuclei by RBL Transfectants. While the release of ^{125}I-DNA into the supernatant was clearly enhanced by granzymes A and B, this is only one criterion of apoptotic death. To confirm that granzyme expression was associated with apoptotic target death characteristics, target cells were marked for identification in the fluorescence microscope by use of a fluorescent lipid analog, thus allowing target nuclei to be examined microscopically for chromatin condensation and nuclear fragmentation after incubation with RBL transfectants. Fig. 8 shows that for two tumor targets, IgE-dependent apoptotic nuclear damage was seen with the triple transfectants but not with RBL-cy. After 4 h, target nuclei showed both chromatin condensation and nuclear fragmentation in those cases in which significant ^{125}I-DNA release had been detected.

Discussion

The studies described here make a strong case for the importance of granzymes in mediating target cell death by cyto-

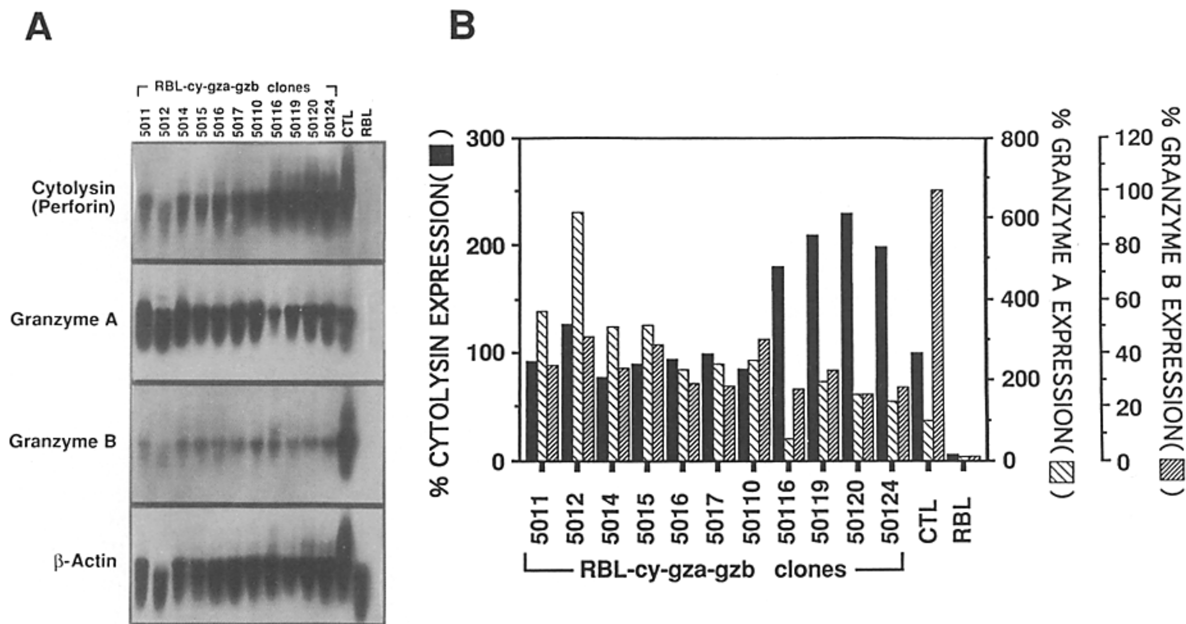

Figure 5. Expression of cytolysin, granzyme A, and granzyme B in individual RBL-cy-gza-gzb clones. As in Fig. 1, *A* shows Northern blot analysis of RNA preparations made for each RBL transfectant clone with the four probes indicated. *B* shows the quantitation of these mRNA expression levels, expressed as a percentage relative to the reference CTL clone.

toxic lymphocytes. They demonstrate the strengths of the approach we have used, that is, the controlled expression of lymphocyte granule components in noncytotoxic cells possessing a regulated secretory pathway. The most striking findings were that, while cytolysin is required for the cytotoxic activity of RBL, granzyme expression is the limiting factor in the cytotoxic activity of the RBL transfectants we have prepared. Furthermore, the cooperative interaction between granzymes A and B in mediating this activity suggests that these granzymes have evolved to maximize this synergistic effect, since these two (or close analogs) seem to be expressed in all cytotoxic lymphocytes studied.

It is clear that granzyme B by itself has the ability to enhance the cytolytic and especially nucleolytic activity of cytolysin-expressing RBL cells. The most active of our RBL-cy-gzb clones were not significantly different in this respect from the most active of our RBL-cy-gza clones, and we speculate that these proteases are roughly equal in their ability to cause lethal target cell damage. However, we have not attempted to compare this activity of granzymes A and B on

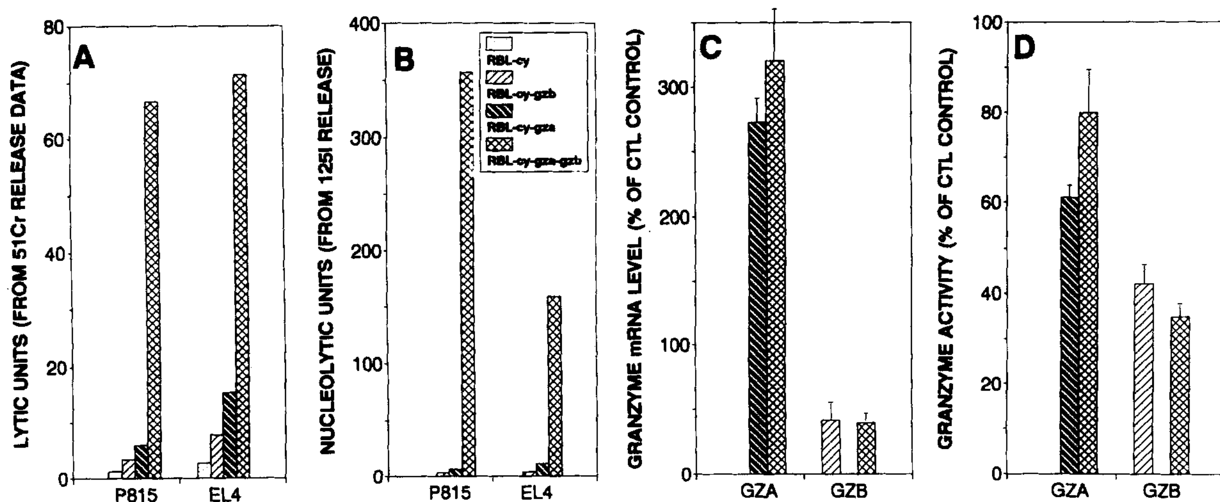

Figure 6. Cytotoxic activity of active RBL transfectant clones expressed as lytic and nucleolytic units. In *A* and *B*, the lytic units were calculated from the data in Fig. 2, with 40% ^{51}Cr release and 30% ^{125}I-DNA release used as the release markers for the lytic and nucleolytic unit definitions, respectively. *C* shows the mean granzyme A and B mRNA expression for the clones compared, while *D* shows their enzymatic (protein) expression levels.

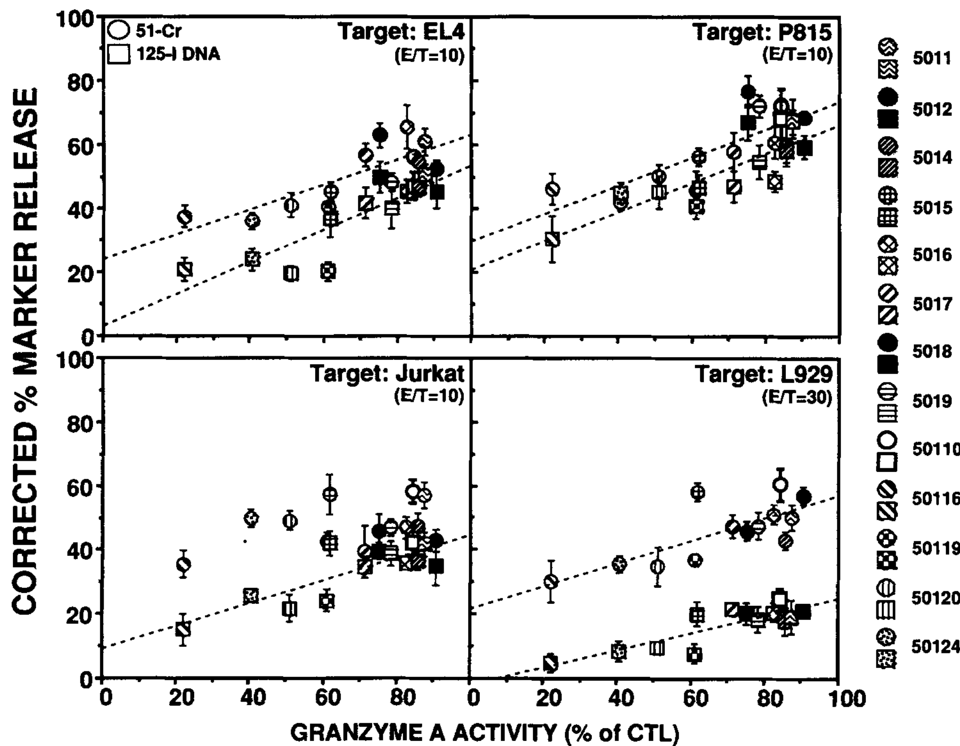

Figure 7. Cytotoxic activity of individual RBL-cy-gza-gzb clones as a function of granzyme A expression. Mean marker release values for experiments for each clone are shown with the following fixed E/T ratios: (*upper left*) EL4 targets, E/T = 10; (*upper right*) P815 targets, E/T = 10; (*lower left*) Jurkat targets, E/T = 10; (*lower right*) L929 targets, E/T = 30.

a molecular basis because of the difficulty in estimating how many protease molecules are being delivered to the target cell. Nevertheless, our results do not support the speculation that granzyme B might be particularly efficient in mediating target death because its preference for Asp at its cleavage site is nominally similar to that of IL-1–converting enzyme, which shows homology to the *Caenorhabditus elegans* death gene *ced*-3 (23, 24). The finding that granzyme B contributes to target cell lethal damage is in agreement with a recent study of granzyme B knockout mice (12), in which primary CTL from such mice killed tumor target cells with slower kinetics than CTL from control mice, with DNA damage being affected more than target lysis.

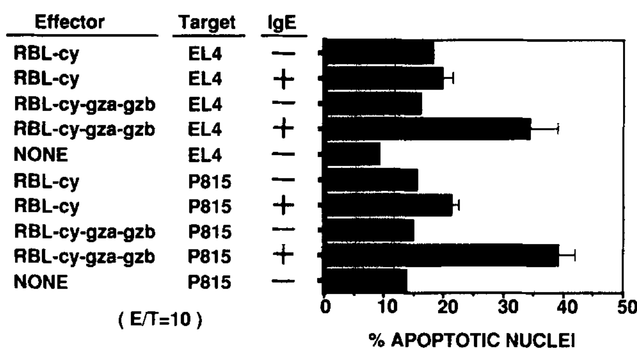

Figure 8. Apoptotic target cell nuclear morphology during cytotoxicity is associated with granzyme expression in RBL transfectants. EL4 and P815 targets were prelabeled with the lipid probe Di-IC_{16} to allow their recognition in the fluorescent microscope, and the standard 4-h cytotoxicity incubation was carried out with and without IgE anti-DNP. Nuclear DNA staining with HOECHST 33342 was carried out, and target nuclei were scored as apoptotic (condensed chromatin and/or nuclear fragmentation) or normal.

By quantitating both the cytotoxic activity of individual clones as well as the expression levels of these granule genes, we were able to look for quantitative correlations and thus implicate the transfected molecules whose expression contributes to the cytotoxic activity. It is clear that cytolysin expression is necessary for cytolytic activity since RBL cells expressing these proteases alone were not cytotoxic (Fig. 2 and reference 15). These results are expected given the general lack of cytotoxic activity of proteases on intact cells. However, within the range of cytolysin mRNA expression in our various RBL-cy transfectants, we observed no overall correlation of cytolytic activity on tumor targets and cytolysin mRNA levels, although at the lowest expression levels there may be an effect (Fig. 4 *A*). In contrast, with RBC targets, the level of cytotoxicity was clearly correlated with cytolysin mRNA levels, although the potent RBC killing activity increased only modestly over the 10-fold range of cytolysin expression of the clones (data not shown). These results bring out the strong differences between red cell and tumor targets and strongly suggest that the modest cytotoxic activity of RBL-cy on tumor targets is unlikely to be due to inadequate delivery of cytolysin protein to the target membrane.

In contrast to cytolysin, the expression levels of granzymes significantly correlated with cytotoxic activities on tumor cell targets. By analysis of RBL-cy-gzb clones, granzyme B expression levels were shown to correlate with both cytolytic

(^{51}Cr release) and nucleolytic (^{125}I-DNA release) activities on two tumor targets (Fig. 4 *B*), while for the RBL-cy-gza-gzb clones derived from one of these, the expression levels of granzyme A correlated with cytotoxic activities on three of four targets and with nucleolytic activity on all four targets (Fig. 7). These results show that the cytotoxic activities of these RBL transfectants are limited by expression of both granzymes, although we were unable to obtain granzyme B expression levels comparable to those of our reference CTL clone.

These results on both tumor and red cell targets are compatible with the granule exocytosis model for cytotoxicity if it is assumed that, with tumor target cells, the role of cytolysin is largely to permeabilize the target membrane to allow access of granzymes to the target cell cytoplasm. Although the original concept behind this model was that cytolysin-induced membrane damage was lethal to the target cell, our results show that this seems to be true only for red cells. The difference between red cell and tumor targets can best be explained by the ability of the latter to repair membrane damage due to inserted pores, as has been clearly demonstrated by studies with complement, in which >10-fold more membrane attack complexes are required to kill nucleated cells than red cells (25). This corresponds to our experience with cytolysin, in which we found that 50–200 times more cytolysin was required to kill nucleated cells than red cells (2). The RBL-cy transfectants deliver enough cytolysin via degranulation to kill red cell targets very efficiently, but tumor cells are quite resistant to these effectors. Previous results showed that roughly equal RBL cell degranulation was triggered by IgE bound to red cell and tumor cell surfaces (13), implying that some property of the target per se is responsible for the difference in cytolytic susceptibility. Membrane damage repair best explains the present finding that nucleated cell killing did not correlate with cytolysin expression in the RBL-cy-gzb clones, even though these clones had a ~10-fold range of cytolysin expression. This could imply that the tumor cell's repair capacity can efficiently handle multiple pores inserted close to each other on a patch of membrane opposite the degranulation event. In contrast, red cells, having no membrane repair capacity, are sensitive to far less cytolysin secreted by RBL effectors.

These results are compatible with a series of experiments in which granzymes have been shown to mediate nuclear damage in permeabilized target cells. We described the ability of granzyme A purified from CTL granules to mediate ^{125}I-DNA release from detergent- or cytolysin-permeabilized cells (6), and Shi et al. (11) described a series of several granzymes from rat NK granules that had a similar ability using cytolysin-permeabilized cells. The active NK granzymes, termed fragmentins, included a granzyme B–like protease with an "Asp-ase" enzymatic specificity, and two granzymes with "tryptase" activity similar to granzyme A. These results are compatible with the finding that both RBL-cy-gza and RBL-cy-gzb cause target death with accompanying DNA damage. Although the molecular relationship between the nucleolytic and cytolytic target cell damage has not been explained, it appears that granzymes trigger a damage pathway in which they are linked.

The expression of seven different granzymes in mouse CTL (8) has been puzzling since their description. If indeed their primary function is in triggering a cytotoxic pathway in target cells, then there are two explanations for granzyme heterogeneity that are not mutually exclusive. One is that the heterogeneity enhances the range of targets in which this efficient cytotoxic mechanism can operate, since the DNA damage in different target cell types appears to have different relative susceptibilities to different granzymes (11). The other explanation is that they interact cooperatively to trigger a death pathway(s) in target cells, as suggested again by experiments showing cooperation between granzyme A– and B–like fragmentins in mediating DNA damage (11). The apparent synergism for both cytolysis and DNA damage in RBL expressing both granzymes A and B shown in Fig. 6 lends further support to the possibility that these two enzymes cooperate in triggering a cell death response in target cells. This finding is interesting in light of the finding that granzymes A and B are the only granzymes expressed in granules from the lytically potent CTL produced in vivo by allogeneic stimulation in the peritoneal cavity (8).

The molecular death pathways triggered by granzymes in nucleated target cells need to be defined. Although the most obvious place to start is the identification of the physiologically relevant molecular substrates cleaved by these granzymes, it is often difficult to establish whether a particular substrate cleaved is required for cell death. The nuclear matrix protein nucleolin has been identified as a granzyme A substrate (26), but the evidence implicating this in target cell death is circumstantial. An interesting effect of the granzyme B–like fragmentin from NK granules is its ability to trigger activation of the protein kinase cdc2, which controls the entry of cells into mitosis (27). Thus, it has been proposed that the nuclear changes characteristic of mitosis could be triggered inappropriately, resulting in a "mitotic catastrophe." Although the precise proteolytic cleavages have not been defined in this system, it is an appealing explanation for the nuclear damage triggered by granzymes. However, while it is often assumed that there is a direct relationship between this apoptotic nuclear damage and cell death, there are examples in which target lysis by CTL can be dissociated from nuclear damage (28–30). Jacobson et al. (31) have recently shown that some stimuli causing apoptotic death also kill enucleated cytoplasts. We are currently using this approach to test whether nuclear events are necessary for the granzyme-mediated cytotoxic effect.

We believe that the studies described here lend substantial support to the granule exocytosis model for lymphocyte-mediated cytotoxicity and further extend our understanding of the basic process of cytotoxic effector function. By expressing the proposed granule mediators in a noncytotoxic effector cell with the appropriate secretory apparatus, we have largely reconstituted the cytotoxic activities of potent cytotoxic lymphocytes and provided strong evidence that the granzymes contribute in an important way to target cell damage.

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