

CYTOLYTIC ACTIVITY OF PURIFIED CYTOPLASMIC GRANULES FROM CYTOTOXIC RAT LARGE GRANULAR LYMPHOCYTE TUMORS

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Despite the efforts of many laboratories over a period of years, the mechanism of lysis by natural killer (NK)¹ cells and other cytotoxic lymphocytes remains controversial. Our previous studies have led us to propose that the cytotoxic process mediated by lymphocytes is basically a secretory phenomenon involving the release of cytolytic material from cytoplasmic granules of the effector lymphocyte after signaling via a receptor on the effector cell surface membrane (1). This proposal was based on electron microscopy (EM) observations showing target membrane-associated ring structures of ~15 nM internal diam (2); these ring structures appeared to arise from material released from cytoplasmic granules of the effector cell (1). Since the size of these ring structures corresponded to that predicted from sieving experiments with target membrane (3), it was proposed that the ring structures are inserted into target membranes in a manner similar to the insertion of the terminal complement components that form an aqueous pore.

These EM observations were recently confirmed and extended by Podack and Dennert (4), who described the formation of the ~15 nM ring structure and also smaller ring structures associated with target membranes after killing by a cloned mouse NK cell line. These structures were interpreted as transmembrane pores that arose from material in the NK cells. The secretory process has also been implicated in the NK lytic mechanism by studies showing that drugs known to inhibit secretion inhibit NK cytotoxicity (5, 6), and by the observations that strontium pretreatment of effector cells caused a depletion of the cytoplasmic granules and a concomitant loss of NK lytic function (7).

To provide a more direct test of the granule secretion hypothesis of lymphocyte cytotoxicity, we have begun a study of the properties of the isolated cytoplasmic granules of large granular lymphocytes (LGL). This subpopulation of lymphocytes is defined by the presence of such granules and contains antibody-dependent, cell-mediated cytotoxicity (ADCC) and NK effector cells (8). Since the above

¹ *Abbreviations used in this paper:* ADCC, antibody-dependent, cell-mediated cytotoxicity; BSA, bovine serum albumin; CTL, cytotoxic T lymphocyte; EM, electron microscopy; E/T, effector/target; HBSS, Hanks' balanced salt solution; LGL, large granular lymphocyte; NK, natural killer; PBS, phosphate-buffered saline; SE, sheep erythrocytes.

hypothesis predicts that LGL granules contain material which damages target cells, we have devised a procedure for the purification of granules from rat LGL tumor cells (9). These cells contain the characteristic cytoplasmic granules, display NK and/or ADCC activity, and share certain surface markers with peripheral blood LGL cells (10, 11). They have been used as a source of LGL granules in order to provide quantities adequate for biochemical and functional studies. As predicted by the above hypothesis, rigorously purified LGL tumor granules displayed a potent cytolytic activity when tested on sheep erythrocytes (9). The purified granules were found to contain prominent protein chains; by biochemical criteria they were clearly distinct from granules found in granulocytes and mast cells. In this report we show that the cytolytic activity of the purified granules has characteristics which are compatible with its role in LGL-mediated cytotoxicity reactions. These properties include the lysis of normal and transformed nucleated cells, rapid kinetics, and calcium dependence. Furthermore, cytoplasmic granules from a number of noncytotoxic cells do not display this lytic activity. Finally, we show that the purified LGL granules form the previously described ring structures visible in the EM under conditions of lytic activity.

Materials and Methods

LGL Tumors, LGL, and Target Cells. Rat LGL tumors were serially passaged in vivo in Fischer (F344) rats. Most experiments used the leukemia form of the tumor, and cells harvested from the spleen as previously described (11). These cells were 80–95% LGL in morphology and contained <5% granulocytes or macrophages as assessed by Wright's stain of smears. Some experiments were also carried out using these LGL tumors passaged in ascites form; these were 90–97% LGL in morphology and contained <5% granulocytes or macrophages. LGL and peripheral T lymphocytes were purified from rat peripheral blood using Percoll density gradient centrifugation (12). The LGL preparations contained about 70% LGL, 30% other lymphocytes, 2% monocytes, and >0.5% granulocytes, while the T cells were >97% small agranular lymphocytes. Granulocytes were prepared from rat peripheral blood as described (12) and were <90% polymorphonuclear leukocytes by morphology. Peritoneal macrophages were harvested from rats or mice previously injected intraperitoneally with thioglycollate or *Corynebacterium parvum*. These macrophages were purified by adherence as described (12). Sheep erythrocytes (SE) were obtained from the NIH Animal Center and stored in 50% Alsevier's solution. Tumor target cells were grown in vitro. Normal spleen lymphocytes were prepared by disruption of spleens and lysis of erythrocytes with ammonium chloride.

Purification of Cytoplasmic Granules. The procedure for purification of cytoplasmic granules of rat LGL tumors has been described in detail (9). In brief, cells were washed in balanced salt serum (HBSS) and resuspended at 1×10^8 /cc in disruption buffer (0.25 M sucrose, 0.01 M HEPES, 4 mM EGTA, 1,000 U/ml heparin [Sigma Chemical Co., St. Louis, MO], pH 7.4). They were lysed by decompression at 0°C after equilibrating at 450 psi nitrogen for 20 min. After the addition of $MgCl_2$ to 5 mM, the homogenate was digested with DNase I (800 U/ml, 22°C, 15 min). Nuclei were removed by filtration through Nuclepore filters (Nuclepore Corp., Pleasanton, CA) of 5 and 3 μ m, and the resulting homogenate was cooled to 0°C. Five-ml aliquots were layered on 20 ml of 48% Percoll (in disruption buffer without heparin) and centrifuged in a 60 Ti rotor at 29,000 rpm for 30 min in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Fullerton, CA). The purified granules were harvested from the visible band near the bottom of the tube. Percoll was largely removed by centrifugation of the granule fraction at 85,000 g for 16 h at 0°C. Granules were recovered as a loose pellet over the hard Percoll pellet.

Cytoplasmic granule preparations from other lymphoid cells were obtained using the identical procedure. Individual 0.8-ml fractions of these gradients were assayed for β -

glucuronidase (13), protein (14), and density (using a refractive index and a previously developed calibration curve). Cytolytic activity of these gradient fractions was determined using SE target cells as described below. A purified preparation of rat liver lysosomes was the kind gift of Dr. Jos van Renswoude and Dr. Richard Klausner, NIH, and purified rat peritoneal mast cell granules were graciously made available by Dr. Fred Atkins and Dr. Dean Metcalfe, NIH.

Assay for Cytolytic Activity. Target cells were labeled by incubation of 10^7 cells with $0.4 \mu\text{g Na}_2^{51}\text{Cr}_2\text{O}_7$ in 1 ml HBSS containing 0.01 M HEPES and 10% fetal calf serum for 45 min at 37°C . Granule preparations were diluted serially in $100 \mu\text{l}$ of calcium-free phosphate-buffered saline (PBS) in U-bottomed microtiter plates. 10^4 target cells in $100 \mu\text{l}$ of HBSS containing 0.01 M HEPES and 2 mg/ml bovine serum albumin (BSA), pH 7.4, were added to each well. (This solution contains the Ca^{2+} required for activity.) After incubation at 37°C for 20 min the plates were centrifuged and the supernatants harvested using a Titertek harvesting apparatus (Flow Laboratories, Inc., McLean, VA). Kinetics of ^{51}Cr release was measured in 1.4-ml conical "Eppendorf" tubes using $200\text{-}\mu\text{l}$ volumes as described above. At the indicated times the tubes were spun at $14,000 g$ for 15 s and $150 \mu\text{l}$ of the supernatant removed and counted. An alternate assay of hemolysis was performed in the U-bottomed microtiter plates as described except that each well contained 5×10^6 SE. After incubation at 37°C for 20 min the plates were centrifuged and $150 \mu\text{l}$ supernatant from each well transferred to a flat-bottomed microtiter well. These plates were read in a Titertek Elisa reader using a 414-nm filter. This assay gave virtually identical results to the ^{51}Cr release assay. 1 U/ml of cytolytic activity is defined as the concentration of granules that will give 50% lysis of SE in the ^{51}Cr release assay.

Electron Microscopy. For electron microscopy, appropriate dilutions of granule suspensions were made in PBS (without added calcium) or HBSS (with calcium) at room temperature. At intervals from 1 to 30 min, $5\text{-}\mu\text{l}$ aliquots were removed and applied to Formvar-coated grids (Ladd Research Industries, Burlington, VT). These dilute granule preparations were negatively stained with 2% sodium phosphotungstate, pH 6.8, or saturated aqueous uranyl formate.

Results

Cytotoxicity of Purified LGL Tumor Granules on Mammalian Cells. The rat LGL tumor cells used for preparations of granules were derived from *in vivo* passages of leukemias shown to possess high NK and/or ADCC activity (10, 11). When cytoplasmic granules were purified by the Percoll gradient technique described, potent cytotoxic activity was routinely observed using SE as target cells (Fig. 1).

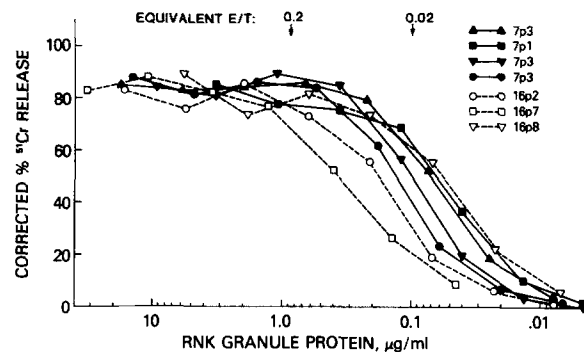


FIGURE 1. Cytolytic activity of purified rat LGL tumor cytoplasmic granules on SE. Titrations of seven independent preparations of purified granules are shown. These preparations are from the RNK 7 and RNK 16 tumor lines (9) whose *in vivo* passage number is indicated by the "p" (10). The basis for calculation of the equivalent E/T ratio is described in Results.

Virtually complete lysis (80–90%) occurred at granule protein concentrations of 1 $\mu\text{g}/\text{ml}$ in all preparations, and detectable lysis occurred at 20 ng/ml in some preparations. Of more than 50 LGL tumor granule preparations, all displayed cytolytic activities comparable to those shown here. For comparison with the cytotoxicity of intact effector cells, an equivalent effector/target (E/T) ratio can be calculated for granule cytotoxicity. Using a granule purification yield of 1 mg protein/ 10^8 RNK tumor cells (9), the value of 1 $\mu\text{g}/\text{ml}$ granule protein shown in Fig. 1 corresponds to an equivalent E/T ratio of 0.2 (i.e., the granule content of one effector cell could kill at least five targets). This rough calculation illustrates the potency of the cytolytic effect. Curves similar to those shown in Fig. 1 were also obtained when hemoglobin release was used as an indicator of target cell lysis. In this case several million SE were used per well, and the hemoglobin release was measured spectrophotometrically. Comparisons of sheep and human erythrocytes as targets in titrations such as those shown in Fig. 1 showed that they are identical in their susceptibility to granule cytolytic activity (data not shown).

Fig. 2 shows the cytolytic effect of LGL tumor granules on a variety of nucleated cells as well as SE. It can be seen that these nucleated cells are more resistant to the granule cytolytic effect than are SE, requiring 30–200-fold more granules to cause lysis. Some other lines of cultured lymphoid cells have subsequently proved to be even more resistant than the EL4 and R8 tumors shown, but all cells tested have been significantly lysed by 100 $\mu\text{g}/\text{ml}$ of the granule preparations that are lytically active on SE. While most cultured lymphoid cells have given lytic curves shaped similarly to those shown here, the shallower slope for spleen cells was a reproducible finding and is presumed to result from the heterogeneity of splenic lymphocytes in their sensitivity to granule cytolytic effects. LGL tumors harvested after passage as ascites tumors yielded granule preparations having similar cytolytic and biochemical properties to those harvested from the spleens of leukemic animals.

Cytolytic Activity of Granule Preparations and Lysosomes from Other Cell Sources. To test the cytolytic activity of cytoplasmic granules of normal LGL, granules were prepared from as many as 5×10^7 LGL purified from rat

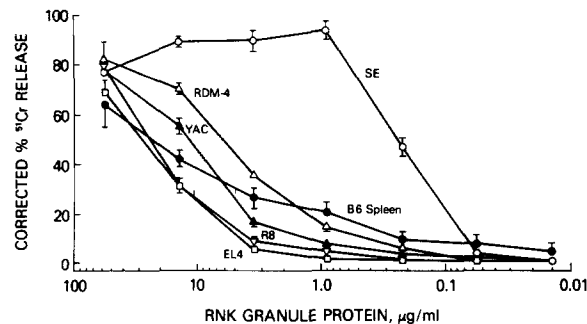


FIGURE 2. Cytolytic activity of purified rat LGL tumor cytoplasmic granules on erythrocytes and nucleated cells. The C57BL/6 spleen cells were freshly prepared, while the murine tumor cells were grown in vitro. (○) SE, (●) C57BL/6 spleen, (Δ) RDM 4, an AKR T cell leukemia, (▲) YAC-1, an A strain lymphoma, (∇) R8, an Abelson virus-induced pre-B cell leukemia, (□) EL-4, a C57BL/6 T cell lymphoma.

peripheral blood using the procedure developed for the LGL tumor granules. In this case a sharp peak of cytolytic activity was found in the granule region, as was seen with the LGL tumors (Fig. 3 A). On a per cell input basis this cytotoxicity was in the range of the LGL tumor granules (Table I). These experiments show that normal LGL display a granule cytolytic activity very similar to that observed in LGL tumors. Difficulties in obtaining large numbers of purified normal LGL prevented further characterization of these granules.

The cytolytic effect seen in cytoplasmic granules from LGL and LGL tumors was not found in comparable preparations of granules from other cell types. As shown in Table I, we have tested the cytolytic activity of granule preparations from a number of types of lymphoid cells prepared by the same protocol used for the LGL tumor granules. These have not had detectable activity, using the sensitive SE target cells. An example of such an experiment is shown in Fig. 3 B, in which the noncytotoxic mouse tumor line EL4 was homogenized and the

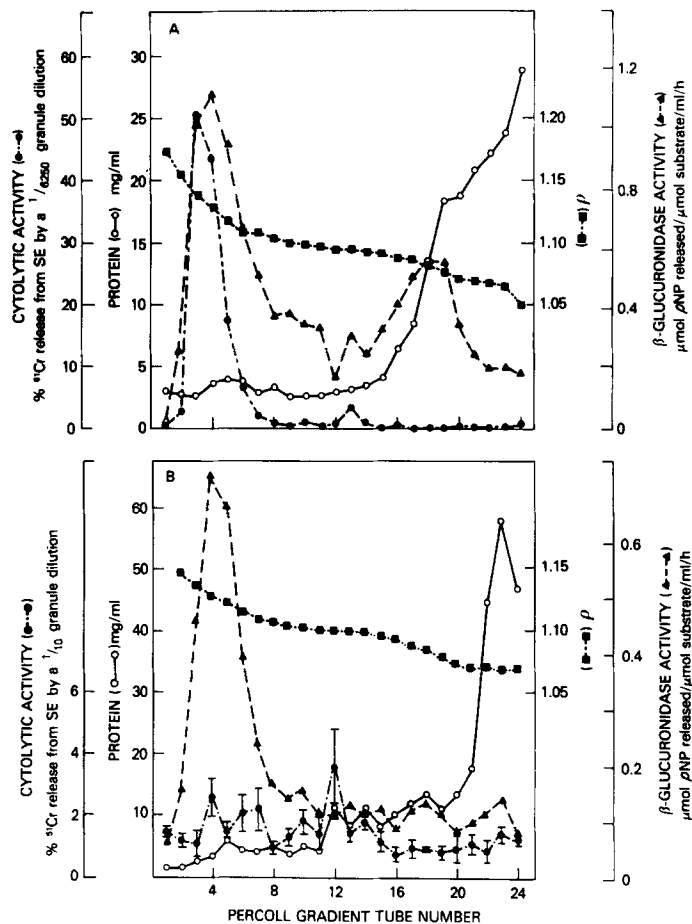


FIGURE 3. Percoll gradient fractionation of the homogenate of the noncytotoxic mouse lymphoma EL4. Each gradient tube contained the homogenate of 5×10^8 cells. (A) Rat LGL tumor, (B) EL-4. In this case the cytolytic activity was also assayed at a final dilution of 1:100 of each fraction, with results similar to those shown here for 1:10. *p*NP, *p*-nitrophenol.

TABLE I
Cytolytic Activity of Purified Cytoplasmic Granules and Lysosomes from Various Cells

Granules from:*	Granule lysosomal enzymes [‡]	Maximal granule protein concentration [§]	Maximum percent SE lysis [¶]	Granule lytic units per 10 ⁶ cells [†]
LGL tumor	+	100 µg/ml	90	1,000–4,000
Peripheral blood LGL	+	ND**	90	3,000
Peripheral blood T cells	+	ND**	3	<10
Spleen	+	120 µg/ml	2	<10
Thymus	–	94 µg/ml	3	<10
EL 4 (mouse) T cell lymphoma	+	500 µg/ml	4	<10
Peripheral blood granulocytes	+	ND**	2	<10
Peritoneal macrophages	–	ND**	3	<10
Liver lysosomes ^{‡‡}	(+)	1 mg/ml	2	ND
Peritoneal mast cells ^{‡‡}	(+)	37 mg/ml	2	ND

* Fresh rat tissue, except EL 4.

‡ Plus sign indicates a peak of β -glucuronidase activity was observed in tubes 2–5.

§ Final protein concentration of 1:10 dilution of mean of granule pool (tubes 2–5) of Percoll gradient.

¶ All tubes of Percoll gradients tested at final dilutions of 1:10 and 1:100.

† Sum of activity in all tubes of gradient divided by cell input.

** Insufficient cells available to determine protein above Percoll interference.

‡‡ Not prepared by the Percoll gradient technique.

homogenate separated on a Percoll gradient under conditions that gave the LGL granules in fractions 2–5. As can be seen in Fig. 3 B, the EL4 homogenate demonstrated a major peak in the activity of the lysosomal enzyme β -glucuronidase and a minor peak of protein in this region, as was also found for the LGL tumor homogenate. When the cytotoxic activity of this fraction was measured at final dilutions of 1:10 and 1:100, no significant ⁵¹Cr release was observed. In contrast, Fig. 3 A shows that the granule fractions (2–5) of LGL tumor homogenates typically showed complete lysis of SE at dilutions of several thousand with the same number of input cells (5×10^8). Similarly, cytolytic activity was not observed in any Percoll gradient fraction of homogenates of rat splenocytes, thymocytes, or peripheral T cells (Table I). Since thymocyte homogenates did not contain detectable β -glucuronidase in any fraction, there is no evidence that any comparable granule fraction exists in these cells. The properties of granules from CTL will be described elsewhere. In addition to these granule preparations from lymphoid cells, we have assayed the cytolytic activity of purified blood polymorphonuclear leukocytes and peritoneal macrophages, since these or similar cells were low-level contaminants in our LGL tumor cell preparations. Table I shows that no cytolytic activity was detectable in the homogenates of these cells. We have also tested preparations of rat liver lysosomes and mast cell granules prepared by standard techniques to see if these well-characterized cytoplasmic granules containing lysosomal enzymes could display activity in our assay. No cytolytic activity was detectable over a thousand-fold range of serial dilutions (Table I).

Kinetics of Granule-mediated Lysis. The kinetics of lysis of SE and nucleated cells is shown in Fig. 4. It is clear that the release of ⁵¹Cr is a rapid event after the mixing of purified granules with target cells in the presence of calcium, since

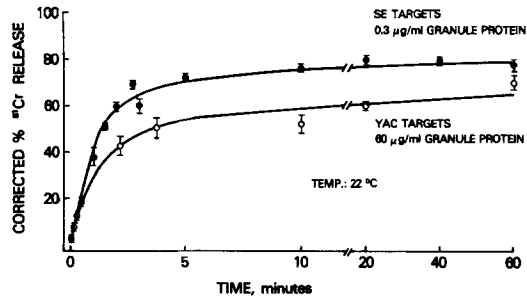


FIGURE 4. Kinetics of lysis of SE and YAC by purified LGL tumor cytoplasmic granules. This experiment was carried out in 200- μ l volumes using 1.5-ml conical test tubes. At time zero, target cells in 100 μ l BSS were added to 100 μ l of diluted granules in PBS. At the indicated time, tubes were spun for 15 s and the supernatant ^{51}Cr was determined. The incubation temperature was 22°C. Points given are the mean and SEM of triplicate determinations.

the ^{51}Cr release was complete within 5 min at room temperature. The reaction proceeded more rapidly at 37°C, but accurate sampling of released label was not achieved before the endpoint. When the release of hemoglobin from SE was measured, approximately the same time course as shown in Fig. 4 was obtained. Other nucleated cells gave data similar to those shown for YAC (data not shown).

Divalent Ion Dependence of LGL Granule Cytolytic Activity. The cytolytic activity of purified LGL tumor granules is highly dependent on divalent ions, as shown in Fig. 5. In the absence of any added divalent cations, cytolytic activity of LGL granules was not detectable. At calcium concentrations of 10^{-4} M, activity was detectable, and it was maximal between 3×10^{-4} M and 10^{-3} M. At higher calcium concentrations there was a tendency of this activity to fall off, as seen in Fig. 5 B; in other experiments with nucleated target cells the decrease in cytolytic activity was more modest, as occurred with SE targets in the experiment shown in Fig. 5 A. Granule cytolytic activity was never observed with Mg^{+2} or Ba^{+2} alone at any concentration with any target. Furthermore, magnesium at 1 mM did not have any detectable influence on the Ca^{+2} titration curve shown in Fig. 5 A (data not shown). The experiments using Sr^{+2} consistently failed to show activity with SE targets (Fig. 5 A). Most nucleated target cells gave Sr^{+2} titration curves similar to that shown in Fig. 5 B, i.e., this ion could replace Ca^{+2} at a 10-fold higher concentration.

A characteristic property of the cytolytic activity of LGL tumor granules is that this activity is not stable in the presence of calcium. When granules were exposed to calcium before contact with the target cells (Fig. 6), activity declined with time. Granule-mediated lysis of nucleated target cells showed a similar calcium-induced inactivation (data not shown). The experiment presented in Fig. 6 was performed at 22°C so that this time course can be compared with the kinetics shown in Fig. 4. This calcium-induced loss of activity was important to consider in preparing LGL granules; the homogenization buffer contains EGTA and all subsequent purification steps have avoided calcium to preserve the cytolytic potential of the purified granules.

Correlation of the Appearance of Ring Structures with the Cytolytic Activity of Isolated Granules. Previous EM studies of lymphocyte-mediated killing had

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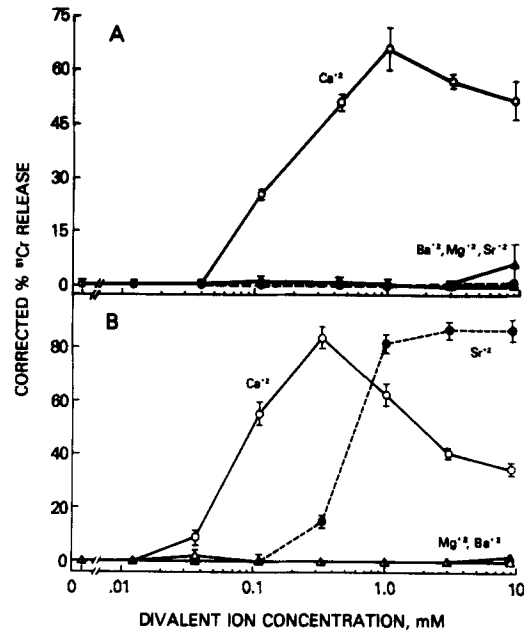


FIGURE 5. Divalent cation dependence of granule cytolytic activity. In these experiments, 100 μ l of target cells in 0.15 M NaCl, 2 mg/ml BSA, 0.01 M HEPES, pH 7.5, was supplemented with divalent ions at twice the concentration indicated. Diluted granules in 100 μ l 0.15 NaCl, 0.01 M HEPES, pH 7.5, were added and mixed. After incubating at 37°C for 30 min, supernatants were harvested and counted. (A) SE targets. Final granule concentration was 8 μ g/ml (this preparation had lost considerable lytic activity). (B) YAC targets. Final granule concentration was 100 μ g/ml.

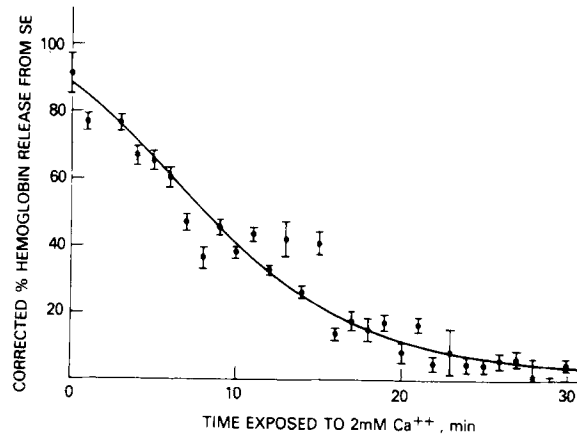


FIGURE 6. Inactivation of granule cytolytic activity by calcium. In this experiment each well of a microtiter plate contained 75 μ l of purified LGL tumor granules at 8 μ g/ml protein in 0.15 M NaCl, 0.01 M HEPES. At 1-min intervals, 25 μ l of 8 mM Ca²⁺ in the above saline was added to each well and mixed. After 30 min, 100 μ l of HBSS containing 2 mg/ml BSA and 5 \times 10⁶ SE were added. Hemoglobin release was measured after 30 min incubation. Granules incubated for 30 min in the absence of Ca²⁺ for 30 min gave 77 \pm 2% hemoglobin release in this experiment.

identified unique ring structures inserted into target membranes, and the rings appeared to originate from the effector cell granules. It was therefore important to determine whether the cytolytic effect of isolated granule material was also associated with the formation of ring structures. Figs. 7 and 8 show that ring structures are formed by isolated granule material under conditions in which it is cytotoxic. Fig. 7 shows electron micrographs of negatively stained preparations of RNK granules after incubation for 20 min at room temperature. Fig. 7 A is from a granule suspension in PBS (without Ca^{+2}). Under these conditions there appeared many electron-opaque spheres a few tenths of a micron in diameter (not shown), which presumably were intact granules that were too thick for the electron beam to penetrate. Interspersed with these structures were a few flat membranes with roughly circular profiles (as shown). Presumably these were the empty membranes of a few disrupted granules. These membrane profiles were smooth except for the presence of contaminating Percoll particles. In granule suspensions that had been exposed to Ca^{+2} before the negatively stained preparation was made, flat membrane profiles were more numerous, and on each membrane were a number of ring structures with outside diameters of 15–28 nm and inside diameters of 5–17 nm. These ring structures resembled very closely the rings seen on the membranes of target cells after attack by effectors of ADCC and NK (2). When samples were taken at various times after mixing the granule suspension with Ca^{+2} , the rings began to appear as quickly as negatively stained preparations could be made (within 2 min after exposure to Ca^{+2}).

When purified granules were mixed with SE in the presence of calcium, the SE underwent lysis as described above. These preparations contained some recognizable profiles of red cell ghosts and many irregular membrane profiles. Although in this preparation it was difficult to be certain which membranes originated from the SE and which from the granule preparation, there were numerous rings on all membranes. An example of such a preparation is shown in Fig. 8. As with the isolated granule preparation alone, no rings were seen when the SE and granules were mixed in the absence of Ca^{+2} and the SE lysed by addition of distilled water. Thus, the calcium requirement and the time course of the appearance of the ring structures closely paralleled the expression of cytolytic activity by isolated RNK granules.

Characterization of LGL Granule Cytolytic Activity. As shown in Table II, the cytolytic activity of LGL granules is unstable at elevated temperatures. At 37°C or higher, all detectable activity was lost within 30 min in the absence of calcium. At 25°C, the stability of granule cytolytic activity was variable from preparation to preparation. In some cases such as the experiment shown in Fig. 6, cytolytic activity was not lost over a period of 0.5 h. In other cases such as the experiment of Table II, a substantial loss of activity occurred during such an incubation. Since this temperature lability was suggestive of a protein requirement for cytolytic function, protease digestions were also carried out. Table II shows that a Pronase concentration of 0.25 mg/ml abolished all detectable activity in 30 min at 25°C while the heat-inactivated enzyme had no effect.

Granule-associated cytolytic activity can be solubilized from the granules, as shown in Table III. Even in granule preparations that had been frozen, cytolytic

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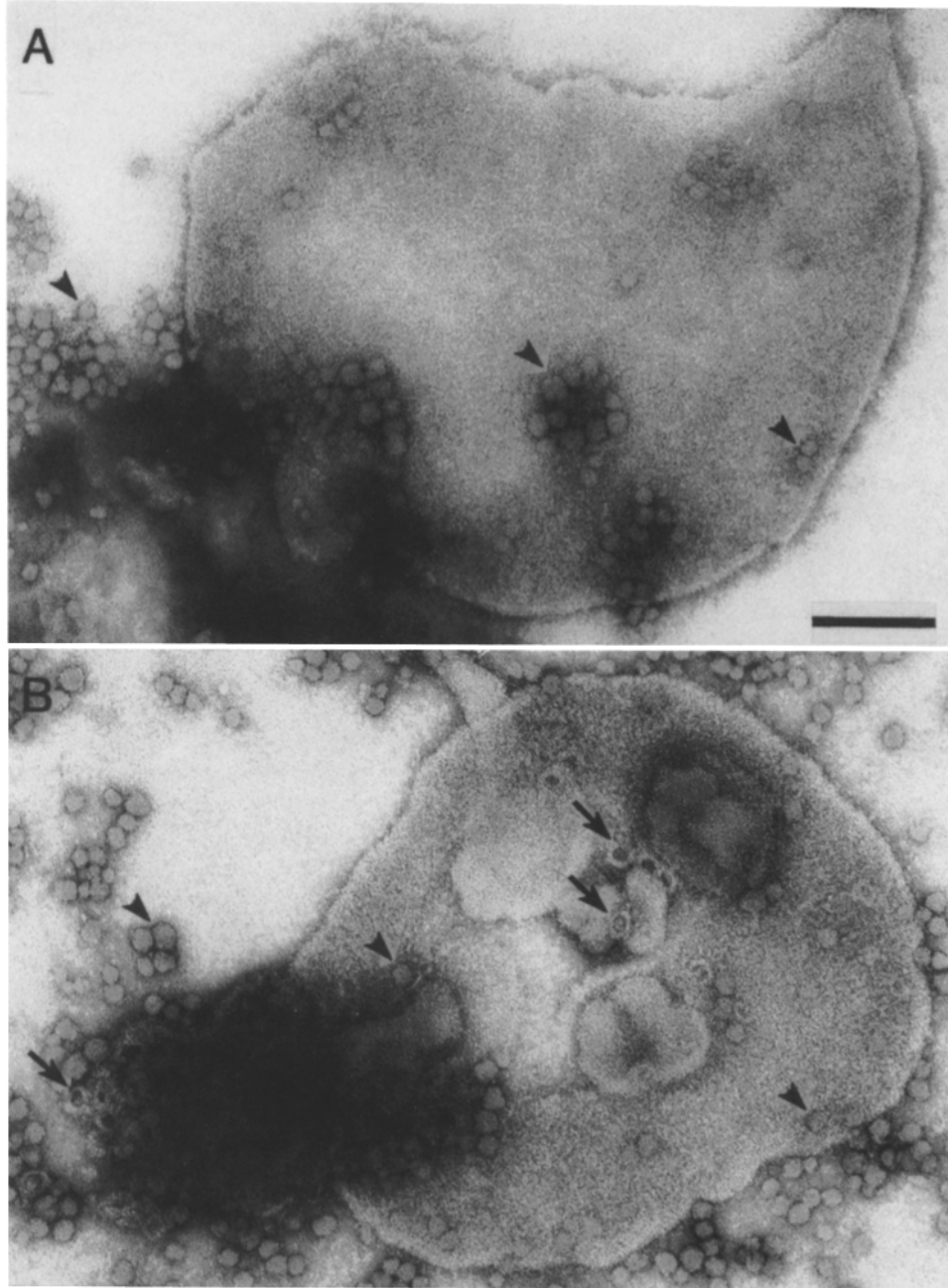


FIGURE 7. Electron micrograph of negatively stained purified LGL tumor granules. Contaminating Percoll particles (arrowheads) appear as light discs surrounded by a border of the dark negative stain in these images. (A) In the absence of calcium the membranes in these preparations are smooth. (B) After exposure to Ca^{2+} , ring structures (arrows) appear on the membranes. The mark indicates 100 nm. $\times 165,000$.

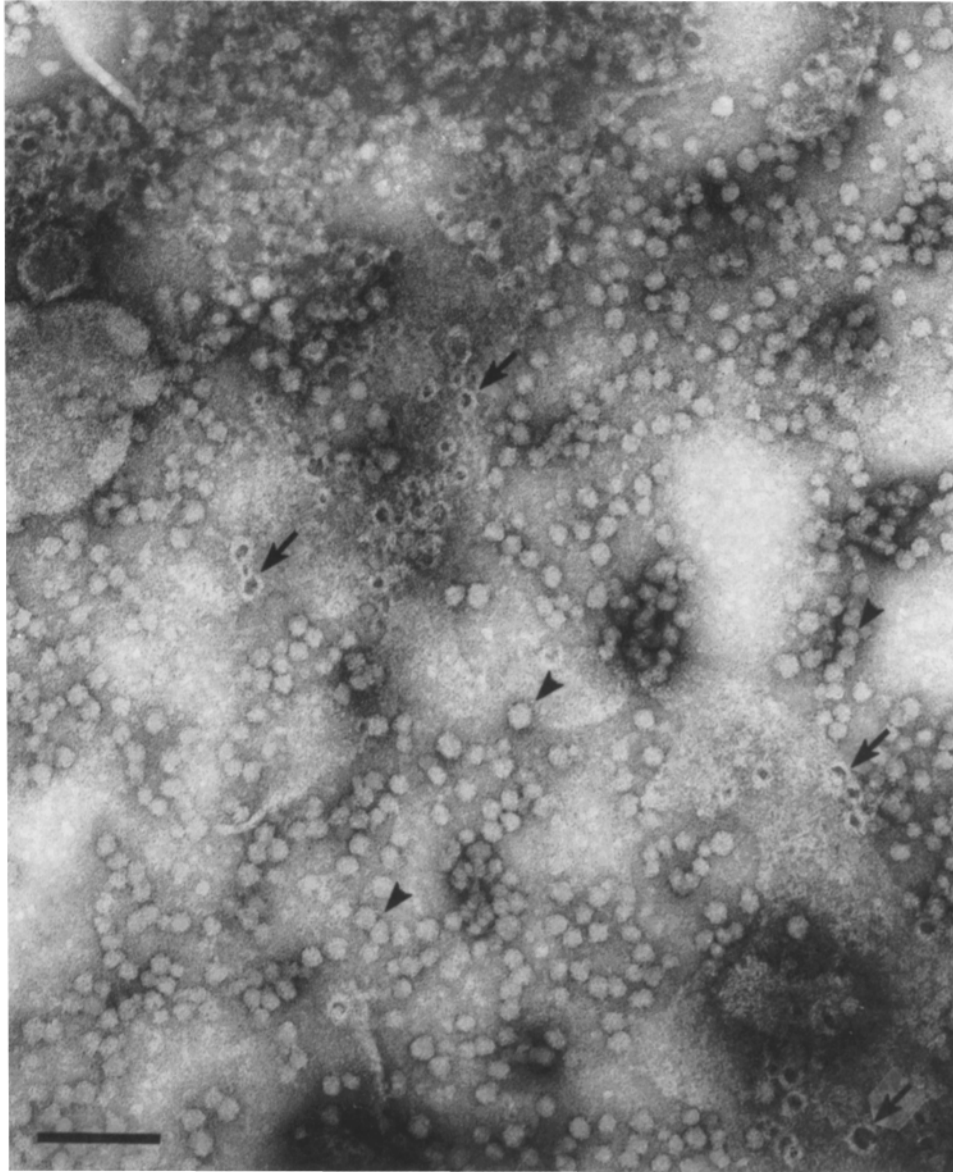


FIGURE 8. Electron micrograph of negatively stained membranes from a preparation of SE lysed by purified granules in the presence of calcium. The arrows indicate examples of the many rings found on all membranes in such preparations. The rings range from 15 to 28 nm outside diam and from 5 to 17 nm inner diam. Rings were not seen when erythrocytes were exposed to granules in the absence of Ca^{2+} . The arrowheads indicate examples of Percoll particles. The mark corresponds to 100 nm. $\times 165,000$.

activity was found in the pellet after a 10,000 *g* centrifugation in low or moderate ionic strength buffers, while a majority of the β -glucuronidase was in the supernatant. Treatment of granules with detergents or sonication failed to solubilize activity, whereas treatment with 2 M NaCl or 2 M KCl did solubilize

TABLE II
Heat and Pronase Sensitivity of LGL Granule Cytolytic Activity

Granule treatment*	Activity U/ml
0°, 30 min	1,900
37°, 30 min	<10
25°, 30 min	410
25°, 0.25 mg/ml Pronase, 30 min	<10
25°, 0.25 mg/ml inactivated [‡] pronase, 30 min	470

* Granules in PBS were treated as indicated before dilution in PBS for the standard assay for SE lysis.

[‡] Boiled 20 min before incubation with granules.

TABLE III
Solubilization of LGL Granule Cytolytic Activity

Solubilizing buffer	Percent activity in supernatant of 10,000 g spin*	
	SE cytotoxicity	β -glucuronidase
0.3 M sucrose	18 [‡]	60
PBS	7 [‡]	74
BSS	4	80
PBS (sonicated 2 min)	8	76
PBS + 2 M NaCl	233	90
PBS + 2 M KCl	233	83
PBS + 1% deoxycholate	0	69
PBS + 1% octylglucoside	12	80
PBS + 1% CHAPS	16	75

* Previously frozen LGL tumor granule preparations. 100% activity corresponds to assays carried out on the original granule suspensions in PBS. Cytolytic and enzyme activities calculated on the basis of activity units.

[‡] In these samples, the cytolytic activity not in the supernatant could be recovered in the pellet.

the activity, yielding about twice as much activity in the supernatant and negligible amounts in the pellet. Cytolytic activity remained in the supernatant of a 100,000 g centrifugation in 2 M NaCl and is currently being purified.

Discussion

The results of this study demonstrate that cytoplasmic granules of LGL tumors and LGL possess a hitherto undescribed cytolytic potential that must be considered as a possible mediator of the lethal damage inflicted on target cells by NK cells, ADCC effectors, and perhaps cytotoxic T lymphocytes (CTL). Many aspects of the physiology of these cytotoxic processes are similar (15, 16) although certain aspects of the initial target cell recognition stage differ (16, 17). It has become clear that ADCC and NK cytotoxicity are mediated by LGL (8), and there is evidence that the lethal damage is inflicted by a common pathway after target cell recognition by different LGL surface receptors (1). This pathway was

proposed (1) to involve a secretory step in which material from the LGL granules is exocytosed into the space between the killer cell and its target. During the secretion event the granule contents were observed to undergo morphological rearrangements so that ring structures were formed; these ring structures appeared to be associated with the target cell membrane, suggesting formation of aqueous pores analogous to those produced by complement (1). Recent EM observations suggest that a similar mechanism may operate in CTL (18). As discussed below, the lytic activity of LGL granules displays properties highly suggestive of their role in the "lethal hit" of LGL-mediated cytotoxicity.

Our conclusion that the observed lytic activity is associated with granules from LGL tumors and LGL rests on several lines of evidence. As previously demonstrated (9), the LGL tumor granule preparation is pure by morphological and several biochemical criteria. The preparation method used insures that any impurities present must be some type of cytoplasmic granule. While non-LGL granule components present at undetectably low levels could conceivably account for the observed cytolytic activity, this is unlikely for several reasons: (a) Activity is found in LGL tumor cells from spleens and from ascites, as well as LGL purified from blood. These different LGL preparations would be expected to have different types of contaminating cells. (b) Other cell types that are the major contaminants of LGL tumors or peripheral blood LGL do not yield cytolytically active granules (Table I). (c) Known toxic components from the granules of polymorphonuclear leukocytes (19) or eosinophils (20) do not show the same characteristics (Figs. 2, 4, and 5) as the lytic activity in LGL granules.

The lytic effects of small quantities of purified LGL tumor granules (Figs. 1 and 2) are clearly compatible with a role for granule-derived cytolytins in the cell-mediated lytic process. EM studies of NK-target conjugates during lysis show that the local concentration of granule material is high in the small space between the effector and target cells (1). It thus seems reasonable that the concentration of 100 $\mu\text{g}/\text{ml}$ required for lysis of most nucleated cells could easily be achieved in that area of the target cell surface. The equivalent E/T ratios given in Fig. 1 are shown for the purpose of demonstrating the potency of this lytic effect, since they compare favorably with the E/T ratios of NK killing by LGL or LGL tumors (10, 11). However, this calculation may be misleading since LGL probably do not use a high percentage of their granules in a single lytic event (1), while the equivalent E/T ratio was based on the presumably high preparative yield of our granule purification. The potency of granule cytolysis appears considerably superior to previously reported cytotoxic phenomena reported from various lymphotoxins or NK cytotoxic factor (21) and to the lytic effects reported from the membranes of cytotoxic lymphocytes (22) and noncytotoxic cells (23). The hemolytic potency of whole granules appears to be comparable to that of purified complement components (24) and superior to staphylococcal α toxin (25). Since the actual lytic agent in the granules may represent only a minor component, it appears that this agent is one of the most cytolytic substances known.

The greater sensitivity of red cells than nucleated cells to granule-mediated cytolysis parallels their relative sensitivity to complement-mediated lysis. The ability of nucleated cells to eliminate complement channels inserted in their membrane (26) may explain at least part of this effect in the case of complement,

and a similar mechanism may operate in the case of granule cytolysis. The relative susceptibility of cells to granule-mediated cytolysis does not reflect the specificity of NK cells, which fail to recognize erythrocytes or normal lymphocytes. There is also no preferential recognition of antibody-coated target cells, reflecting an Fc receptor-mediated ADCC specificity (data not shown). These results presumably reflect the absence on the granules of LGL surface receptors, which play an important role in target cell recognition. The mechanisms responsible for the varied susceptibility of tumor cells to granule-mediated lysis are currently under study and may provide insights into the resistance of some tumor cells to NK lysis.

The lack of cytolytic activity of granule preparations of noncytotoxic lymphoid cells (Table I) suggests that the cytolytic potential of cytoplasmic granules may be the result of a product(s) of lymphocyte differentiation specialized for the cytotoxic function. It seems unlikely that normal lysosomal enzyme activities can account for granule-mediated cytolytic activity, since some of the noncytotoxic cells in Table I gave granule fractions containing levels of lysosomal enzymes at least as high as those in LGL tumors. The activity of such lysosomal enzymes has been suggested to be responsible for the lethal hit in NK (5, 27) and CTL cytotoxicity (28), but we favor the idea that the lysosomal enzymes present in these granules are not responsible for either granule- or cell-mediated lytic activity. In this regard we would point out that lysosomal enzymes in eosinophil granules (29) and mast cell granules (30) are not known to play a functional role after secretion. The calcium requirement of granule-mediated cytolytic activity also casts doubt on the role of lysosomal enzymes in this process, since known lysosomal enzymes do not show this property.

The kinetics of granule-mediated cytolysis of both red cells and nucleated cells is strikingly rapid (Fig. 4), showing complete release of ^{51}Cr within 1 min at 37°C . The speed of this lysis is greater than might be predicted for a selective small ion permeability increase leading to colloid osmotic lysis, and is more compatible with a detergent-like membrane disruption or with the creation of large pores that release macromolecules directly. Such large pores were detected in a red cell ghost membrane attacked by LGL in ADCC (3) and are the most obvious interpretation of the ring structures visible in the EM. However, studies of the NK lytic process (16) have demonstrated a "killer cell-independent lysis" period in which the ^{51}Cr release from target cells is delayed after removal of effector cells. For the NK lytic process this stage has been found to be <30 min, but would not have been detected if it were as fast as 1 min. Thus the speed of granule-mediated cytolysis is faster than would be predicted for a physiological "lethal hit" process, which is more compatible with a small pore. However, there are a number of differences between the presentation of granule material to target cells by NK cells during the lytic process and presentation by the addition of purified granules to a suspension of target cells. In the former case the granule material comes into contact with a limited portion of the target cell surface, and in this area the local concentration of granule material is very high. When purified granules are added to a suspension of target cells, a large dilution results and the target cells are exposed to lytic material from all sides. It is possible that such differences can affect the assembly of membrane pores; in the case of

complement there is evidence that the membrane pore size is controlled by the number of C9 molecules (31). Size heterogeneity of LGL-killing-associated ring structures have been reported (2, 4), and it seems possible that LGL-delivered pores are smaller than those induced by isolated granules. It is also possible that repair processes of the target cell are capable of acting more efficiently on a localized area of membrane undergoing attack by granule components released from an effector cell, and that such repair processes retard the rate of target cell lysis. A more detailed analysis of individual granule components and their functional activity is necessary to address these possibilities.

A characteristic feature of granule-mediated cytolytic activity is its regulation by divalent cations (Fig. 5). It is notable that a strong Ca^{+2} dependence is also a characteristic of the postbinding lethal hit phase of lymphocyte-mediated cytotoxicity. Since all known secretory processes triggered by cell surface receptors require calcium and thus show a similar calcium dependence, many investigators have considered the possibility that the lethal hit involves a secretory event (32). While EM studies have shown that a secretory event indeed takes place, the results presented here suggest that a second calcium-dependent process may occur during or soon after the secretion of LGL granule contents. It is striking that the calcium titration curves shown in Fig. 5 *B* are virtually identical to those described for human ADCC (33) and very similar to those demonstrated for NK killing (34). Furthermore, both calcium and strontium curves in Fig. 5 *B* are identical to those for the mouse CTL lethal hit (35). The difference in strontium effects on granule-mediated lysis of nucleated cells and red cells (Fig. 5 *A* vs. 5 *B*) is not currently understood. One interpretation is that these targets are lysed by different components of the granules. However, this seems unlikely in view of the similarities of all other properties of granule-mediated lysis of these different targets.

The loss of granule cytolytic activity in the presence of calcium (Fig. 6) reveals a second aspect of the interaction of the cytolytic granule component(s) with calcium. One straightforward interpretation of this phenomenon is that calcium activates the cytolytic component but the activated species is unstable and decomposes if it does not interact with a target cell. A similar phenomenon is known to occur (24) in the "membrane attack" part of the complement pathway. In this case the instability is generally interpreted as the creation of an amphipathic species that will insert into a nearby hydrophobic moiety if no target membrane is available. Such an interpretation is also plausible for the granule cytolytic activity.

LGL granule cytolytic activity is heat labile and protease sensitive (Table II) and can be solubilized from the granules by high salt (Table III). These findings suggest that a soluble protein is required for this activity, and biochemical purification experiments currently underway confirm these results. The solubilized granule cytolsin may be hypothesized to act via a mechanism similar to complement, in which hydrophilic proteins become amphipathic during an activation process (36). As pointed out previously (9), sodium dodecyl sulfate gels of these granules do not reveal any major protein chains that correspond to the complement proteins C5b, C6, C7, C8, or C9. For this and other reasons we

feel it is unlikely that complement proteins are involved in granule-mediated lytic activity.

The finding that ring structures become visible in the EM when purified granules are treated with Ca^{+2} (Fig. 7) is significant for a number of reasons. First, these results directly confirm the previous EM observations (1) that ring structures arise from granule material during the secretory process. Such ring structures are associated with target membranes after lymphocyte-mediated cytotoxicity (2, 4, 18). These combined observations show that lymphocytes secrete material from cytoplasmic granules that is transferred to target cell membranes during the cytotoxic process, as predicted by the secretion model. Second, the EM observations reported here strongly suggest that the ring structures are responsible for granule cytolytic activity since there is an absolute calcium requirement for both ring formation and lysis. Other strong correlations suggesting that these ring structures represent lytic membrane pores include the observation of a 15-nm maximal pore diameter from marker sieving experiments on ADCC attacked red cell membranes (3) corresponding to the maximal internal diameter observed in the ring structures. Complement-associated ring structures, which are generally similar in appearance, have a smaller diameter in the EM (2), corresponding to a smaller maximal pore diameter observed in marker sieving experiments (3). Ring structures have now been associated with the cytolytic activity of purified LGL granules, LGL (1, 2, 4), T lymphocytes (18), complement (36), and bacterial toxins (25). More direct evidence for the role of granule-derived ring structures in LGL- and granule-mediated cytolytic activity must await biochemical purification of the lytic component. Nevertheless, we feel that the present results provide strong suggestive evidence for this hypothesis. Further evidence has come from recent experiments demonstrating that anti-LGL tumor granule antibodies inhibit LGL-mediated cytotoxicity in a postbinding step of the lytic process (manuscript in preparation).

Summary

Purified cytoplasmic granules from cytotoxic rat large granular lymphocytes (LGL) tumors were cytolytic to erythrocytes, splenocytes, and a number of different lymphoid tumor cells. Granule concentrations of $\sim 1 \mu\text{g/ml}$ granule protein were adequate to lyse 100% of the erythrocytes, while the nucleated cells required up to $100 \mu\text{g/ml}$ granule protein to achieve complete lysis. Cytoplasmic granules purified from noncytotoxic lymphoid cells did not contain detectable cytolytic activity; purified granules from rat mast cells and rat liver lysosomes likewise failed to display cytolytic activity. However, granules prepared from normal rat peripheral blood LGL were cytolytic. Granule-mediated lysis of erythrocytes and nucleated cells was complete within 3 min at room temperature. The lytic activity required calcium at concentrations of 10^{-4} – 10^{-2} M; magnesium or barium failed to replace calcium, while strontium could replace calcium at 10^{-3} – 10^{-2} M when nucleated cells were the target. Exposure of LGL tumor granules to calcium before the addition of target cells resulted in an inactivation of granule cytolytic activity over the course of 20 min at room temperature. Granule cytolytic activity was heat and Pronase sensitive, and could be solubilized by 2 M salt. Examination of granules exposed to calcium in the electron

microscope using negative staining showed that calcium treatment of granules results in the formation of ring-shaped structures previously described to be associated with LGL-mediated cytotoxicity.

These results provide support for the hypothesis that the cytotoxic processes mediated by LGL are a secretory event characterized by the release of cytolytic material from the cytoplasmic granules after triggering by a surface receptor. The results further suggest that the ring structures visible in the electron microscope are associated with the lytic event.

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