Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 5551–5555, September 1984
Immunology

Liposomes as targets for granule cytolysin from cytotoxic large granular lymphocyte tumors

(natural killer cells/lethal hit/membrane damage/pores/calcium)

R. BLUMENTHAL*, P. J. MILLARD†, M. P. HENKART†, C. W. REYNOLDS‡, AND P. A. HENKART†

*Laboratory of Theoretical Biology, National Cancer Institute, Building 10, Room 4B56, and †Immunology Branch, National Cancer Institute, Bethesda, MD 20205; and ‡Biological Therapeutics Branch, NCI Frederick Cancer Research Center, Frederick, MD 21701

Communicated by Manfred M. Mayer, May 29, 1984

ABSTRACT **Purified cytoplasmic granules from rat large granular lymphocyte tumors having natural killer activity and/or antibody-dependent cell-mediated cytotoxicity induced a rapid, dose-dependent release of the water-soluble marker carboxyfluorescein from liposomes made of phosphatidylcholine. A solubilized, partially purified cytolytic preparation termed "cytolysin" from these granules showed identical properties. Marker release induced by granules or the cytolysin was strongly dependent on the presence of Ca^{2+} at a concentration of 0.1 mM or higher in the medium; Ca^{2+} could be replaced by higher concentration of Sr^{2+} but not by Ba^{2+} or by Mg^{2+}. These properties strikingly parallel the lytic effects that granules and granule cytolysin exert on cells. Marker release from liposomes was stopped instantaneously when an excess of EGTA was added to the medium. The remaining carboxyfluorescein inside the liposomes was present at the original internal concentration, indicating that marker release was all-or-none from individual liposomes. Liposomes comprised of lipid in the solid phase released marker more slowly than did comparable liposomes containing fluid-phase lipids. Variation of the lipid headgroup had only minor effects on the cytolysin-induced marker release. Electron microscopy of liposomes exposed to cytolysin in the presence of Ca^{2+} showed cylindrical structures of 15-nm diameter inserted into the membrane concomitant with the penetration of negative stain into the liposome. These properties of large granular lymphocyte granule cytolysin strongly suggest that it operates through a mechanism similar to the membrane attack of complement.**

Cytotoxic lymphocytes are a major component of the effector arm of immune responses to foreign tissue, virally infected cells, and probably tumor cells. Several types of such "killer cells" bind to their target cells by specific receptors, inflict a lethal injury, and then detach from the dying target cell, often to repeat the process with another target cell (1). Large granular lymphocytes (LGL) are a subpopulation of blood lymphocytes, which contain the natural killer (NK) effector cells and those mediating antibody-dependent cell-mediated cytotoxicity (2). Based on morphological studies of the LGL-mediated cytotoxic process, we have proposed (3) that target cell lysis results from secretion by the effector lymphocytes of the contents of cytoplasmic granules into the space between the killer cell and its target. The granules were proposed to contain material which forms membrane pores in the target cell membrane; these pores were previously studied by functional (4) and electron microscopic (5, 6) techniques.

We recently have described the purification of cytoplasmic granules from rat LGL tumors that display natural killer activity and/or antibody-dependent cell-mediated cytotoxicity (7). These granules were shown to mediate a potent, rapid, Ca^{2+}-dependent cytolytic activity on erythrocytes and nucleated cells, and this lytic effect was accompanied by the formation of membrane-associated ring structures visible in the electron microscope (8). This lytic activity was shown to be due to a protein termed "cytolysin" that could be solubilized from the granules. While these experiments yielded results that strongly supported the above model for lymphocyte cytotoxicity, they were not designed to probe the mechanism of target-cell damage by the cytolysin. In the present study we show that purified LGL tumor granules and solubilized granule cytolysin induce a rapid, Ca^{2+}-dependent release of the water-soluble marker carboxyfluorescein from liposomes. [We use the term liposome to include both the small unilamellar vesicle (SUV) and the reverse-phase evaporation vesicle (REV).] The affected liposomes contain pore-like structures inserted into their lipid bilayer as seen by electron microscopy. These results suggest that the lytic action of granule cytolysin occurs through the insertion of the pore-forming cytolysin molecules into the target lipid bilayer, in a mechanism generally analogous to that of complement (9).

MATERIALS AND METHODS

Preparation of Liposomes. Phospholipids were obtained from Avanti Biochemicals, cholesterol from Calbiochem–Behring, and cholesteryl hemisuccinate from Sigma. SUVs (approximately 30 nm in diameter) containing carboxyfluorescein were prepared as previously described (10, 11) by sonication in the presence of 80 mM carboxyfluorescein (pH 7.4), followed by elution from a Sephadex G-25 PD-10 column (Pharmacia) into 145 mM NaCl/10 mM Hepes, pH 7.4. REVs were prepared by reverse-phase evaporation according to Szoka and Papahadjopoulos (12) with 80 mM carboxyfluorescein (pH 7.4) in the aqueous phase. The vesicles were extruded through polycarbonate membranes (Nuclepore) with 0.4-μm and 0.2-μm pores under argon pressure. The vesicles were separated from nonencapsulated carboxyfluorescein on a PD-10 column as described above. REVs prepared in this way are a few tenths of a micrometer in diameter.

Carboxyfluorescein Release. The assay for release makes use of the self-quenching properties of the highly water-soluble fluorescent dye carboxyfluorescein (10, 11), which was purified as described (13). Fluorescence intensity of the dye encapsulated in vesicles at 80 mM concentration is about 3% of that obtained when the dye is released and diluted into the medium. The percentage of dye release by various combinations of granules and Ca^{2+} was determined by measuring fluorescence before and after adding Triton X-100. Recordings were done at an excitation maximum of 490 nm and an emission maximum of 520 nm wavelength with a Perkin–Elmer

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PtdCho, phosphatidylcholine; LGL, large granular lymphocyte; REV, reverse-phase evaporation vesicle; SUV, small unilamellar vesicle.

model MPF 44B spectrofluorometer. An aliquot of 1–50 μl of granule suspension or cytolysin was added to 2.5 ml of a vesicle suspension (0.1 μM in lipid) in NaCl/Hepes buffer, pH 7.4 (containing 1 mM Ca^{2+} unless otherwise specified), in a 1 × 1 cm cuvette and was mixed. All experiments were conducted at room temperature.

LGL Tumor Cytoplasmic Granules and Cytolysin. Cytoplasmic granules from rat LGL tumors were prepared by disruption of the cells by nitrogen cavitation and separation of the homogenate on a Percoll gradient (7). Some experiments were carried out with solubilized cytolysin, prepared by extraction of previously frozen purified granules with 2 M NaCl/3 mM phosphate/0.5 mM EDTA, pH 7.4, at 0°C, followed by gel filtration on ACA 54 (LKB) in this buffer. The cytolytic activity against sheep erythrocytes is recovered in excellent yield in a single retarded peak and retains its characteristic calcium dependence. This peak also contains all detectable cytolytic activity against nucleated cells, but lacks most of the lysosomal enzymes associated with the granules.

Electron Microscopy. For electron microscopy, liposomes were suspended at a final lipid concentration of 50 μM, with partially purified cytolysin at a final concentration of about 60 μg/ml of protein. Hepes buffer (pH 7.4) was added to a final concentration of 30 mM, and at zero time $CaCl_2$ was added to some samples to a final concentration of 10 mM. The mixtures were incubated at room temperature for 10 min, after which 5-μl samples were applied to Formvar-coated grids and negatively stained with 2% sodium phosphotungstate (pH 6.8). SUVs were examined after dilution in NaCl/Hepes with and without Ca^{2+} in the presence and absence of cytolysin. Likewise, cytolysin was examined with or without Ca^{2+} in the absence of liposomes.

To verify the Ca^{2+}-dependent, cytolysin-mediated release of carboxyfluorescein from these liposomes, 2-μl aliquots of each suspension were diluted to 2.5 ml, and fluorescence was read in the fluorometer before and after adding Triton X-100.

RESULTS

Granule and Cytolysin-Mediated Carboxyfluorescein Release from Liposomes. The purified LGL granules mediated a rapid release of carboxyfluorescein from REVs in medium containing 1 mM $CaCl_2$ (Fig. 1*A*). At a sufficiently high granule-to-vesicle ratio, essentially all of the encapsulated carboxyfluorescein was released within 1 min. At lower granule-to-vesicle ratios, the rate of release was slower and the extent of release was less. The initial slope of these carboxyfluorescein-release curves was linear with granule concentration (Fig. 1*B*) over the range studied. In some experiments such as that shown in Fig. 1*A*, a slight lag in marker release was observed. Granule-induced carboxyfluorescein release from SUV was similar to that from REVs, except that lower rates were observed at comparable granule-to-lipid ratios (Fig. 1*B*). Soluble partially purified cytolysin behaved like whole granules in releasing carboxyfluorescein from liposomes, except that less protein (1/3 to 1/10) was required to achieve the same rates of carboxyfluorescein release (see Fig. 4 below). For both types of liposomes, the rate of release declined with time; at low granule or cytolysin concentrations, only partial marker release was observed at long incubation times (Fig. 1). In these cases, further addition of cytolysin induced release of the remaining marker (data not shown). This indicates that all of the original cytolysin had been irreversibly inactivated.

Divalent Ion Dependence of Carboxyfluorescein Release. In the absence of divalent cations, granules or cytolysin did not cause detectable carboxyfluorescein release from liposomes. Curves similar to those shown in Fig. 1*A* were obtained by addition of Ca^{2+} to cuvettes containing cytolysin in Ca^{2+}-free buffer. Fig. 2*A* shows the rate constant for carboxyfluorescein release as a function of Ca^{2+} concentration. The concentration for half-maximal release was about 0.1 mM Ca^{2+}. Fig. 2*B* shows an experiment in which granules were incubated with liposomes and various divalent ions for 10 min. The granule-to-liposome ratio was such that a maximal release of 70% was obtained. With Ba^{2+} and Mg^{2+}, there was no release at any of the divalent cation concentrations up to 10 mM. In the presence of Sr^{2+}, maximal release was obtained, but the threshold was shifted from 0.1 mM (in the case of Ca^{2+}) to about 1 mM and a decrease was observed at higher concentration. These data are very similar to our previously reported data (8) on divalent cation dependence of granule-mediated lysis of nucleated target cells and to the divalent ion requirements for the lethal-hit phase of cytotoxic T-lymphocyte killing (14).

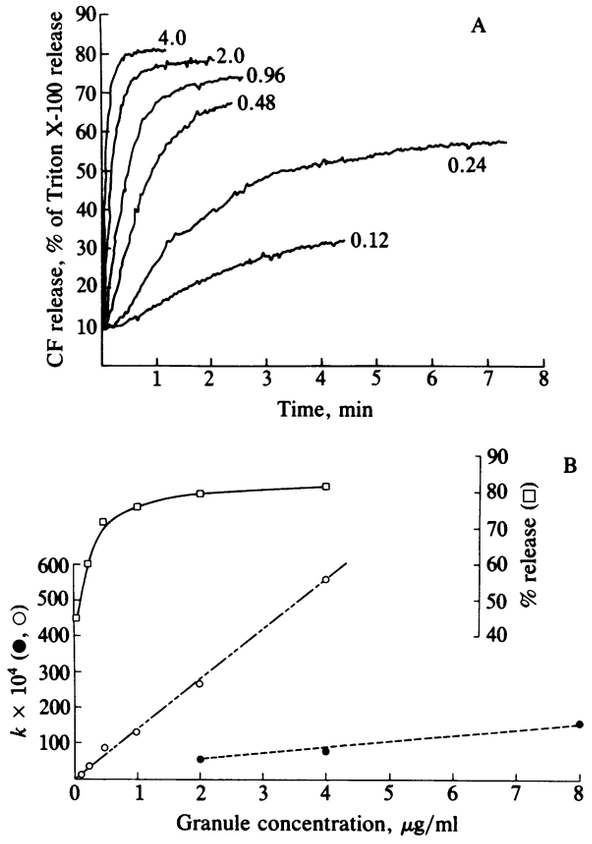

FIG. 1. Release of carboxyfluorescein (CF) from liposomes induced by LGL tumor granules. The liposomes were composed of egg PtdCho/cholesterol, 2:1 mol ratio. (*A*) Time course; the amount of LGL tumor granules is shown in μg/ml. (*B*) Rate constant for release, *k*, calculated from $(\Delta F/\Delta t)/Ft$, where *F* is fluorescence at time *t*, and *Ft* is total fluorescence released by Triton X-100. ○, REVs; ●, SUV; □, the final percentage of Triton-releasable CF from REVs as a function of granule concentration. Granule concentration is expressed as granule protein concentration; the lipid concentration was 0.1 μM. These experiments and the others in this paper were carried out at room temperature.

Mechanism of Carboxyfluorescein Release from Liposomes. Granule- or cytolysin-mediated carboxyfluorescein release was stopped rapidly by adding an excess of EGTA to the cuvette (Fig. 3), demonstrating that no long-lived reactive species are detectable when Ca^{2+} is removed. This rapid means of halting marker release was used in experiments designed to test whether granule-induced carboxyfluorescein release was graded or was all-or-none from individual liposomes (i.e., when half of the carboxyfluorescein was re-

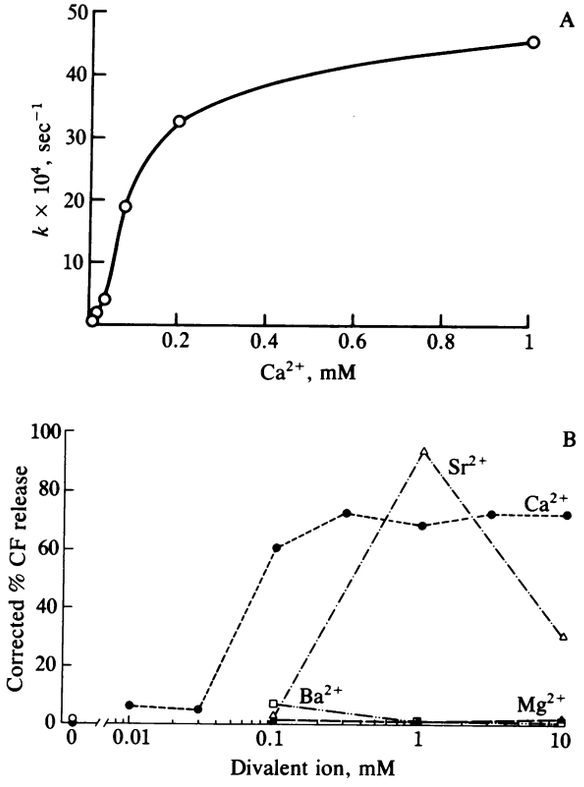

FIG. 2. Divalent cation-dependence of the release of carboxyfluorescein from REVs induced by granules. (*A*) Rate constant k (calculated as in Fig. 1) as a function of Ca^{2+} concentration. (*B*) Extent of release after 15 min with different divalent cations. ●, Ca^{2+}; △, Sr^{2+}; □, Ba^{2+}; ▲, Mg^{2+}. The liposomes had the same lipid composition as in Fig. 1.

leased, had all the vesicles lost half of their carboxyfluorescein or had half the vesicles lost all of their carboxyfluorescein?). This experiment is possible since the efficiency of carboxyfluorescein fluorescence varies with its local concentration, being "self quenched" at high concentrations such as the original carboxyfluorescein concentration inside the liposomes (12). Thus, the level of fluorescence of carboxyfluorescein remaining in liposomes after partial cytolysin-mediated release could be calculated from the internal carboxyfluorescein concentrations predicted by the two hypotheses by using the standard carboxyfluorescein quenching curve previously determined.

Table 1 shows three examples of such experiments, for three different degrees of carboxyfluorescein release at the time of EGTA addition (column 1). For each case, the level of carboxyfluorescein fluorescence expected for a graded release (in which the local carboxyfluorescein concentration inside the liposomes will decrease) is shown in column 2. The level of carboxyfluorescein fluorescence expected by the all-or-none hypothesis is the same as the original (highly quenched) level because the local carboxyfluorescein concentration inside liposomes would not change (column 3).§ In order to measure the level of carboxyfluorescein fluorescence for the marker remaining in the liposomes after cytolysin-induced leakage, the liposomes containing residual carboxyfluorescein were separated from released carboxyfluorescein on a PD-10 column, and their carboxyfluorescein fluorescence was determined before and after addition of Triton X-100 (Table 1, column 4). These results were close to the predicted values for the all-or-none hypotheses (column 3) and clearly very different from the graded-release prediction (column 2). Thus, individual vesicles appear to lose their entire carboxyfluorescein content when granule cytolysin acts on them, as would be expected from the insertion of a pore into the liposome.

§This assumes no size change in liposomes such as would occur with vesicle fusion. Absence of such fusion was indicated by a failure to detect fluorescent energy transfer between fluorescent lipid probes, incorporated into two different liposome populations, after mixing the two liposome populations and cytolysin in the presence of calcium (data not shown).

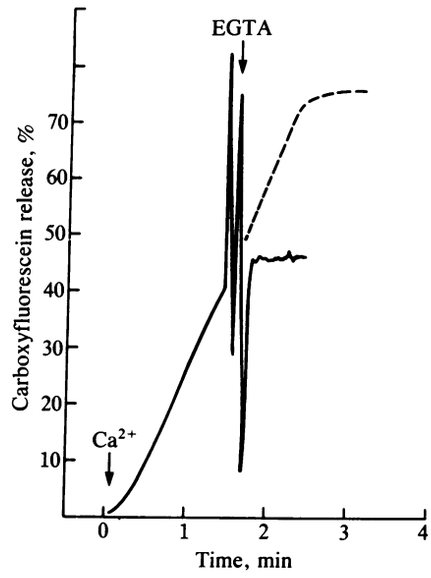

FIG. 3. Control of granule-mediated carboxyfluorescein release from liposomes by Ca^{2+}. In this experiment granules and liposomes were mixed in the NaCl/Hepes buffer. In the absence of Ca^{2+}, no carboxyfluorescein release was observed. At $t = 0$ Ca^{2+} was added and mixed, giving a final concentration of 1 mM. ---, Complete course of carboxyfluorescein release from a previous experiment; —, result of an experiment in which EGTA was added prior to completion of carboxyfluorescein release. The steeply fluctuating pen resulted from the opening of the cuvette chamber and insertion of the micropipette tip into the light path. The liposomes had the same lipid composition as in Fig. 1.

Variation of Liposome Lipid Composition. Vesicles with different lipid composition were tested as targets for granule- or cytolysin-induced carboxyfluorescein release. The liposomes used in the experiment shown in Fig. 1 contained 31 mol% cholesterol. However, the rate of granule-induced carboxyfluorescein release was not detectably altered with liposomes containing no cholesterol or with inclusion of cholesterol up to 40 mol% (data not shown). Dipalmitoyl phospha-

Table 1. Internal fluorescence after partial cytolysin-induced release from liposomes

% CF release by granules before EGTA addition	% CF fluorescence remaining in SUV*		
	Predicted: all SUV leak CF†	Predicted: all-or-none loss‡	Experimental result§
0	3.3	3.3	3.3
51	15	3.3	3.3
63	20	3.3	3.6
70	29	3.3	3.3

*Percentage of carboxyfluorescein (CF) fluorescence in the presence of Triton X-100; egg PtdCho SUV were used in these experiments.
†Values obtained from CF fluorescence quenching curve (12); assumes all vesicles are equal and release the same amount of CF.
‡Assumes some vesicles lost all CF, others lost none.
§Determined after removal of released CF with a PD-10 column.

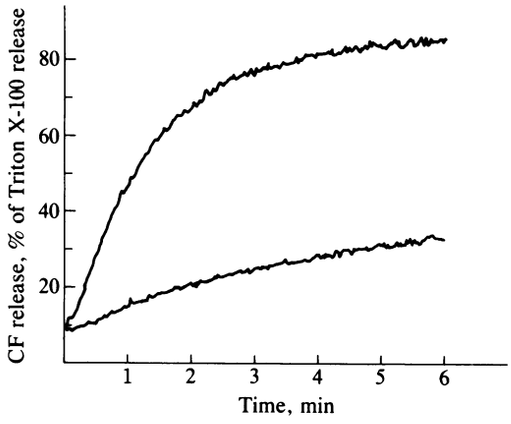

FIG. 4. Carboxyfluorescein (CF) release from dipalmitoyl (lower tracing) and dioleoyl (upper tracing) PtdCho SUV by partially purified cytolysin. This experiment was performed at room temperature, where dioleoyl PtdCho is in the fluid phase and dipalmitoyl PtdCho is in the solid phase. No cholesterol was present in these liposomes. The final cytolysin and lipid concentrations were 200 ng/ml and 0.1 mM, respectively.

tidylcholine (dipalmitoyl PtdCho) SUV, whose lipids are in the solid phase at room temperature (15), exhibited a Ca^{2+}-dependent, cytolysin-induced carboxyfluorescein release, albeit at a markedly slower rate than did dioleoyl PtdCho or egg PtdCho SUV, whose lipids are in the fluid phase at room temperature (15) (Fig. 4). To examine headgroup specificity, we tested REVs made of phosphatidylethanolamine and cholesteryl hemisuccinate (7:3) (16) with no PtdCho. Carboxyfluorescein release from these vesicles was not significantly different from REVs made of pure PtdCho, release indicating no detectable headgroup specificity (data not shown).

Appearance of Liposomes Treated with Granule Cytolysin. To determine the nature of the lesion formed by the interaction of granule cytolysin and liposomes, we examined liposomes exposed to partially purified cytolysin by electron microscopy with negative staining. Fig. 5 shows examples of SUV exposed to different preparations of solubilized cytolysin in the presence of 10 mM Ca^{2+} for 10 min at room temperature (cf. Fig. 1). Ring structures similar to those previously described (3, 5, 6) can be seen associated with SUV (arrows) in Fig. 5*A*. The rings have outer diameters of 15–20 nm and inner diameters of 7–15 nm. Examples of the "ring" structures in side view are shown in Fig. 5 *B* and *C*; this reveals the rings to be short cylinders with diameters of 15–20 nm that apparently insert into the lipid bilayer and extend 7–10 nm above the outer edge of the membrane. Liposomes that bear these structures (arrows with tails) are filled with negative stain and, thus, appear to have dark interiors. Liposomes that did not contain inserted cylindrical structures did not have dark interiors (Fig. 5*B*). This correlation of negative-stain penetration and inserted cylindrical structures is similar to that reported for the C5b-9 complex by Bhakdi and Tranum-Jensen (9). The appearance of these structures was dependent on Ca^{2+}: when parallel samples of liposomes and cytolysin were incubated without Ca^{2+}, no cylindrical structures were observed. Liposomes exposed to Ca^{2+} in the absence of cytolysin never had such structures, nor were they seen in preparations of cytolysin alone with or without Ca^{2+}. Thus, the appearance of these pore-like structures correlates with the release of carboxyfluorescein from these liposomes.

DISCUSSION

Our results show that the cytolysin in the cytoplasmic granules of cytotoxic LGL tumor cells mediates a rapid, Ca^{2+}-dependent permeability increase in liposomes and that ring structures visible in the electron microscope are inserted into liposomes concomitant with this increase in permeability. These results are strikingly similar to those we obtained in a study of the action of purified granules on cells (8), where we described a similar dependence of the lytic activity on divalent cations, similar kinetics and the formation of ring structures under conditions leading to cell lysis. The similarity of cytolysin action on liposomes to its action on cells implies that the mechanisms of these processes are similar and that the nonlipid components of cell targets are not required for cytolysin interaction.

Our previous studies of the cytolytic activity of LGL tumor granules revealed that Ca^{2+} plays a complex role in regulating this phenomenon. Not only is it absolutely required for lysis, parallel to the requirements for liposome marker release, but Ca^{2+} was shown to cause an inactivation of the lytic capacity when incubated with granules prior to exposure to target cells (8). Thus, it is not surprising that the rate of marker release from liposomes declined with time, as seen in Figs. 1 and 4. The present study provides additional insights into the interaction of Ca^{2+} and cytolysin. As seen in Fig. 3, chelation of Ca^{2+} rapidly stops marker release from liposomes, showing that no stable reactive intermediate is generated by the interaction of Ca^{2+} and cytolysin. Furthermore, studies with partially purified cytolysin make it clear that Ca^{2+} does not induce cylindrical structures to form in the absence of lipid. Thus, a complex interaction between

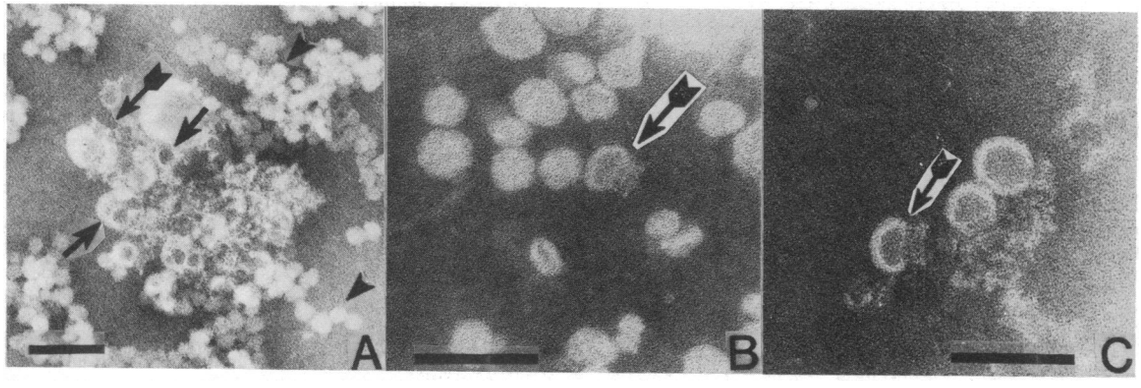

FIG. 5. Appearance of cytolysin-treated liposomes in the electron microscope. Electron micrographs of examples of negatively stained SUV incubated with different soluble cytolysin preparations in the presence of Ca^{2+} as described in the text. Arrows indicate ring structures. Arrows with tails indicate ring structures viewed from the side, which shows them to be short cylinders inserted into the lipid bilayer and extending above the edge of the vesicle membrane. Arrowheads in *A* indicate contaminating Percoll particles that were present in the cytolysin preparation used in this experiment. The liposomes had the same lipid composition as in Fig. 1. (Bar = 60 nm.) (*A*, ×165,000; *B* and *C*, ×275,000.)

Ca^{2+}, lipid, and cytolysin appears to be required for the formation of these pore-like structures.

By varying the structure of the lipids in the liposomes, we have concluded that lipid headgroup structure plays at most a minor role in the ability of the cytolysin to interact with membranes. On the other hand, our data show that when the fatty acid chains are in the solid phase, cytolysin mediates carboxyfluorescein release less efficiently than when they are in the more physiological fluid phase. This suggests that insertion of protein into the hydrophobic portion of the lipid is required for the permeability increase. Such an insertion of cytolysin protein into lipid is also suggested by the images shown in Fig. 5, where one end of the cylinders appears to be inserted into the bilayer of these small liposomes.

These results lend strong support to the hypothesis that the cytolysin in LGL granules acts through the insertion of a protein pore into the lipid bilayer. Such pores were proposed to explain the results of our previous experiments in which lymphocytes were shown to increase the ionic permeability of planar lipid bilayers modeling antibody-dependent cell-mediated cytotoxicity (17), but subsequent studies of the action of cytotoxic lymphocytes on liposomes have proven disappointing. One explanation of this is that liposomes are much smaller targets than cells and that any given number of lethal units will cause a smaller percentage of total marker release than would be the case for larger targets (4). This effect may also explain why cytolysin-induced carboxyfluorescein release from small unilamellar liposomes is much slower than from larger unilamellar liposomes (Fig. 1*B*).

The pore insertion model for cell-mediated cytotoxicity has received recent support because of the general similarity of ring structures identified in the electron microscope as a result of the lytic action of complement (9, 18), LGL (3, 5, 6), and cytotoxic T-lymphocytes (19). Extensive studies of the action of complement on liposomes (20) helped to establish the concept that the membrane attack occurs by the creation of amphipathic proteins during activation, and the insertion of these into lipid bilayers to form pores (9). Since purified LGL granules do not contain major protein bands similar to those of the complement C5b-9 complex (7), it does not appear that granule cytolysin and complement utilize common components. Nevertheless, the present results suggest that these two cytolytic effector pathways of the immune system share common mechanistic features, as suggested by Mayer (21). It remains possible, however, that granule components other than the cytolysin may contribute to target cell damage and that the creation of membrane pores allows the penetration of these components into the target cell.

We thank Drs. J. N. Weinstein, D. M. Segal, and S. J. Shaw for valuable suggestions in the preparation of this manuscript.

1. Henney, C. S. & Gillis, S. (1984) in *Fundamental Immunology*, ed. Paul, W. E. (Raven, New York), pp. 669–684.
2. Timonen, T., Ortaldo, J. R. & Herberman, R. B. (1981) *J. Exp. Med.* **153**, 569–582.
3. Henkart, M. P. & Henkart, P. A. (1982) in *Mechanisms of Cell-Mediated Cytotoxicity*, eds. Clark, W. R. & Golstein, P. (Plenum, New York), pp. 227–242.
4. Simone, C. B. & Henkart, P. A. (1980) *J. Immunol.* **124**, 952–963.
5. Dourmashkin, R. R., Deteix, P., Simone, C. B. & Henkart, P. A. (1980) *Clin. Exp. Immunol.* **43**, 554–650.
6. Podack, E. R. & Dennert, G. (1983) *Nature (London)* **302**, 442–445.
7. Millard, P. J., Henkart, M. P., Reynolds, C. W. & Henkart, P. A. (1984) *J. Immunol.* **132**, 3197–3204.
8. Henkart, P. A., Millard, P. J., Reynolds, C. W. & Henkart, M. P. (1984) *J. Exp. Med.* **160**, 75–93.
9. Bhakdi, S. & Tranum-Jensen, J. (1983) *Biochim. Biophys. Acta* **737**, 343–372.
10. Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R. & Hagins, W. A. (1977) *Science* **195**, 489–492.
11. Weinstein, J. N., Klausner, R. D., Innerarity, T., Ralston, E. & Blumenthal, R. (1981) *Biochim. Biophys. Acta* **647**, 270–284.
12. Szoka, F. C. & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4194–4198.
13. Ralston, E., Hjelmeland, L. M., Klausner, R. D., Weinstein, J. N. & Blumenthal, R. (1981) *Biochim. Biophys. Acta* **649**, 133–137.
14. Gately, M. K. & Martz, E. (1979) *J. Immunol.* **122**, 482–489.
15. Chapman, D. (1975) *Quart. Rev. Biophys.* **8**, 185–235.
16. Ellens, H., Bentz, J. & Szoka, F. C. (1984) *Biochemistry* **23**, 1532–1538.
17. Henkart, P. & Blumenthal, R. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2789–2793.
18. Podack, E. R. & Tschopp, J. (1982) *J. Biol. Chem.* **257**, 15204–15212.
19. Dennert, G. & Podack, E. R. (1983) *J. Exp. Med.* **157**, 1483–1495.
20. Kinsky, S. C. & Nicolotti, R. A. (1977) *Annu. Rev. Biochem.* **46**, 49–67.
21. Mayer, M. M. (1977) *J. Immunol.* **119**, 1195–1203.