

The Lytic Granules of Natural Killer Cells Are Dual-Function Organelles Combining Secretory and Pre-lysosomal Compartments

Janis K. Burkhardt, Susan Hester, Cheryl K. Lapham, and Yair Argon

Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

Abstract. Cytolytic lymphocytes contain specialized lytic granules whose secretion during cell-mediated cytotoxicity results in target cell death. Using serial section EM of RNK-16, a natural killer cell line, we show that there are structurally distinct types of granules. Each type is composed of varying proportions of a dense core domain and a multivesicular cortical domain. The dense core domains contain secretory proteins thought to play a role in cytotoxicity, including cytotoxicin and chondroitin sulfate proteoglycan. In contrast, the multivesicular domains contain lysosomal proteins, including acid phosphatase, α -glucosidase, cathepsin D, and LGP-120. In addition to their protein content, the lytic granules have other properties in

common with lysosomes. The multivesicular regions of the granules have an acidic pH, comparable to that of endosomes and lysosomes. The granules take up exogenous cationized ferritin with lysosome-like kinetics, and this uptake is blocked by weak bases and low temperature. The multivesicular domains of the granules are rich in the 270-kD mannose-6-phosphate receptor, a marker which is absent from mature lysosomes but present in earlier endocytic compartments. Thus, the natural killer granules represent an unusual dual-function organelle, where a regulated secretory compartment, the dense core, is contained within a pre-lysosomal compartment, the multivesicular domain.

DIRECTED secretion of specialized granules is a major mechanism by which natural killer (NK) cells and cytotoxic lymphocytes (CTL) kill their target cells. Upon recognition of an appropriate target, the killer cell granules realign to face the target, fuse with the plasma membrane of the killer cell, and release their contents (29). Two sets of proteins are contained in the granules and secreted in this way. One set consists of proteins expressed specifically by cytotoxic lymphocytes. Foremost among these is cytotoxicin (also called perforin), which assembles into amphipathic pore complexes, capable of damaging the target cell membrane (6, 45). Other members of this set are lymphocyte-specific serine proteases (31, 57) and a chondroitin sulfate proteoglycan (35). Although the functions of all the proteases and the proteoglycan are not known, the protease granzyme A may be involved in breakdown of the target nucleus (28). The second set of proteins that is released from NK cells and CTL during cytotoxicity consists of lysosomal hydrolases, including acid phosphatase, arylsulfatase, and β -glucuronidase (19, 42, 61, 63). The role of these lysosomal enzymes in cytotoxicity is not clear. One hypothesis is that their secretion aids the destruction of target cells, but other functions are equally possible.

At the EM level it is clear that there is morphological heterogeneity among the granules. This is particularly obvious in human large granular lymphocytes (LGL) that are highly enriched in NK activity (12, 40, 62). Neighbour et al. (40) distinguished two major types of granules. Type I granules are round, with a homogeneous electron-dense core surrounded by a thin cortex. By contrast, type II granules are larger, more irregularly shaped, and contain lamellar and vesicular material. These cells also contain granules with an intermediate morphology; these have one or more small dense cores surrounded by a large multivesicular cortex. Granules with similar morphologies are present in normal rat and mouse LGL and in several CTL and NK cell lines (13, 19, 26, 38, 43).

The relationship among the granule subtypes is not understood. It is not known if the various types of granules are truly distinct, or if they actually result from different planes of section through one nonhomogeneous organelle. If the subtypes are distinct, two questions arise: What function does each perform, and are they somehow derived from one another? Finally, while it is known that at least some granules contain lysosomal hydrolases (19, 42, 61, 63), the source of the hydrolases that are secreted during cytotoxicity has not been identified. This in turn raises the question: Are the granules lysosomes, secretory granules, or both?

To approach these problems, we took advantage of the rat leukemic clonal line RNK-16, which has the structure of an LGL, surface markers characteristic of NK cells, and the ac-

1. *Abbreviations used in this paper:* CF, cationized ferritin; CTL, cytotoxic T lymphocyte; DAMP, 3-(2,4-dinitro-anilino)-3-amino-N-methyldipropylamine; LGL, large granular lymphocyte; man-6-P, mannose-6-phosphate; NK, natural killer.

tivity of an NK cell (48). The granules of RNK-16 exhibit the morphological heterogeneity described for polyclonal populations of NK and CTL (11, 38). We have previously shown that two granule secretory components, cytolysin and the serine proteases, are confined to the core regions of the granules (11). In the present study, we have determined the distributions of several other proteins with respect to the granule subtypes and structural domains. We also asked if any of the granules have functional properties of lysosomal compartments. These studies indicate specialization of granule compartments, and suggest that lytic granules have a secretory domain and a domain which lies along the endosomal-lysosomal traffic pathway.

Materials and Methods

Cell Lines

RNK-16 clone CRC⁻ is a clonal NK cell line derived from an LGL tumor that arose spontaneously in aging Fisher rats (reference 48; gift of C. Reynolds, National Cancer Institute, Frederick, MD). This clone retains NK morphology and surface markers, and does not require or respond to interleukin-2 (48). The cells were grown in RPMI 1640 medium supplemented with pyruvate, nonessential amino acids, glutamine, β -mercaptoethanol, penicillin/streptomycin, and 5% FCS. Clone 33 is a mouse CTL line specific for the N protein of vesicular stomatitis virus, and alloreactive with the H-2K^b major histocompatibility complex antigen (54). These cells were obtained from Dr. J. Sheil (University of West Virginia, Morgantown, West Virginia) as cultures stimulated with irradiated C57BL/6 mouse splenocytes. Clone 33 cells were analyzed at peak activity, 4–5 d after stimulation.

Isolation of Rat LGL

Normal rat LGL were isolated from either spleens or heparinized peripheral blood from retired breeder PVG rats (bred in the colony of Dr. A. Sanfilippo, Duke University, Durham, North Carolina), using a modification of the procedure of Reynolds et al. (47). Leukocytes were purified by centrifugation through Ficoll-Hypaque density gradients and adherent cells were removed using nylon wool. LGL were purified further by density gradient centrifugation through Percoll (Pharmacia Fine Chemicals, Piscataway, NJ). The Percoll was adjusted to 300 mosM with 10 \times PBS, and four-step gradients with Percoll densities of 1.072, 1.069, 1.066, and 1.054 g/ml were prepared. These gradients were overlaid with leukocytes and spun at room temperature for 30 min at 1,200 rpm in a centrifuge (model RC-3; DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Wilmington, DE). Cells banding at each interface were harvested, washed, and tested for cytotoxicity against YAC-1 target cells. The majority of the cells with lytic activity banded at the 1.054/1.066 and 1.066/1.069 interfaces, and the vast majority of LGL in these fractions were positive for the NK surface marker 3.2.3 (14), as judged by immunoelectron microscopy. These enriched rat LGL were prepared for EM as described below.

Cytotoxic activity of all lymphocytes was monitored by measuring chromium release from YAC-1 (for RNK-16 and polyclonal LGL) or VSV-infected EL-4 (for clone 33) targets. Typical lysis by RNK-16 was 25% in 4 h at an effector/target ratio of 25:1, in good agreement with the killing ability of normal rat LGL (47). Typical lysis by clone 33 cells was 60% at an effector/target ratio of 4:1.

Conventional Electron Microscopy

Conventional EM was performed as described previously (11). Briefly, cell suspensions were fixed in 2% glutaraldehyde/2.5 mM CaCl₂/0.15 M sodium cacodylate, pH 7.4. Cells were fixed at room temperature except in the case of timed uptake experiments, when they were fixed at 4°C. Cells were postfixed with 2% OsO₄/0.5% potassium ferrocyanide in the same buffer, stained en bloc with 1% uranyl acetate/0.2 M sodium acetate, pH 5.5, dehydrated with graded ethanols, and embedded in EMBED 812 (EM Sciences, Fort Washington, PA). Silver sections were cut using a Reichert Ultracut E, and examined with an electron microscope (Philips EM300) at 80 kV.

Serial Section Analysis

Ribbons of pale gold sections were collected on formvar-coated slot grids. Three series of 15–26 consecutive sections, each series representing 1.3–2.3 μ m total thickness, were analyzed. Since the largest granules have a diameter of \sim 1 μ m, many granules were contained completely within each series. Four cells, exhibiting a variety of granule types, were selected and photographed at a magnification of 9,000 in each consecutive section. Individual granules were identified in the micrographs and only those granules that were completely contained within the series were evaluated. A granule was scored as type I, type II, or intermediate in each section, independently of its score in other sections.

Uptake of Cationized Ferritin

RNK-16 cells were washed twice with serum-free RPMI, resuspended at 5 \times 10⁶ cells/ml in ice cold serum-free RPMI, and transferred to flat-sided culture tubes (Nunc, Newbury, CA). Cationized ferritin (CF; Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 0.5 mg/ml and the cells were incubated for 10 min on ice to allow the CF to adhere to cell surfaces. Samples were then quickly warmed to either 20°C or 37°C, as required, and cultured for up to 3 h. Some cultures were then diluted with serum-containing medium, and incubated further for a total of either 5 or 22 h. The viability after these incubations was better than 70%. At the end of each incubation, cells were washed twice with an excess of ice cold RPMI and fixed for EM as described above. Lysosomes and granules containing ferritin were quantified on randomly selected cells examined at 17,000 \times . Round, multivesicular organelles, smaller than 500 nm, were considered lysosomes and those larger than 500 nm were scored as granules. 20–200 profiles in each category were counted.

Acid Phosphatase Cytochemistry

Acid phosphatase cytochemistry was performed using the method of Hand and Oliver (27). Cells were fixed for 10 min in suspension with 1% paraformaldehyde/1% glutaraldehyde/0.05% CaCl₂/0.1 M sodium cacodylate, pH 7.4, pelleted, embedded in agar, and stored overnight in cacodylate buffer containing 7% sucrose. Blocks were washed with 5% sucrose/20 mM sodium acetate, pH 5.0, and incubated for 1 h at 37°C in 0.1% cytidine 5'-monophosphate/3.6 mM lead nitrate/5% sucrose/20 mM sodium acetate, pH 5.0. They were treated briefly with 1% ammonium sulfide, and postfixed and processed as described above. The specificity of the reaction was verified by omission of substrate.

Immunoelectron Microscopy

Immunoelectron microscopy was performed using the method of Griffiths et al. (24). Cells were first fixed for 10 min with 4% paraformaldehyde/150 mM Pipes, pH 7.0, and then fixed for several days with 8% paraformaldehyde in the same buffer. Samples were cryoprotected by infusion with 2.1 M sucrose in PBS and frozen in liquid nitrogen. Ultrathin frozen sections were cut using a cryo system (Reichert FC4E) and transferred to grids in a drop of 2.3 M sucrose in PBS. Before labeling, sections were blocked by floating on 5% FCS/PBS. Grids were incubated for 30 min in primary antibody, washed with PBS, incubated for 20 min with colloidal gold secondary reagents, washed with PBS and water, coated with 2% methylcellulose/0.3% uranyl acetate, and dried.

Primary and secondary reagents were diluted in 5% FCS/PBS. Rabbit antiserum to cytolysin (reference 49; gift of P. Henkart, National Cancer Institute, Bethesda, MD) was diluted 1:30. Rabbit antiserum to cathepsin D (reference 7; gift of T. Brulke, Institut für Biochemie, Göttingen, FRG) was diluted 1:100. Rabbit antiserum to α -glucosidase and two rabbit antisera to the cation-independent mannose-6-phosphate (man-6-P) receptor (reference 8; all gifts of W. Brown, Cornell University, Ithaca, NY) were diluted 1:40, 1:100, and 1:100, respectively. Rabbit antiserum to lysosomal glycoprotein 120 (reference 3; gift of I. Mellman, Yale University, New Haven, CT) was used at a dilution of 1:30. Chondroitin sulfate proteoglycan was detected with mouse mAb CS56 (reference 3; Sigma Chemical Co.) at 1:200. Rabbit antisera were detected with protein A-colloidal gold (5 nm or 10 nm), prepared according to Slot and Geuze (55), and used at dilutions of 1:15 and 1:5, respectively. Antibody CS56, and IgM, was detected with goat anti-mouse IgM/10-nm colloidal gold (Janssen Life Sciences Products, Piscataway, NJ), at a dilution of 1:10.

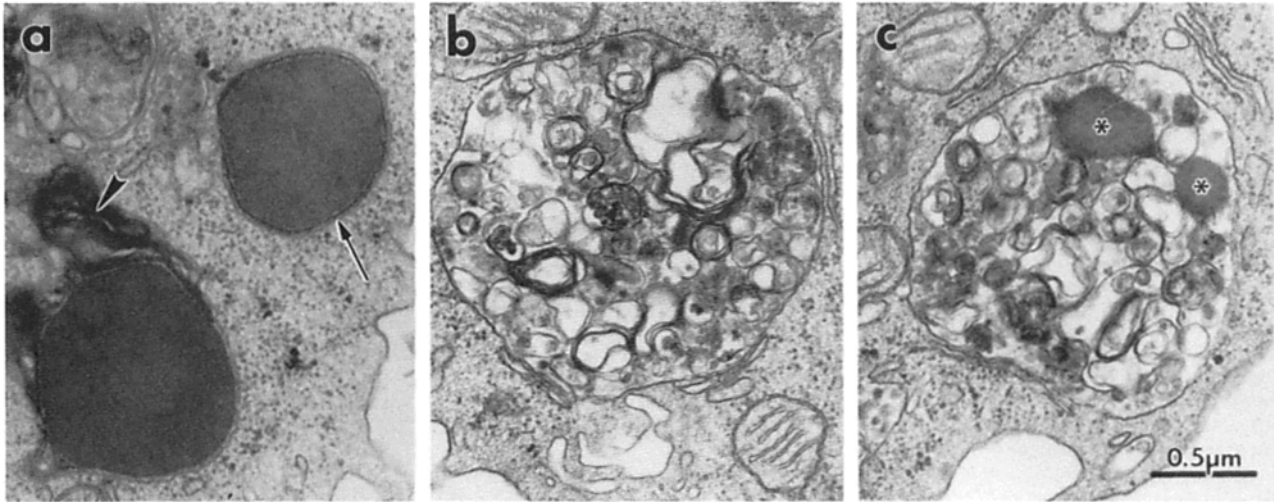


Figure 1. Electron micrographs showing the heterogeneity of RNK-16 granules. (a) Type I granules. Note the thick membrane surrounding the core (*arrow*). Arrowhead indicates membrane whorl within the cortical rim. (b) A type II granule. (c) An intermediate type granule with small cores (*). Note the apparent absence of a membrane around these cores.

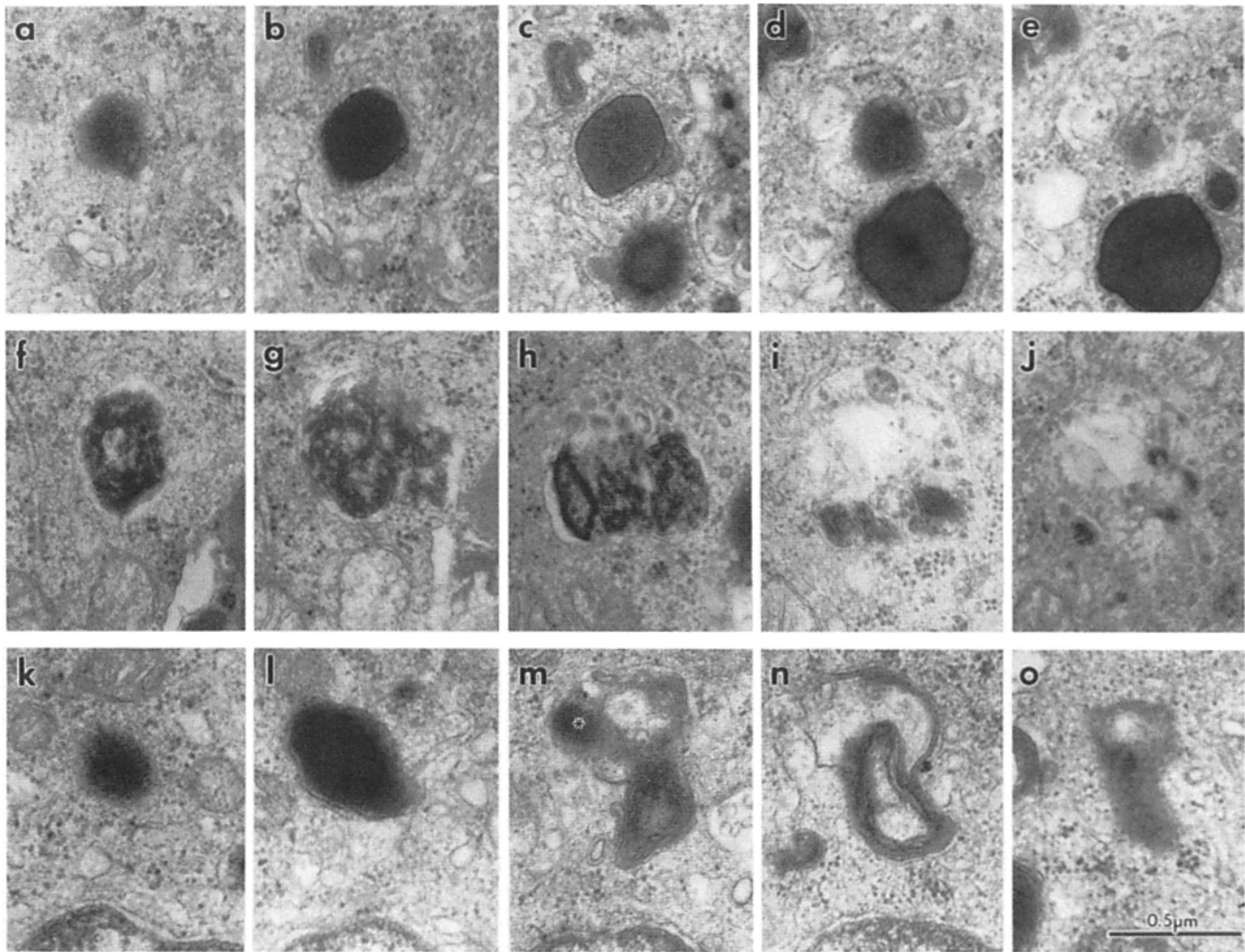


Figure 2. Electron micrographs showing selected serial sections through type I (*a-e*), type II (*f-j*), and intermediate-type (*k-o*) granules. * indicates small dense core surrounded by lamellar material. Note that in *k* and *l*, this granule would be scored as type I, while in *n* and *o* it would be scored as type II.

Estimation of Granule pH

Granule pH was measured using a modification of the technique of Anderson et al. (2). RNK-16 cells were brought to 5×10^6 cells/ml in complete medium, and incubated for 30 min at 37°C with 50 μ M 3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP; Oxford Biomedical, Oxford, MI). Excess DAMP was removed by washing cells twice with serum-free RPMI. The cells were then fixed with 2% glutaraldehyde/2.5 mM $\text{CaCl}_2/0.15$ M sodium cacodylate, pH 7.4, and otherwise processed as described above for immunoelectron microscopy. Grids were immunolabeled with HDPI anti-DNP monoclonal (Oxford Biomedical) at a dilution of 1:3, followed by protein A-10-nm colloidal gold. The low dilution of anti-DNP was chosen intentionally to give a background of nonspecific labeling. The gold particles over nuclei were then quantified and the pH represented by this level of label was set equal to 7.0 (1). The quantitation of DAMP was performed by enumerating gold particles which fell within various structures and measuring the area of these structures using a digital planimeter (model 1224; Numonics Corp., Lansdale, PA). The pH was estimated by the following formula: $\text{pH} = 7.0 - \ln(x/y)$, where x is the density of DAMP labeling over the structure of interest and y is the density of DAMP labeling over the nucleus (1).

Results

Granules Can Be Divided into Three Distinct Types Based on the Proportion of Cores and Multivesicular Domains

The granules of RNK-16, like those of other NK cells, are heterogeneous. Based upon their appearance in random EM sections, they can be divided into three groups, corresponding to the groups described by Neighbour et al. (40). Some consist of a predominant dense core, surrounded by a thin cortical rim of membraneous material (Fig. 1 a; type I). The thickness of this lamellar rim is somewhat variable, and in some areas it widens to include a small multivesicular region, or a whorl of myelin-like membrane (Fig. 1 a, arrowhead). With the exception of the cortical rim, these granules closely resemble dense core secretory granules of other cells. Two other groups of granules are characterized by a large amount of vesicular and lamellar material (Fig. 1, b and c). These are generally larger and more pleomorphic than the dense core granules. Profiles of some of these multivesicular granules show the presence of small dense cores, structurally similar to those of type I granules (Fig. 1 c). Those multivesicular granules lacking an apparent dense core have been designated type II, while those with both core and multivesicular domains have termed intermediate type (40).

It has been assumed that the three types of granules observed in random EM sections represent distinct structures. However, the possibility that they result from different planes of section through one or two complex organelles has never been excluded. It is particularly important to resolve this question in light of the existence of structures and proteins (see below) common to the different granule types.

We evaluated 27 granules throughout their length by serial sections. Seven of the granules consisted throughout of a predominant dense core (type I). Selected sections of one such granule are shown in Fig. 2, a-e. Three of the granules we evaluated contained vesicular material throughout their entirety, and never contained discernible dense cores (type II; Fig. 2, f-j). Seventeen of the granules were classified as intermediate type at some point along their length; i.e., there was a core surrounded by a substantial multivesicular cortex in some sections. Of these, five had small cores embedded

throughout the granule length (intermediate type throughout). In four others, subsequent sections showed a portion of the granule which consisted of one dominant core surrounded by a thin cortex (intermediate - type I). In seven more, subsequent sections revealed multivesicular regions lacking cores (intermediate - type II). Finally, serial sections of one granule revealed a conversion from a type I morphology, through an intermediate type structure, to a type II morphology (Fig. 2, k-o).

Two conclusions can be drawn from this analysis. First, the three types of granules that are seen in random EM sections do in fact exist as distinct structures. Second, we conclude that the three granule types are related. Any granule may, in subsequent sections, "convert" to a different morphology, so that classifying granules based on their appearance in any one section may be misleading. The various morphologies are due to two structural domains, dense cores and

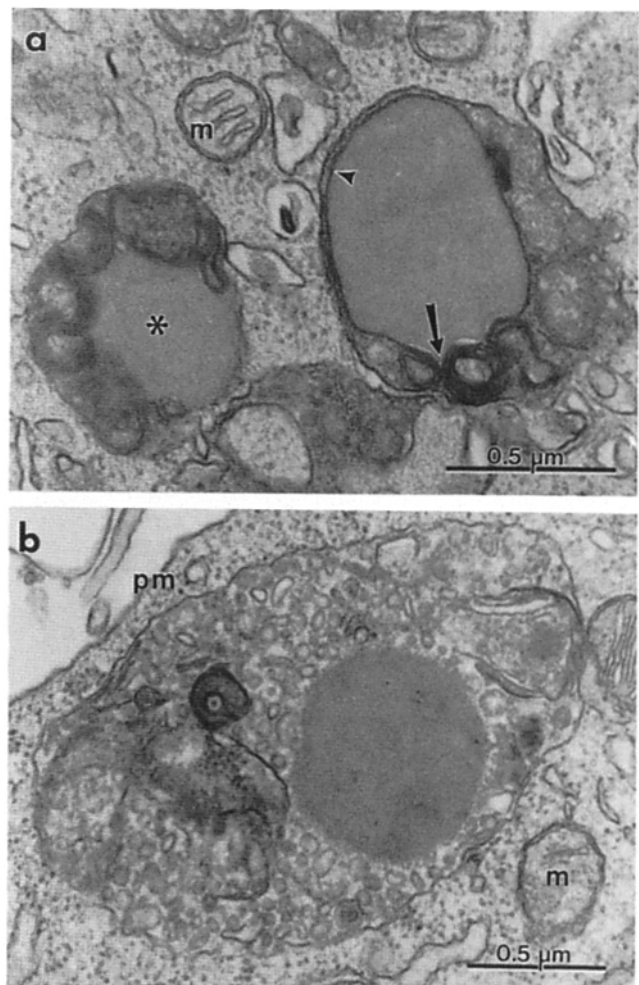


Figure 3. Electron micrographs showing the presence or absence of membrane around the granule cores. (a) Two intermediate-type profiles of granules from RNK-16. The core of one granule is circled by a membrane (arrowhead), while another core (*) is devoid of a membrane. Arrow indicates apparent discontinuity in the core membrane. (b) An intermediate-type profile from clone 33 cells, showing the absence of membrane around the core. *m*, mitochondrion; *pm*, plasma membrane.

multivesicular regions, which are present in varying proportion in each granule.

To understand how the two domains are organized within the same granule, we looked for a membrane separating them. In most of the granules we observed (e.g., Fig. 3 *a*, *; Fig. 1 *c*), the cores and cortices did not appear to be separated by a membrane. This was also true in samples prepared for cryoelectron microscopy (see Fig. 5). However, we did observe delimiting membranes in some profiles (Fig. 3 *a*, arrowhead). When present, the core membranes sometimes appeared to be thicker or more complex than a "unit membrane" (Fig. 1 *a*, arrow), and discontinuities in them were often apparent (e.g., Fig. 3 *a*, arrow). This suggests that in at least some cases membranes do not actually envelope the core, but are simply formed by local apposition of cortical lamellae.

Peters et al. (43) have described granules having an intermediate-type structure in CTL, and found that their cores and cortices are separated by a membrane. We therefore compared the granule structure of RNK-16 cells with that of clone 33, a murine CTL line (Fig. 3 *b*). Each of the granule types described above for RNK-16 was found in clone 33 cells. The main difference was that in clone 33, the cortical domains exhibited a more regular structure, with more vesicles and fewer membrane whorls than in RNK-16. Membranes separating the granule domains were very rare in clone 33 cells (Fig. 3 *b*). This observation suggests that in clone 33, as in RNK-16, a delimiting membrane is not necessary for segregation of granule domains.

Secretory and Lysosomal Components Are Segregated within Granules

We have previously shown that cytolysin and the serine proteases, proteins expressed specifically by cytolytic lymphocytes and secreted during cytolysis, are restricted in their distribution within granules. These proteins are present in the dense core regions of both type I and intermediate type granules, but are not found in the multivesicular cortical regions of any granule type (11). We have now determined the subcellular distribution of a battery of other granule proteins, both cytolytic and lysosomal. Our main objective was to determine whether each protein is concentrated within the dense granule cores or the multivesicular regions.

Fig. 4 *a* shows an immunolabeled cryosection demonstrating the presence of cytolysin in the dense cores. An identical distribution is observed for granule serine proteases (11). Labeling of a third secretory component associated with cytolysis, a chondroitin sulfate proteoglycan, shows that it too is concentrated in the same compartment (Fig. 4 *b*). None of these regulated secretory products was detected in the multivesicular cortices.

The multivesicular domains of the granules not only have a different ultrastructure from the cores, but they also contain a different set of proteins, lysosomal proteins. Fig. 5 shows the distribution of several lysosomal proteins localized by immunolabeling and enzyme cytochemistry. Each protein was abundant in the granule cortical regions. As shown in Fig. 5, *a-c*, cathepsin D was primarily found in the multivesicular regions of granules. The density of label was similar over all multivesicular domains, whether or not the

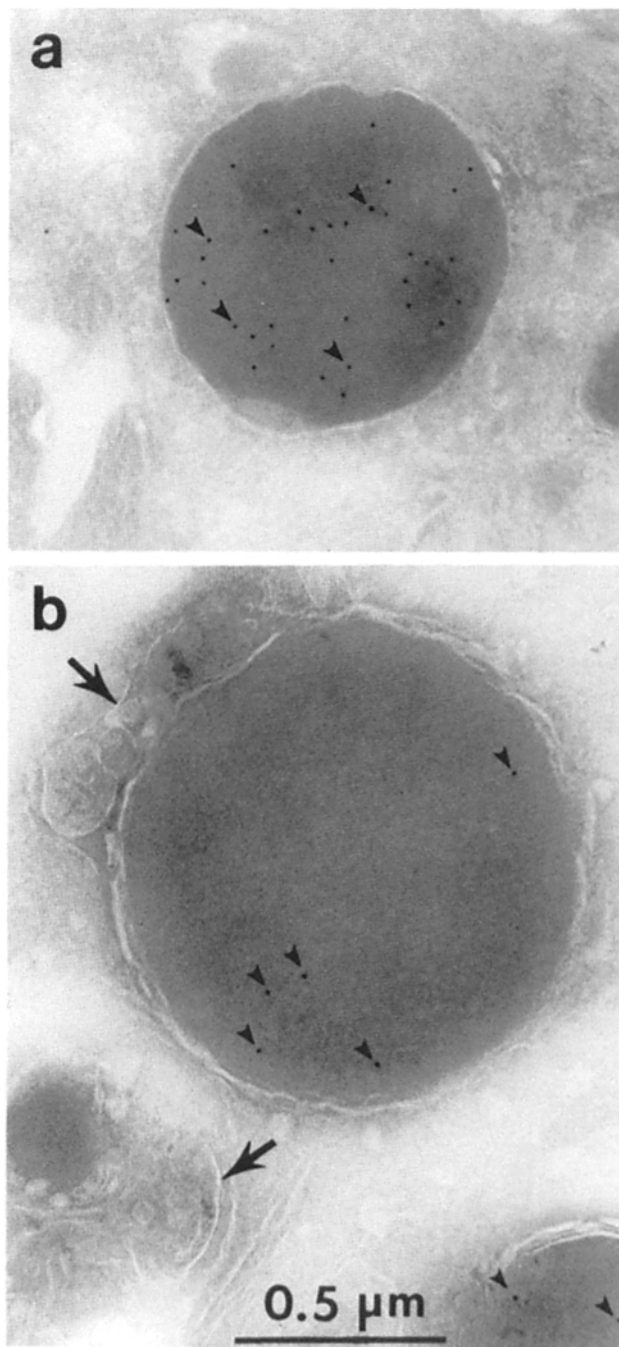
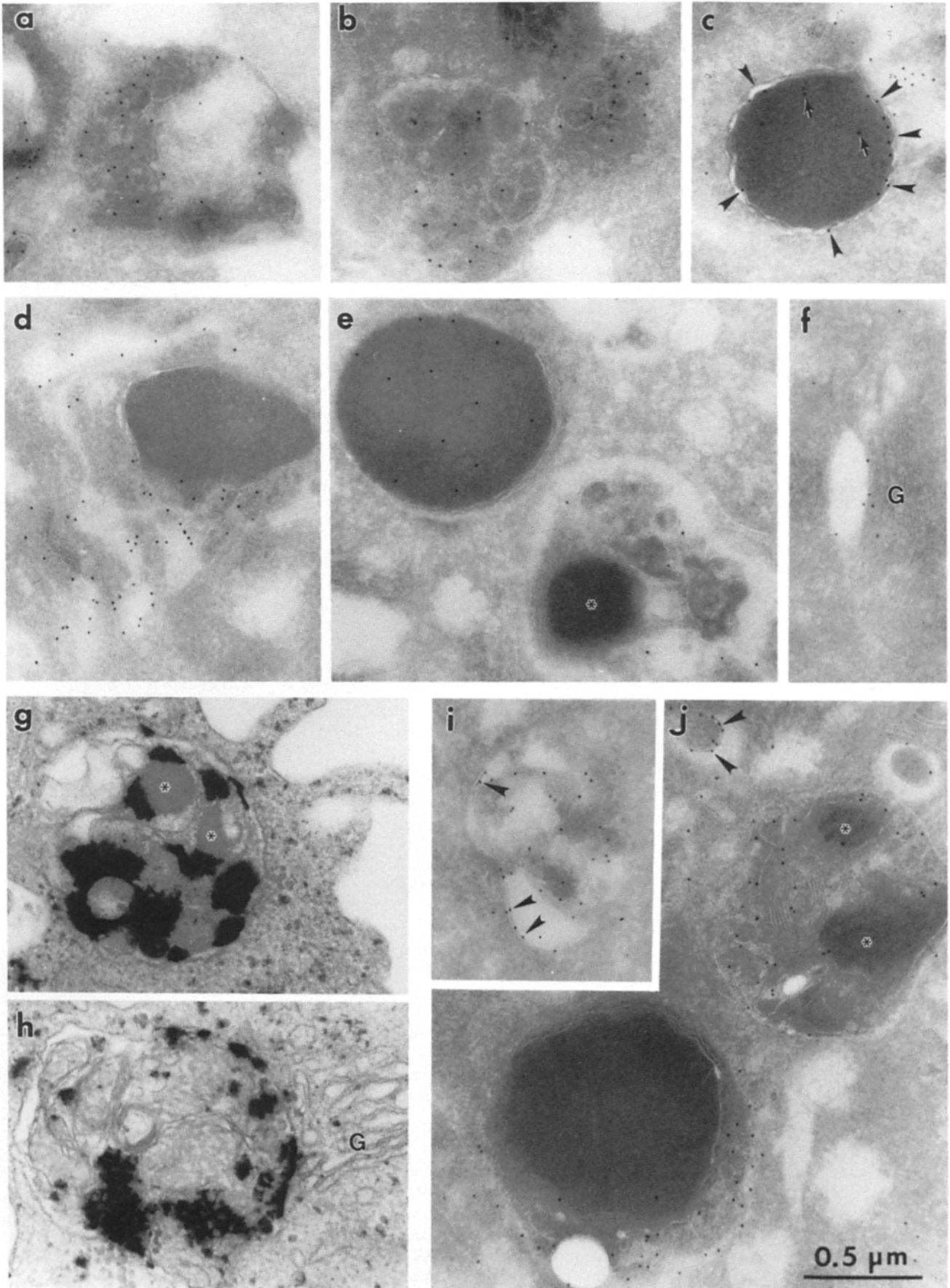


Figure 4. Immunolocalization of secretory proteins within the granules of RNK-16. (a) An ultrathin cryosection labeled with anti-cytolysin and 10-nm colloidal gold. (b) A similar cryosection labeled with antichondroitin sulfate proteoglycan and colloidal gold. Note the presence of gold over the granule cores (arrowheads) and its absence over the multivesicular cortical regions (arrows).

granule profile contained core material. Interestingly, the thin cortical regions of type I profiles were also positive for cathepsin D; this could often be seen as a ring of gold particles decorating the periphery of the dense cores (Fig. 5 *c*, arrowheads). Occasional labeling of the dense cores themselves was also observed, albeit at much lower density (Fig. 5 *c*, arrows). This labeling of the cores could result from



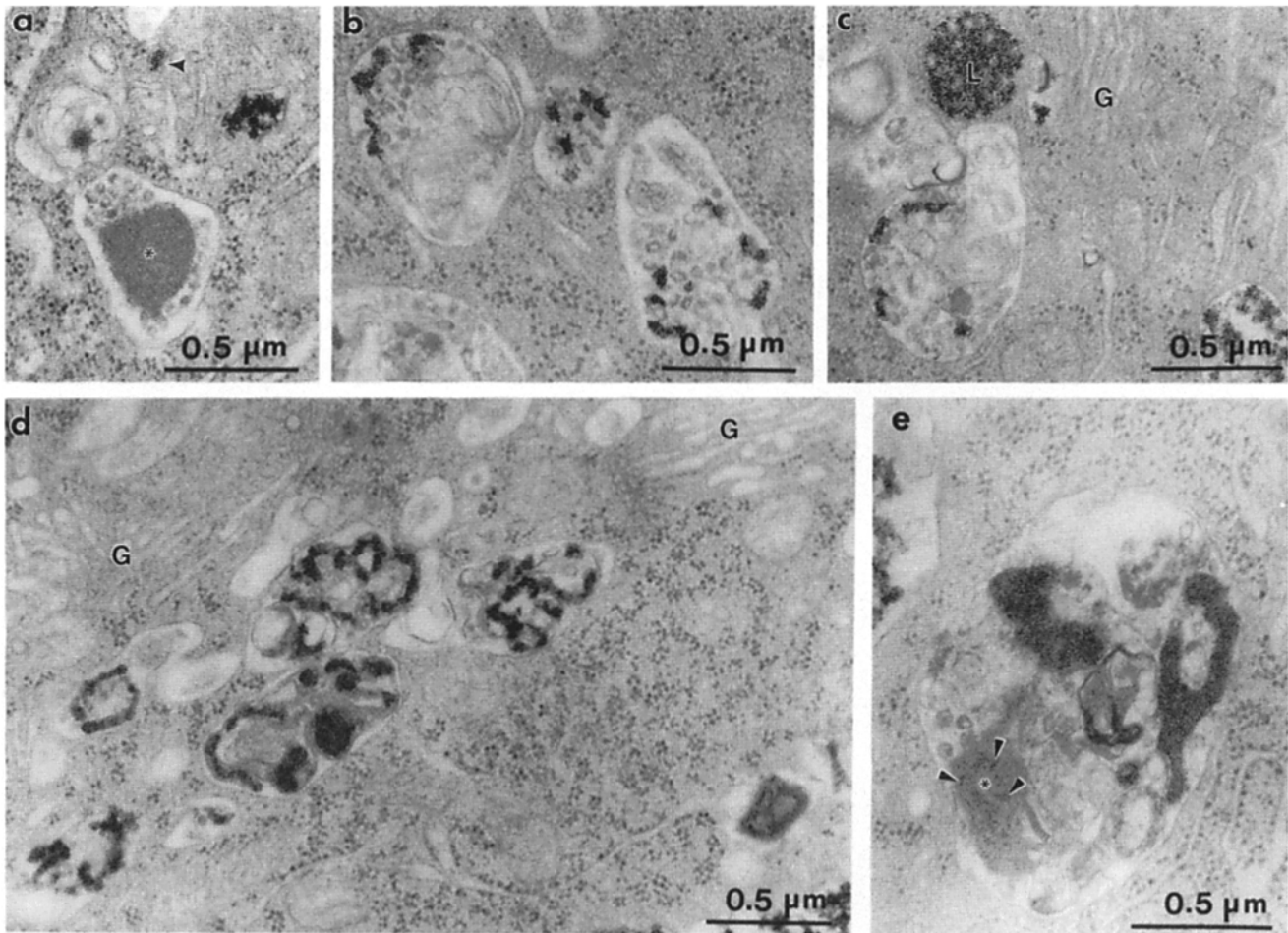


Figure 6. Time course of endocytic uptake of CF into lytic granules. CF was bound to cell surfaces at 4°C, and cells were warmed to 37°C for 15 min (a), 1 h (b and c), and 3 h (d and e). G, Golgi complex; L, lysosomes; *, core.

weak cross-reaction of the anticathepsin antibody with the serine proteases, which are related proteins. However, as discussed below, other unrelated hydrolases were also present at low levels in the granule cores.

The distribution of α -glucosidase, another lysosomal hydrolase, was similar to that of cathepsin D. Most of the label was found over the multivesicular regions of both type II and intermediate granule profiles (Fig. 5 d and lower granule in 5 e), and over the thin cortex of type I profiles (not shown). Significant levels of label were also present over one face of the Golgi stack (Fig. 5 f). Fig. 5 e also shows a rare α -glucosidase-positive dense core. Because such cores were observed reproducibly with antisera to different lysosomal proteins, it seems that lysosomal enzymes are present within a few of the cores, although the vast majority of each enzyme is always concentrated in the multivesicular regions.

A third lysosomal hydrolase, acid phosphatase, was localized by its cytochemical reaction product. As with the other hydrolases, acid phosphatase reaction product was found in the multivesicular regions, whether or not they contained cores (Fig. 5, g and h). Reaction product was also present as a thin rim around type I granules (not shown), but it was always excluded from the dense core regions.

The segregation of lysosomal proteins to the multivesicular regions of the granules was not limited to soluble lysosomal hydrolases. The membrane-spanning protein LGP-120, a membrane marker for lysosomes and endosomes (34), had the same distribution as the soluble lysosomal enzymes. It was found in the multivesicular regions and not in the cores (Fig. 5, i and j). As expected, labeling of LGP-120 differed from that of the soluble hydrolases in that gold particles decorated granule membranes (Fig. 5, i and j; arrowheads).

Figure 5. Localization of lysosomal proteins within the granules of RNK-16. (a-c) Ultrathin cryosections labeled with anticathepsin D and 10-nm colloidal gold. Arrowheads in c point to labeling of the thin cortical rim of a type I granule. Arrows show occasional gold particles labeling this granule core. (d-f) Cryosections labeled with anti- α -glucosidase. The granule in d and one of the granules in e represent the typical labeling pattern, in that the cortices are labeled and the cores are unlabeled. Labeled cores like the one shown in e are rare. G, Golgi stack. (g and h) Epoxy sections showing cytochemical localization of acid phosphatase. Note the exclusion of reaction product from the granule cores (*). (i and j) Cryosections labeled with antibody to the *trans*-membrane protein LGP-120. Arrowheads point to gold particles decorating membranes. * indicates dense cores of intermediate-type granule.

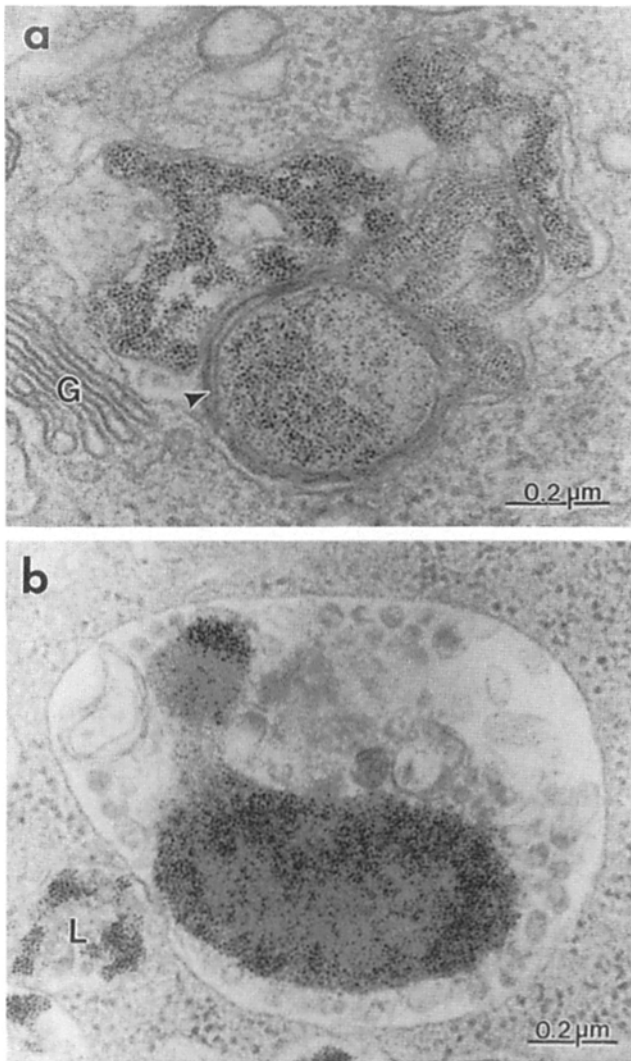


Figure 7. Uptake of CF into the granule cores. (a) Granule containing CF in both its core and cortex. (b) Granule in which CF is concentrated in the core. Both distribution patterns were observed at times longer than 3 h. Note the membrane around the core in a (arrowhead), and the lack of a membrane in b. G, Golgi complex; L, lysosome.

These results show that, based on both morphology and protein content, the three types of lytic granules are composed of two distinct domains. The dense cores of type I and intermediate type granules are equivalent. They contain the components that are unique to cytolytic cells and that are secreted in a regulated fashion during cell-mediated cytotoxicity. Similarly, the multivesicular regions of type II and intermediate type granules and the membranous rims of type I granules are all equivalent. These regions contain both membrane-bound and soluble lysosomal proteins, some of which are also secreted during cytotoxicity.

Lytic Granules Accumulate Exogenous Ligands

The similarities between multivesicular granule domains and lysosomes led us to test whether granules could perform lysosomal functions. We therefore asked whether the granules are capable of taking up CF, a nonspecific membrane

tracer. CF was bound to cell surfaces at 4°C, and the cells were then warmed to 37°C for various times to allow its internalization. After 15 min of uptake, CF was present in endosomal vesicles and tubules at the cell periphery. It was absent from lysosomal structures, and from the lytic granules (Fig. 6 a). By 1 h of uptake (the time required for uptake into lysosomes in other cells; references 41, 60), CF was abundant in most lysosomal structures (Fig. 6 c). At this time CF was present in 45% of the multivesicular granule regions (Fig. 6, b and c), while cores were usually devoid of CF. Uptake of CF into the multivesicular regions (both type II and intermediate profiles) reached saturation after 3 h, at a level of 80–90% (Fig. 6 d). It is important to note that the Golgi complex never contained CF (Fig. 6, c and d). Thus, the presence of CF in the granules cannot be explained by exocytic traffic through this organelle.

Interestingly, by 3 h a significant fraction (10–15%) of granule cores in intermediate profiles contained CF (Fig. 6

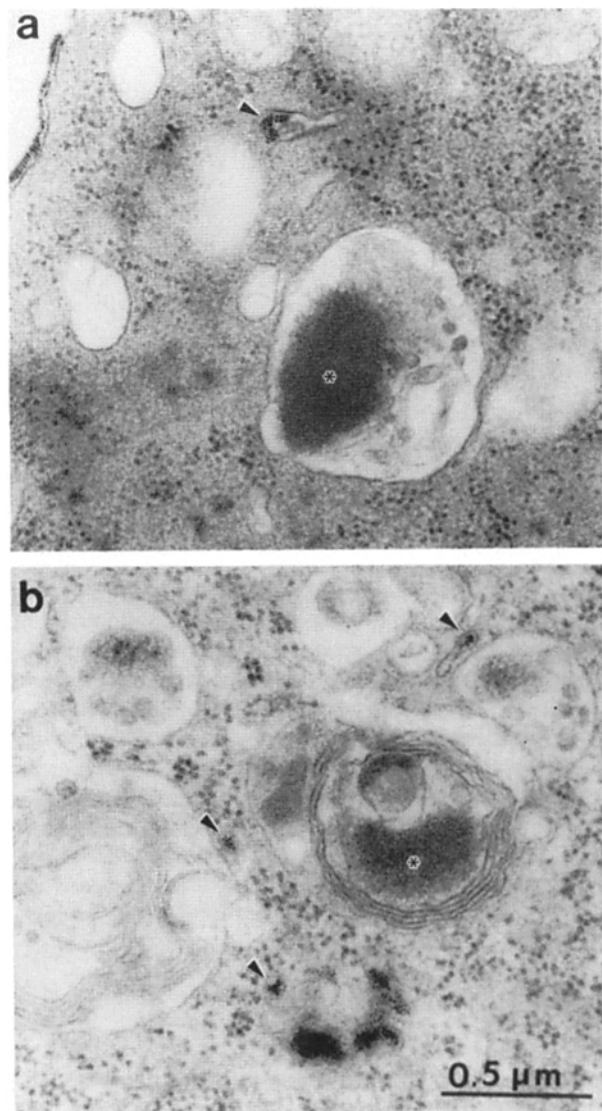


Figure 8. Inhibition of CF uptake into granules. RNK-16 cells were allowed to take up CF for 3 h at either 20°C (a), or at 37°C in the presence of 10 μM CCCP (b). Arrowheads indicate CF in endosomal tubules. * indicates cores.

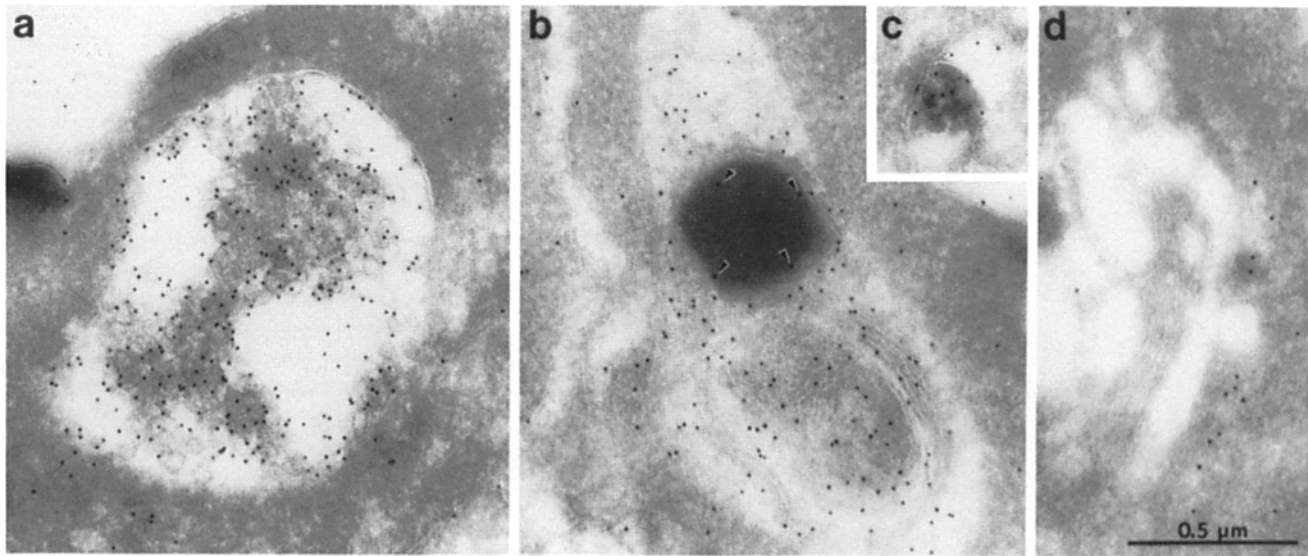


Figure 9 Electron micrographs of cryosections showing the localization of the pH-sensitive tracer DAMP. (a) Multivesicular granule. (b) Intermediate-type granule showing abundant DAMP label in the cortical region and less in the core. Arrowheads show gold particles around the periphery of the core. (c) A more classical lysosomal structure. (d) Golgi stack, labeled on one (presumably the *trans*) side.

e). In some profiles, CF was present in both the multivesicular domain and the core (Fig. 7 a). In others, CF was concentrated in the core (Fig. 7 b). Movement of CF into the cores lagged behind uptake into the multivesicular domains, suggesting that CF first enters the cortex of the granule and gradually finds its way into the core. The finding that a significant percentage of cores contain CF (up to 56% after a 22-h incubation) supports the idea that there is no barrier membrane between the granule domains. Indeed, even cores that appeared to be surrounded by a membrane could take up ferritin (e.g., Fig. 7 a).

As with uptake into lysosomes in other cell types (20, 22, 36), the uptake of CF into the granules was sensitive to low temperature and to agents that neutralize acidic compartments. When CF was endocytosed at 20°C, it was present in peripheral endosomes but was absent from the granules, even after 3 h (Fig. 8 a). In cells which were subsequently shifted to 37°C, the tracer moved into the granule compartment (data not shown). Similarly, in the presence of the lysosomotropic ionophore carbonylcyanide *m*-chlorophenylhydrazide, CF entered endosomes but did not reach the granules (Fig. 8 b). As expected, incubation at 20°C or treatment with carbonylcyanide *m*-chlorophenylhydrazide also inhibited CF uptake into classical lysosomal structures in RNK-16 (data not shown). All these properties support the notion that the lytic granules, and in particular their multivesicular cortices, serve as lysosomes.

The Intra-granule pH Is Acidic

The second lysosomal property we tested was the pH of the granules. Endosomes, lysosomes, and secretory granules are all known to be acidic organelles, with lysosomes possessing the lowest pH (1, 37). To estimate the acidity of the granule domains, we used the DAMP technique (2). DAMP, a weak base with a dinitrophenol group, diffuses freely across cell membranes until it reaches an acidic compartment, where its protonated form becomes trapped. Fig. 9

shows RNK-16 cells which had been incubated with DAMP, and the cryosections labeled with anti-DNP and colloidal gold. As expected, the granules were positive for DAMP. Both the cores and the multivesicular regions were labeled more heavily than the cytoplasm, mitochondria, or nuclei (Fig. 9, a and b). The multivesicular domains were labeled with DAMP to the same extent as smaller lysosomal structures (Fig. 9 c), indicating a similar pH. By comparison, the mildly acidic *trans*-Golgi elements were labeled less heavily (Fig. 9 d).

The density of DAMP labeling was quantified and used to estimate the pH, as described by Anderson and Orci (1). We estimate that the multivesicular regions of the granules have a pH of 5.4–5.5. In the same samples we estimate the pH of endosomal/lysosomal structures to be 5.5. Thus, the DAMP labeling extends the structural and biochemical similarities between the multivesicular granules and lysosomes. It should be emphasized that because of their large size, these multivesicular domains are the major acidic compartment in RNK-16 cells.

A consistent and as yet unexplained observation was that within the granules, the multivesicular regions labeled more heavily with DAMP than the cores (Fig. 9 b). In addition, the labeling of the cores was nonhomogeneous, with much of it over the circumference (Fig. 9 b, *arrowheads*). Taken at face value, this observation suggests that the cores are less acidic than the multivesicular regions. Alternatively, this pattern could be due to failure of DAMP to penetrate the cores or to be fixed there quantitatively. At present, we can only conclude that the cores seem to be mildly acidic, without estimating their pH.

The Same Ultrastructure and Protein Segregation Exists in Normal Rat LGL

One explanation for the presence of a secretory domain within a lysosomal domain is that this structure arises during degradation of type I (secretory) granules by type II (lyso-

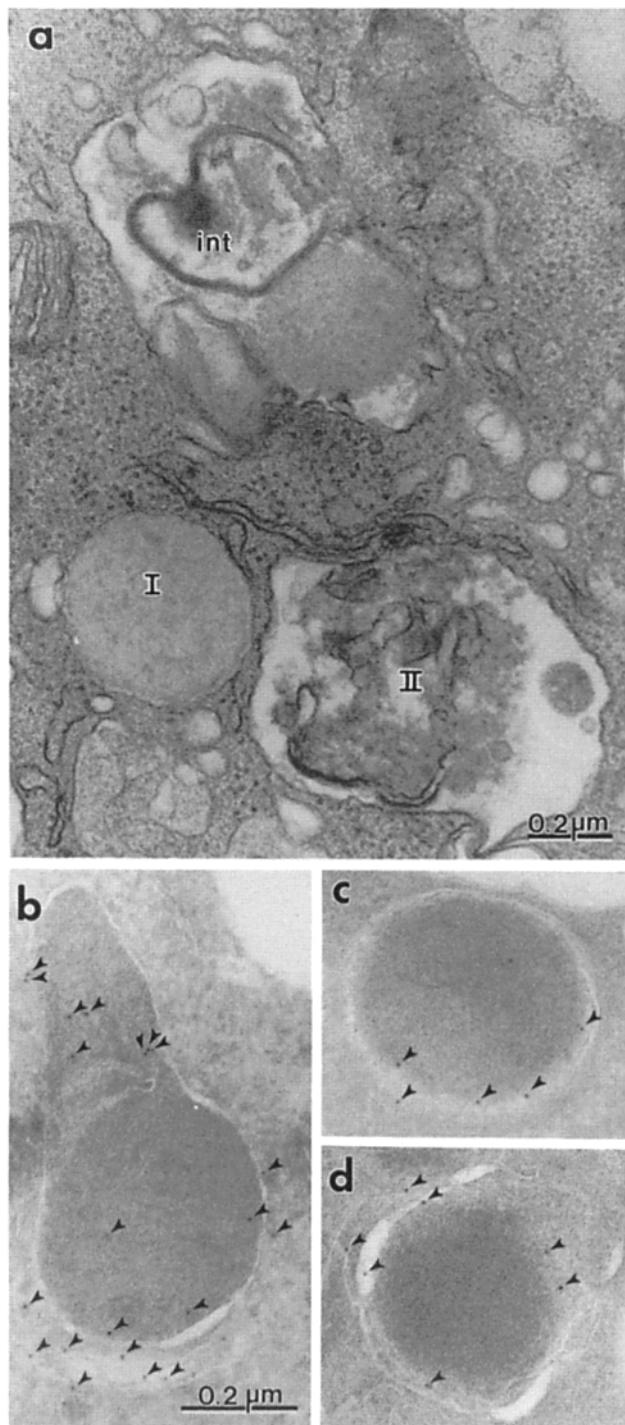


Figure 10. Granules of rat peripheral blood LGL. (a) Epoxy section showing profiles of the three morphological types, similar to those seen in RNK-16 cells. I, type I; II, type II; *int*, intermediate type. (b–d) Cryosections labeled with anti-LGP-120, as above, and 5 nm protein A–colloidal gold. Each gold particle is marked by an arrowhead.

somal) granules. This process, known as crinophagy, occurs normally in a variety of secretory tissues (15, 52, 56), but is sometimes enhanced when these tissues are cultured in vitro (46). To determine whether the structures we describe are unusual to cultured NK cells and CTL, we examined the

granules of normal rat LGL with NK activity. Peripheral blood or spleens were fractionated to obtain leukocyte preparations that contained 50–80% LGL. These preparations were highly enriched in natural killing activity and the granular cells within them were positive for the rat NK surface marker 3.2.3. The granules of these LGL displayed type I, type II, and intermediate morphologies, essentially as described for the granules of RNK-16 (Fig. 10 a). The normal LGL differed from RNK-16 in that their type I granules were considerably more abundant than multivesicular granules. As in RNK-16, the multivesicular domains of normal LGL were endocytic, because they frequently contained Percoll particles (not shown). The distribution of lysosomal proteins in the LGL granules was similar to that described above. As in RNK-16, anti-LGP-120 labeled the multivesicular regions, and not the cores (Fig. 10 b). Most importantly, the core of virtually every type I granule was surrounded by an LGP-120–positive rim (Fig. 10, b–d). The dearth of granules that lack lysosomal markers argues against crinophagy as the sole basis for generating the two-domain structure.

Lytic Granules Contain the man-6-P Receptor

To better characterize the lytic granules as an endocytic compartment, we localized the 270-kD cation-independent man-6-P receptor. In other cells, this receptor is present in the Golgi complex and in a variety of endosomal compartments but is absent from mature lysosomes (9, 21, 25, 51, 59). In RNK-16 cells, the highest level of the receptor was in the multivesicular regions of granules (Fig. 11, a–c, and e). Lower levels of label were present over Golgi-associated membranes (most likely the *trans*-Golgi reticulum, Fig. 11 d) and peripheral endosomal structures (not shown). man-6-P receptor was detected over occasional granule cores. These labeled much less frequently than the multivesicular regions, but their labeling was reproducible. Fig. 11, e and f, shows semi-consecutive sections through a granule containing a man-6-P receptor–positive core. It is not clear why the receptor should be present in some core regions and not in others, but its presence there, along with low levels of some lysosomal enzymes, may provide clues about granule biogenesis.

Given the absence of man-6-P receptor from mature lysosomes in other cell types, we were surprised to find it in any of the granule domains. Nonetheless, the same distribution was obtained with two different antireceptor antibodies and two different secondary gold reagents (data not shown). The presence of the man-6-P receptor has been used to distinguish “pre-lysosomes” from mature lysosomes (25). By this definition, the multivesicular regions of lytic granules should be considered a pre-lysosomal compartment.

Discussion

The granules of cytotoxic lymphocytes are complex structures which mediate the ability of these cells to kill other cells. The structural, biochemical, and functional analyses presented here provide evidence that the granules have a dual function and are composed of two domains. They contain dense cores which resemble other secretory granules, and multivesicular cortices which resemble lysosomes, but whose contents are secretable.

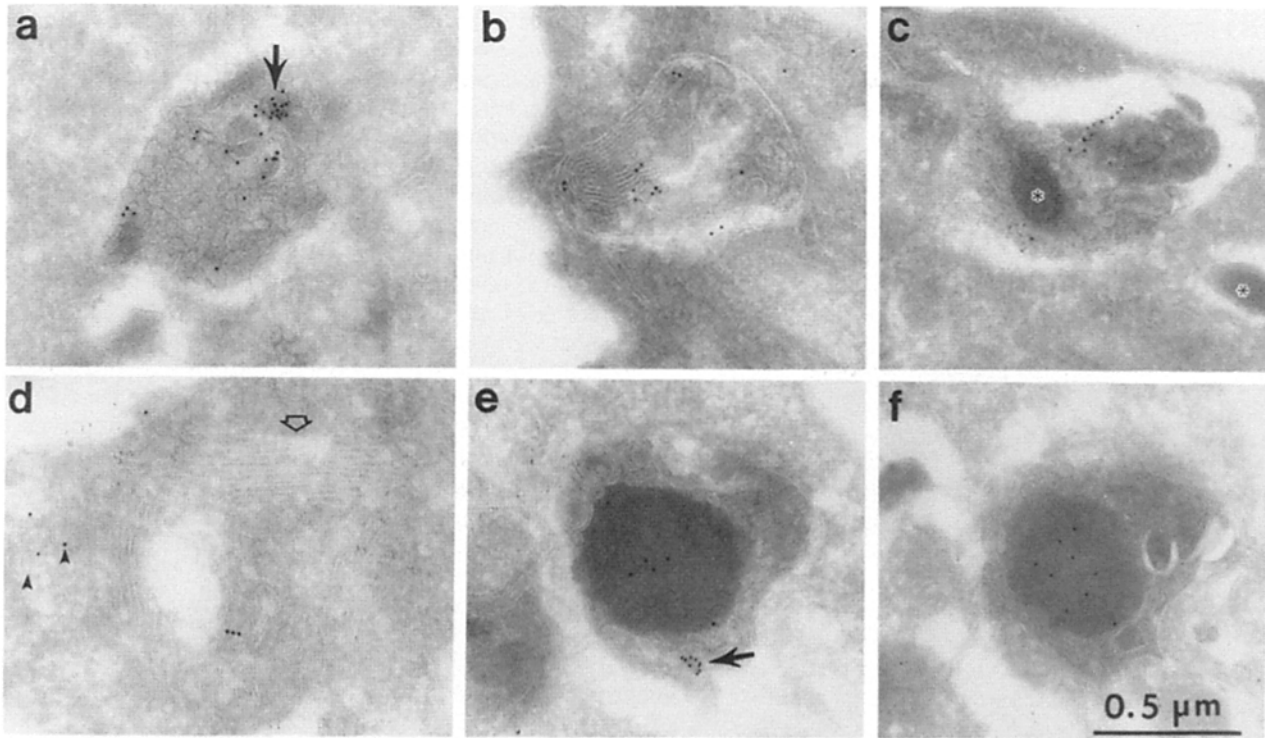


Figure 11. Immunoelectron micrograph showing localization of the cation-independent man-6-P receptor to lytic granules. *a-c* show the most typical observation, labeled multivesicular granule regions and unlabeled cores (*). Clumps of gold over multivesicular granule regions were often observed (*a* and *e*, arrows). *d* shows a Golgi complex. Arrowheads indicate man-6-P receptor-positive tubules or vesicles in the Golgi region. Open arrow indicates unlabeled region of the Golgi stack. Note that the Golgi complex is less heavily labeled than the granules. *e* and *f* show semi-serial cryosections of the same granule, with label over the core. Such granules were unusual, but were observed consistently.

Our reconstruction of RNK-16 granules substantiates their division into three morphological types (40). Granules containing predominantly a dense core and granules containing only multivesicular material do in fact exist as distinct entities, along with granules that contain both compartments. On the other hand, it is clear that all the various granules are related. Since even type I granules have a thin cortical region, the main distinction between type I, intermediate, and type II granules is the relative proportions of the two domains.

The dense cores of type I and intermediate granules are equivalent, and have all the hallmarks of regulated secretory organelles (17). First, these regions are morphologically similar to dense core secretory granules of endocrine and exocrine cells. Second, they contain proteins that are secreted during cytolysis and inflict damage on the target cell. We have previously shown that cytolysin and lymphocyte-specific serine proteases reside in the dense cores of NK granules (11). Studies localizing these proteins in murine CTL have revealed the same distribution (26, 30). We now show that a chondroitin sulfate proteoglycan, another secreted granule component (35), is restricted to the same domain. The presence of proteoglycan in the cores is consistent with their specialization for secretion, since similar proteoglycans are found in virtually all secretory granules (10). A third feature of the cores which is consistent with a secretory role is that they are at least mildly acidic; most dense core secretory granules are acidic to some degree (1, 37).

In contrast to the dense cores, the multivesicular regions

of all three granule types resemble lysosomes. These regions contain a variety of lysosomal proteins, both soluble hydrolases and membrane proteins. They have an acidic pH comparable to that of lysosomes, and they participate in the endocytosis of exogenous ligands. All these properties suggest that one function of the lytic granules is lysosomal degradation. In fact, since the granules are much larger than lysosomes, they probably constitute the major degradative compartment in these cells. This specialization may be required, for example, to clear debris from target cells after repeated rounds of cytolysis, or to protect the cytolytic cell from its own lytic machinery.

The multivesicular domains of the granules differ from "conventional" lysosomes in two important ways. First, they are larger and more structurally complex than most classical lysosomes. Second, they are positive for the 270-kD man-6-P receptor, which lysosomes lack (25). In fibroblasts, an acidic compartment with extensive reticular structure, positive for both LGP-120 and man-6-P receptor, has been recently characterized by Griffiths et al. (25) as pre-lysosomal. The numerous tubular and vesicular profiles observed in the multivesicular granule domains are very similar to the structure of the pre-lysosomal compartment in fibroblasts. Our data, therefore, are entirely consistent with the interpretation that the multivesicular domains of lytic granules are an extensive pre-lysosomal compartment of NK cells and CTL.

An important feature that distinguishes the multivesicular domains from both conventional lysosomes and pre-lysosomes is their ability to be secreted in a regulated fashion.

There is ample evidence that lysosomal hydrolases are secreted during cytolysis (19, 42, 61, 63). While it is reasonable to assume that some of the secreted hydrolases derive from the rims of type I granules, there is microscopic evidence that multivesicular granules also fuse with the plasma membrane during cytolysis (40, 43; Hester, S., unpublished results). There are a number of reasons why it may be advantageous for NK cells and CTL to secrete lysosomal hydrolases along with cytolytic proteins. The hydrolases may improve the efficiency of cytolysis by damaging the target cell. This is consistent with the finding that targets are killed more efficiently by total granule contents than by purified cytolysin (39). Alternatively, the secreted hydrolases may protect the effector cell from harmful target cell debris. This could be important, for example, when the target is a virus-infected cell.

In essence, the intermediate and type I granules consist of a regulated secretory granule enclosed within a pre-lysosome. How then do these organelles arise? One possible way is by crinophagy, degradation of secretory granules by lysosomes (15). As in other regulated secretory cells, where newly synthesized granules are secreted preferentially (e.g., reference 50), crinophagy in cytolytic cells could serve to regulate secretion by degrading unused lytic granules. In a model based on crinophagy, type II granules would degrade type I granules, giving rise to the intermediate morphology. However, several pieces of evidence argue that this is not the major route by which intermediate granules form. First, the crinophagy model predicts the existence of newly synthesized secretory granules which are not yet being degraded. These granules should be particularly abundant in cells with high lytic activity, such as the peripheral blood LGL. Yet virtually all of the type I granules we observed, both in RNK-16 and in the polyclonal LGL, contained a rim of lysosomal components. A second argument against the crinophagy model is that multivesicular granules fuse with the plasma membrane and release their contents. Even lytic granules which contain exogenous markers can fuse with the plasma membrane (43; and our unpublished observations). Such secretion would not be expected if the multivesicular granules play a purely degradative role. Third, crinophagic structures are thought to be mature secondary lysosomes (4, 15, 52), but the lytic granules contain the man-6-P receptor, a marker for less mature endocytic compartments. Although there is evidence that pre-lysosomal compartments can engage in autophagy of cytosolic proteins (23), there is no evidence that pre-lysosomes or endosomes can participate in crinophagy.

An alternative explanation for the biogenesis of the lytic granules is that the three subtypes represent a maturation sequence of a specialized secretory compartment. The intermediate granules may be immature structures, analogous to condensing vacuoles in exocrine and endocrine cells, where cores grow by a process of condensation. Cytolysin and the granule proteases are basic proteins, which may condense with the proteoglycan as the granule acidifies, thus segregating core proteins from lysosomal proteins. Indeed, several granule proteases were shown to associate with the proteoglycan via ionic interactions (32). Type I and type II granules might then arise from the intermediate-type precursors, for example by fission.

This model for granule biogenesis accounts for several unexpected observations. It explains why there is a man-6-P

modification on the core serine proteases (11) and why the multivesicular domains are rich in man-6-P receptor. The serine proteases could use the same man-6-P receptor-dependent mechanism used by lysosomal hydrolases for transport from the Golgi complex to intermediate-type granules. This model also explains our observation that lysosomal hydrolases are present at low levels in a few granule cores. These cores could be immature ones, where segregation of proteins is incomplete.

One important issue in this context of granule biogenesis is whether or not the cores are enveloped by a membrane. Peters et al. (43) have described such a membrane in the granules of human CTL. Indeed, its existence could account for the observed segregation of proteins and for the differences in DAMP labeling between the cores and cortices. However, in our hands most cores were not enveloped by a continuous membrane. This was especially true in the clone 33 CTL line. Even in RNK-16, where some cores did appear to be surrounded by membrane, these cores were permeable to CF after the tracer had accumulated in the cortical domains. When a membrane is apparent, it could form by coalescence of cortical vesicles, as suggested by Peters et al. (43), or by apposition of cortical lamellae. Either mechanism could produce a noncontinuous membrane. Since Peters et al. have hypothesized that a delimiting membrane confers cytolytic specificity on the granule cores (43), this unresolved issue has important implications for granule function as well as biogenesis.

While the dual nature of NK granules described here is surprising, there are parallels in other systems. Azurophilic granules of neutrophils look like, and usually function as, a degradative intracellular compartment, but they can be secreted in response to some stimuli (16). Conversely, there are examples of organelles that are primarily secretory, which have properties of lysosomes. The crystalline secretory granules of eosinophils and the renin-containing granules of juxtaglomerular epithelioid cells contain lysosomal hydrolases (5, 53, 58). Similarly, the prespore vesicles of *Dictyostelium* contain both secreted spore coat proteins and lysosomal enzymes (33). Like the NK granules, the renin granules of epithelioid cells take up exogenous ligands (58). Furthermore, Faust et al. showed that renin, like the serine proteases of NK cells, bears man-6-P (18). These examples all point out that secretory granules and lysosomes may be more closely related than is commonly appreciated. Other general similarities, such as their acidic pH and the fact that proteins of both organelles are sorted from the *trans*-Golgi reticulum via clathrin-coated vesicles (44), also point to their relatedness. Cytolytic cells may make use of the similarities of secretory granules and lysosomes to create a dual function organelle. Such an organelle may serve either to enhance the efficiency of target cell destruction, or to ensure the disposal of target cell debris during membrane reclamation after degranulation.

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