Cytotoxic T Lymphocyte Granules Are Secretory Lysosomes, Containing Both Perforin and Granzymes

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Summary

Cytotoxic T lymphocytes (CTL) contain granules that are exocytosed during specific interaction with target cells (TC). In this process, the granule contents, including the lethal protein perforin, as well as granzymes, a family of serine esterases, are delivered to the TC. Information regarding the routing of these proteins towards the granule and their exact localization within the granule is of primary importance to resolve the mechanism of granule-mediated TC killing. In this study, the subcellular localization of perforin, granzymes, and known endosomal and lysosomal marker proteins was determined in human and murine CTL, by immunogold labeling of ultrathin cryosections followed by electron microscopy. Perforin and granzymes can be detected in rough endoplasmic reticulum, Golgi complex, *trans*-Golgi reticulum, and in all cytotoxic granules. Within the granules, they have a similar distribution and are localized not only in the so-called dense core but also over the region containing small internal vesicles. This finding implies that perforin and granzymes can be released in membrane-enveloped and/or -associated form into the intercellular cleft formed upon CTL-TC interaction. On the basis of the present evidence, additional release of these molecules in soluble form cannot be excluded. The lysosomal membrane glycoproteins lamp-1, lamp-2, and CD63, are abundantly present on the granule-delimiting outer membrane, which becomes incorporated into the CTL plasma membrane during lethal hit delivery. In contrast, the cation-independent mannose 6-phosphate receptor, known to be present in endosomes and absent from lysosomes, is found only in a minority of the granules. Together with our previous findings that the granules are acidic and connected to the endocytic pathway, these observations define CTL granules as secretory lysosomes.

Upon activation, peripheral blood T lymphocytes may differentiate into CTL, containing characteristic granules. Triggering of the TCR/CD3 complex in CTL results in granule redistribution and exocytosis towards the target cell (TC)[1]. Although there is evidence for granule-independent killing mechanisms (1–4), it is clear that delivery of lytic components by degranulation is an effective mechanism for TC destruction (5–11).

Perforin, granzymes, and proteoglycans are the major components isolated from granule fractions (8). Perforin is thought to bind to the TC membrane and assemble into multimeric complexes causing tubular lesions (8–12). Part of the perforin molecule has sequence homology with the complement component C9 in a region that might fold into an amphipathic α helix (13–16). Purified perforin induces lysis of a variety of tumor cells by inserting directly into the plasma membrane (8). The perforin protein is only found in CTL and NK cells (8). Although it has been argued that perforin expression may only be inherent to long-term in vitro cultured cells (1), perforin mRNA expression has also been observed in peritoneal exudate lymphocytes (17), in mice infected with choriomeningitis virus (18, 19), and in mice suffering from autoimmune disease (20). Strong evidence in favor of the role of perforin in TC killing comes from recent studies where antisense oligonucleotides hybridizing with perforin mRNA significantly reduced killing (21). Murine CTL contain eight highly homologous serine esterases termed granzymes A–G (22–24); human CTL contain three granzymes, designated

[1] *Abbreviations used in this paper:* CI-MPR, cation-independent mannose 6-phosphate receptor; RER, rough endoplasmic reticulum; TC, target cell, TGR, *trans*-Golgi reticulum.

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A, B, and 3 (8, 25, 26). Their role in lethal hit delivery is unknown, although it has been suggested that granzyme A is implicated in DNA breakdown in the TC (27, 28). The granule proteoglycans are thought to be involved in packaging of the perforin and granzyme molecules (29–32).

Ultrastructural studies on the granule structure of human CTL have indicated that cytotoxic granules are membrane enveloped and contain many small internal vesicles and a dense core (11, 12, 33–35). Upon lethal hit delivery, the granules undergo exocytosis, whereby the dense core and small internal vesicles are released into the intercellular cleft formed between the CTL and TC and can be seen to interact with the TC membrane (12, 34, 35). The dense core and small internal vesicles expose the TCR/CD3 complex and CD8 (34). Perforin and granzymes contain a hydrophobic signal peptide and bear N-linked oligosaccharides as demonstrated for rat NK cells (36), indicating that they enter the rough endoplasmic reticulum (RER) and therewith would pass through the Golgi complex. However, this has not yet been demonstrated directly. In addition, it is unclear along which pathway they travel towards the granule. Murine perforin (33) and granzymes D/E/F (24) have previously been detected in the dense core of CTL granules, but not in other compartments. Also, various authors (2, 8, 37, 38) have suggested that different granule subtypes may exist, containing either perforin or granzymes. To understand the mechanism of lethal hit delivery, we have investigated the localization within the biosynthetic route and within the granule of proteins that may inflict TC damage. In the present immunocytochemical study, it is shown that the granules are a homogeneous population with respect to the distribution of perforin and granzymes. These proteins were detected within the dense core of the granule and distributed over the region containing the small internal vesicles. Also specific marker proteins for lysosomes and endosomes were localized to define the cell biological nature of the CTL granule. Detected were the three integral lysosomal membrane proteins, lamp-1,-2 (40), CD63 (41), and the cation-independent mannose 6-phosphate receptor (CI-MPR), a marker for endosomes that is absent from lysosomes (42–44). The CI-MPR/ligand complex is delivered from the *trans*-Golgi reticulum (TGR) (46) to an acid pre-lysosomal compartment where dissociation of the ligand occurs (42–44). The CTL granules appeared to contain lamp-1,-2 as well as CD63 in the granule outer membrane. In addition, the majority of the granules were devoid of CI-MPR. These characteristics classify CTL granules as relatives of lysosomes.

Materials and Methods

Cells. The murine CTL clone 860/4 is a C57BP/6 (H-2^b) anti-DBA/2 (H-2^d) clone kindly provided by Dr. J. C. Cerottini, Ludwig Institute, Lausanne, Switzerland. B6.1 is a H-2^d-specific murine CTL clone. Both clones were cultured as described (22). The human CTL clones JS-132 and JS-136 express TCR-α/β and the CD8 and CD4 accessory molecules, respectively. They were derived from donor JS (HLA-A3,3; B7,7; DR2,2) by MLC with the EBV-transformed B cell line JY (HLA-A2,2; B7,7; DR4,6) and are directed against determinants associated with HLA-A2 (JS-132) and HLA-DR6 (JS-136) (47, 48). The clones were maintained by weekly stimulation with a feeder cell mixture containing irradiated allogeneic PBMC and JY cells, PHA, and rIL-2, essentially as described (48), and cultured in Yssel's medium (49), supplemented with 2% human serum, in round-bottomed wells. The human B cell line JY was cultured in Iscove's modification of MEM, and supplemented with 5% FCS. Before fixation for immuno-EM, cells were washed with serum-free medium at room temperature.

Antibodies. Rabbit anti–murine perforin serum was raised against purified protein (5). When the antiserum was tested by Western blot analysis of total granule extract, one protein with the appropriate molecular mass of perforin was detected (Tschopp, unpublished results). A rabbit antiserum, recognizing granzymes D/E/F, has been described (22, 25). Rabbit anti–human granzyme B serum was raised against recombinant human granzyme B (22). Rabbit anti–human lamp-1,-2 (40), murine anti–human CD63 mAb (RUU-SP 2.28) (41), rabbit anti-cathepsin D, and rabbit anti–human CI-MPR sera (44) have been described. Rabbit sera and murine ascites fluid were used at dilutions of ~1:300.

Immuno-EM. Cell samples were fixed in either 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 37°C or in a mixture of 1% formaldehyde and 1% acrolein in the same buffer for 2 h at 37°C, and 2 d at 4°C. Samples were washed in PBS containing 0.15 M glycine to block free aldehyde groups of the fixative. The cells were embedded in 10% gelatin and processed in a cold room. Small gelatin blocks were incubated overnight in 2.3 M sucrose at 4°C and then frozen in liquid nitrogen. The ultrathin cryo-sectioning and immunogold labeling have been described by Slot et al. (50). Briefly, ~80-nm-thin cryo-sections were made with a cryo-nova ultra-microtome (Reichert-LKB) using a Diatome diamond knife and incubated at room temperature with the polyclonal antisera for 30–60 min followed by incubating with protein A–gold complex (51) for 20 min. Purified rabbit anti–mouse Ig (at 2 μg/ml) was used after the mAb and before protein A–gold. In the case of double labeling, sections were incubated with 1% glutaraldehyde for 10 min after the first gold incubation to prevent interaction between both immunoreagents (51a). At optimal antiserum dilutions, virtually no background labeling over nuclei and mitochondria was observed. Irrelevant control antibodies did not give any labeling. An electron microscope (1200 EX; JEOL) was used for examination.

Quantitative Analysis. Quantitative analysis of immunogold labeling was carried out by calculating the relative distribution of the label over the compartments shown in Table 1. For each of the three proteins, gold particles were counted at high magnification in 30 medial cell profiles, which were randomly selected at low magnification. Background labeling was determined by counting gold particles over nucleus, mitochondria, and cytosol, and comparing to the total gold labeling. The density of lamp-1 labeling per unit membrane length was determined by using an X/Y tablet attached to an IBM computer.

Results

Morphology of Murine and Human CTL Granules. We have first examined the fine structure of CTL granules. Because of possible clone-specific differences in granule micro-architecture, seven human and seven murine CTL clones were studied. Each human or murine cell contained 15–25 granules, as estimated by extrapolating the number of granules in a cross-section to the total cell volume. The granules had diameters of ~700 nm, but, particularly in the murine clone

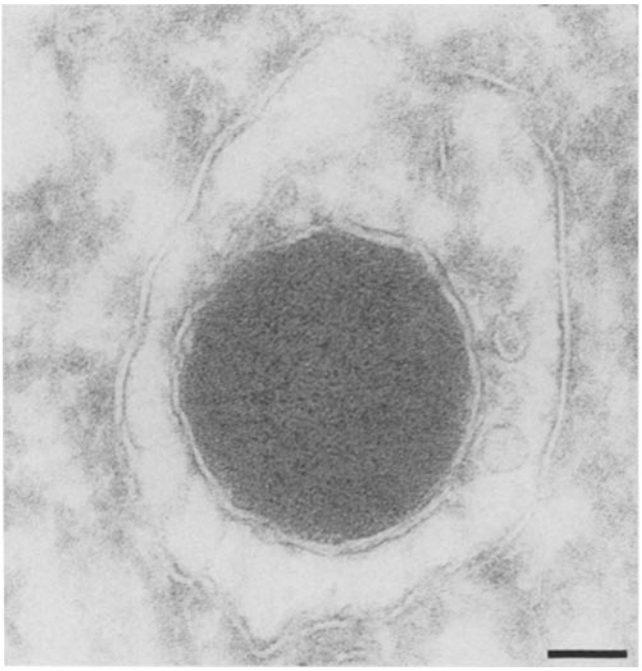


Figure 1. Unlabeled ultrathin cryo-section of a CTL granule from human clone JS-132 showing the membrane around the electron-dense core. Only if the granule core is cut equatorially continuous membranes can be seen. Bar, 100 nm.

B6.1, very large granules with diameters >5 μm were found. Generally, the cytotoxic granules contained many small internal vesicles (40–70 nm in size) and one, or sometimes two or three, large electron dense core(s) that comprised up to about one-third of the granule volume. The dense cores in human CTL granules showed a limiting membrane (Figs. 1, 2, and 3, *A* and *D*), but those in the murine CTL granules hardly ever showed a membrane in cryo-sections (Figs. 4, *B* and *C*, and 5). The internal vesicles in murine CTL granules were much more abundant and smaller, whereas the dense cores were larger than in human CTL granules. Apart from the large cores, murine granules occasionally showed relatively small membrane enveloped dense core-like structures (Fig. 4, *B* and *C*). Remnants of mitochondria and membrane sheets were occasionally observed within granules (data not shown), indicative of autophagocytic activity. Granule profiles often showed only small internal vesicles (Fig. 4 *A*) or dense core structures. Since the dense core only comprises about one-third of the total granule volume, tangential sectioning can in many cases give an incomplete image of the granule. We assume that human and murine CTL clones contain a morphologically homogeneous granule population.

Perforin and Granzymes Colocalize in the Biosynthetic Pathway and within the Cytotoxic Granule. To better understand intracellular transport pathways of perforin and granzymes towards the granules, we have determined their precise localization. Since no anti-human perforin reagent was available, perforin localization was only studied in murine CTL. As can be seen in Fig. 4 *A*, murine perforin and granzymes D/E/F colocalized to the Golgi stack of cisternae and to the TGR with associated small vesicles. Quantitative analysis revealed that only a minority of granzymes D/E/F or perforin molecules was present in RER and Golgi, whereas the majority was found in the granule (Table 1). Plasma membrane and cytosol were devoid of label (Fig. 4, *B* and *C*). Background label was very low, as can be seen on the nucleus (Fig. 4 *B* and Table 1). It can be concluded that perforin and granzymes D/E/F share the biosynthetic pathway.

Within the cytotoxic granules, perforin and granzymes D/E/F displayed an identical distribution. Granzymes D/E/F labeling was distributed randomly over the dense core and over the region containing the small internal vesicles (Fig. 4 *B*). A relatively small part of the gold particles was distributed over the dense core, while the majority was distributed over the region containing the small internal vesicles (see Fig. 4 *B* and Table 1). It was impossible to establish whether the proteins reside within the vesicles or are associated with the small internal vesicles at the outside, because the thickness of the cryo-sections was in the order of the diameter of the small internal vesicles and because the small internal vesicles in the murine CTL are so tightly packed (see Discussion). Double labeling showed that each cytotoxic granule contained perforin as well as granzymes D/E/F (Fig. 4 *C*). We con-

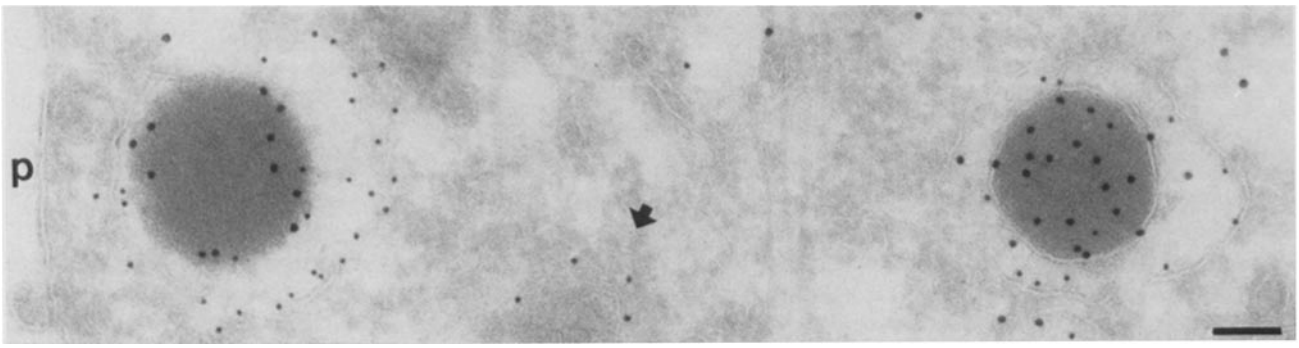


Figure 2. Double labeling of lamp-1 (10 nm gold) and granzyme B (15 nm gold) in CTL clone JS-136. Granzyme B labeling is mainly present within the electron-dense core. lamp-1 is predominantly present at the outer membrane of the granules. Only a few gold particles detecting lamp-1 can be seen at the membrane around the electron-dense core of the granule on the right. The arrow points to some lamp-1 labeling, at what might be a top plane of a granule. The plasma membrane (p) does not contain label for lamp-1 or granzyme B. Bar, 100 nm.

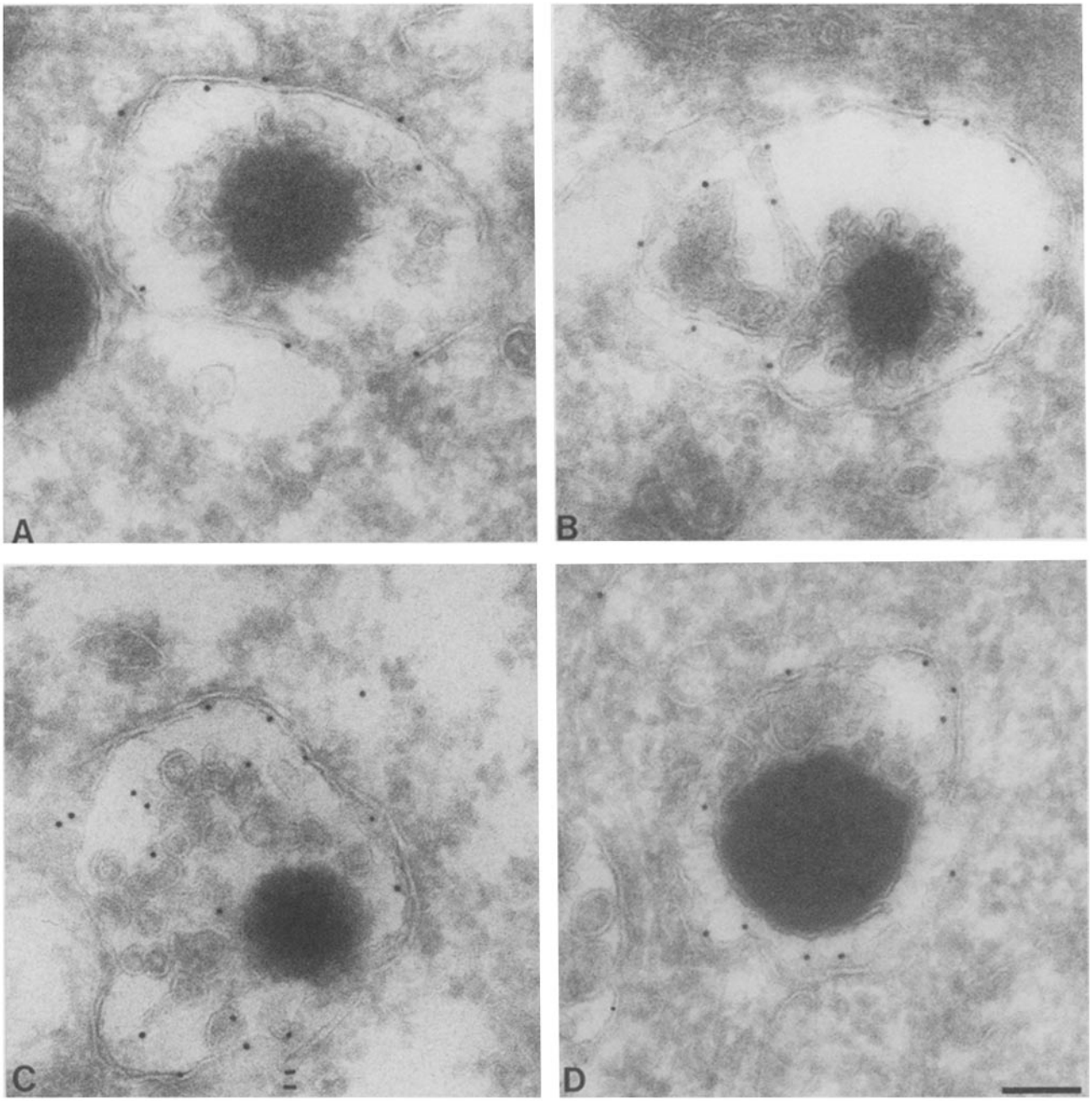
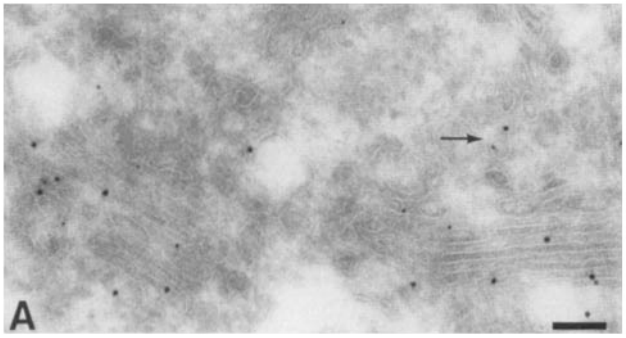


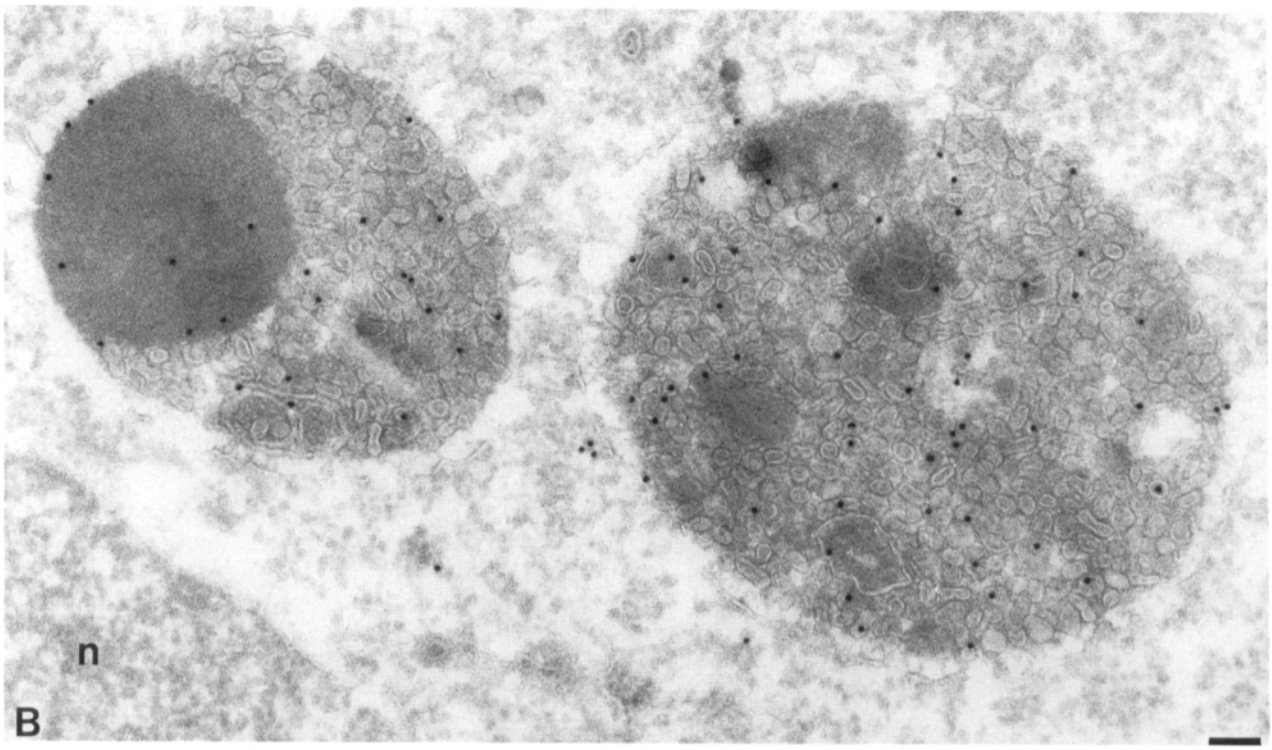
Figure 3. (*A* and *B*) Single labeling of lamp-1; (*C* and *D*) single labeling of lamp-2 in CTL clone JS-136. Both proteins are mainly localized at the outer membrane of the granules and to a lesser extent at the membrane of the small internal vesicles. The vesicles in B suggest fusion with each other and the electron-dense core. Bar, 100 nm.

clude that murine perforin and granzymes colocalize in the biosynthetic route as well as within the cytotoxic granule.

In human CTL, also a minority of granzyme B was present in RER and Golgi (Table 1), whereas the majority was demonstrated within the granule (Fig. 2). However, in contrast to murine CTL, the granule area containing internal vesicles only occasionally showed label for granzyme B. The majority of the label was restricted to the membrane-enveloped dense core (see Table 1 and Fig. 2). Granzyme B was absent from the plasma membrane (Fig. 2).

CTL Granules Contain Proteins Characteristic for Lysosomes. The distribution of proteins known to be specifically associated with lysosomes and endosomes was studied to define the cell biological nature of the cytotoxic granule. The human lysosomal membrane glycoproteins lamp-1 (Figs. 2 and 3, *A* and *B*), lamp-2 (Fig. 3, *C* and *D*), and CD63 (Fig. 6) were mainly found at the outer membrane of all granules and occurred occasionally on the membranes of a few small internal vesicles. The differential distribution of lamp-1 at the outer membrane of the granule and granzyme B within the membrane-envel-





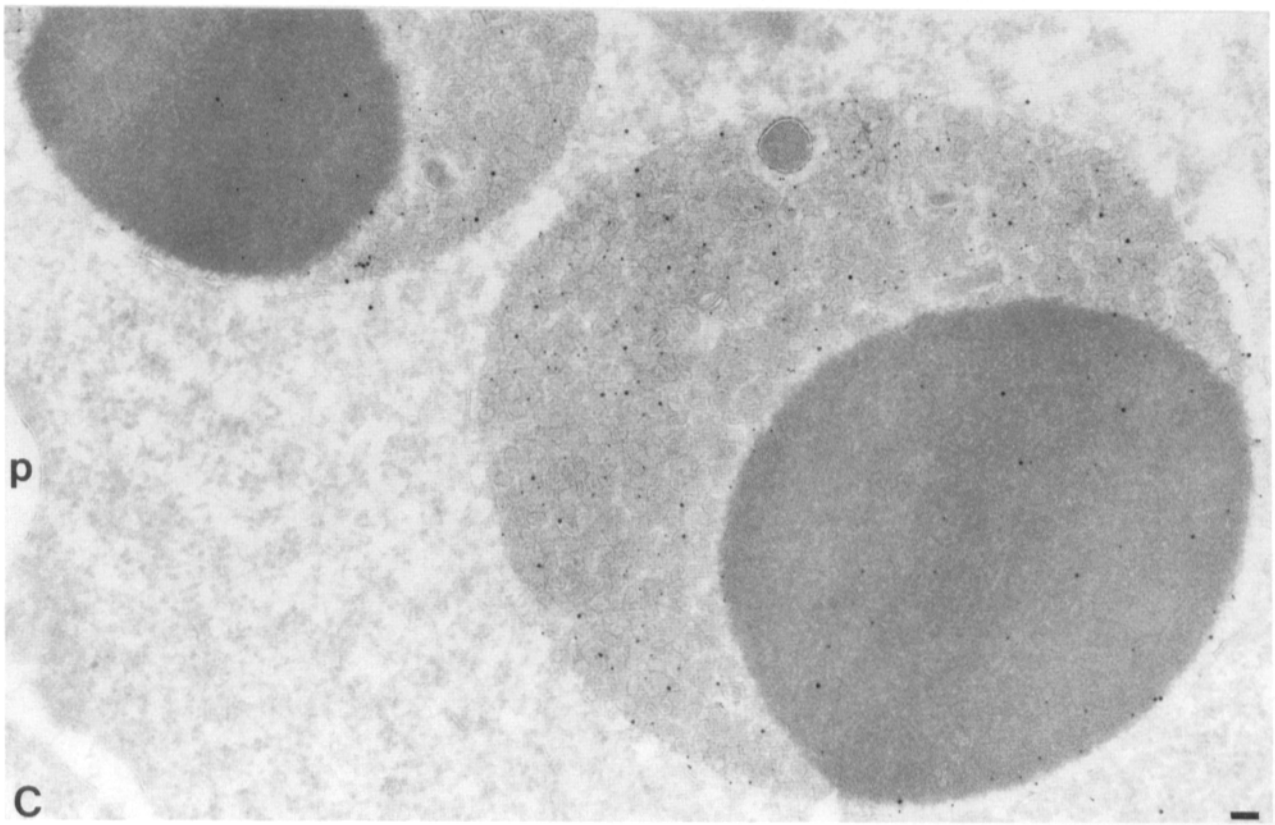


Figure 4. (*A*) Ultrathin cryo-section of murine clone B6.1 showing distribution of perforin (10 nm gold) and granzyme D/E/F (15 nm gold) throughout the Golgi complex and in vesicles (*arrow*) in the *trans*-Golgi region. Bar, 100 nm. (*B*) Ultrathin cryo-section of cytotoxic granules in murine clone B6.1 showing that granzymes D/E/F are localized in the region containing the small internal vesicles and randomly distributed over the dense core. There is virtually no background labeling over the nucleus (n) and the cytosol (poly-ribosomes). Section most probably made under a plane that grazes through the extreme pole of the electron-dense core in the granule on the right. Bar, 100 nm. (*C*) Double labeling of granzymes D/E/F (10 nm gold) and perforin (15 nm gold) of CTL clone 860/4. Both granules are labeled as in *B*, and perforin and granzyme colocalize within the same granule. Bar, 100 nm.

Table 1. *Relative Distribution of Gold Particles for Murine Perforin, Murine Granzymes D/E/F, and Human Granzyme B*

	Murine perforin	Murine granzymes D/E/F	Human granzyme B
		%	
RER	7	7	5
Golgi, TGR	4	4	3
Dense core	12	13	81
Region of small internal vesicles	72	71	9
Background	3	2	2

The murine B6.1 CTL clone was used to quantitate granzymes D/E/F and perforin, and the human JS-132 CTL clone for quantitation of granzyme B.

oped dense core is particularly evident in Fig. 2. The CTL plasma membrane was very sparsely labeled for lamp-1 (Fig. 2) and CD63 (Fig. 6). Quantitative analysis of the label detecting lamp-1 revealed 450 gold particles/100 μm of granule outer membrane as compared with 27 gold particles/100 μm of plasma membrane. The plasma membrane of the TC, the B cell line JY, did not label for CD63 (Fig. 6) or lamp-1 (51b). Since it is known that CTL clones release granzymes spontaneously, the 27 gold particles/100 μm for lamp-1 on the CTL plasma membrane might be a result of spontaneous degranulation.

We have previously demonstrated that in human CTL, a major lysosomal enzyme, cathepsin D, occurs in the granule lumen between electron-dense core and small internal vesicles (34). Also in murine CTL granules, the dense core did not label for cathepsin D. The gold particles were restricted to the region containing the small internal vesicles (data not shown). Given the limited space between the vesicles in the murine granules, it was not possible to establish whether cathepsin D was exclusively present in between the internal vesicles and dense core, as shown for human CTL (34).

CI-MPR is known to occur in endosomes, but not in lysosomes (42, 43). In human CTL, CI-MPR was found in TGR (Fig. 5), while only ~10% of the cytotoxic granules were clearly positive. Granules positive for CI-MPR are shown (Fig. 5).

These data, together with our previous observations that the granules are acidic and connected to the endocytic route (32, 34), classify the majority of the cytotoxic granules as lysosomes (i.e., CI-MPR-negative, and lamp-1,-2 and CD63-

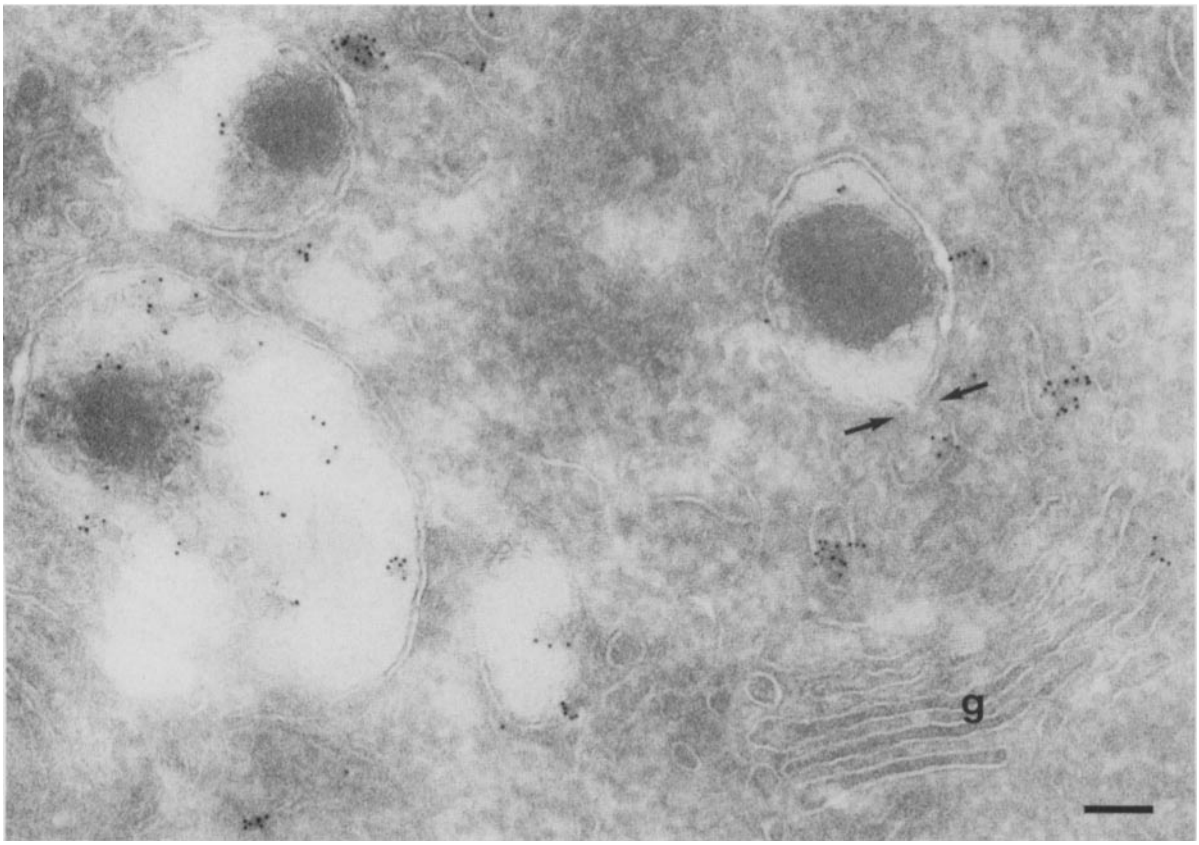


Figure 5. Labeling of CI-MPR in JS-132 in the TGR and associated small vesicles and in some granules. The arrows point to a profile suggestive for fusion of CI-MPR-positive vesicles with a granule. The micro-architecture of the granule is not as well preserved as in other samples because of the weak fixative used, which was necessary to retain antigenicity of CI-MPR. Bar, 100 nm.

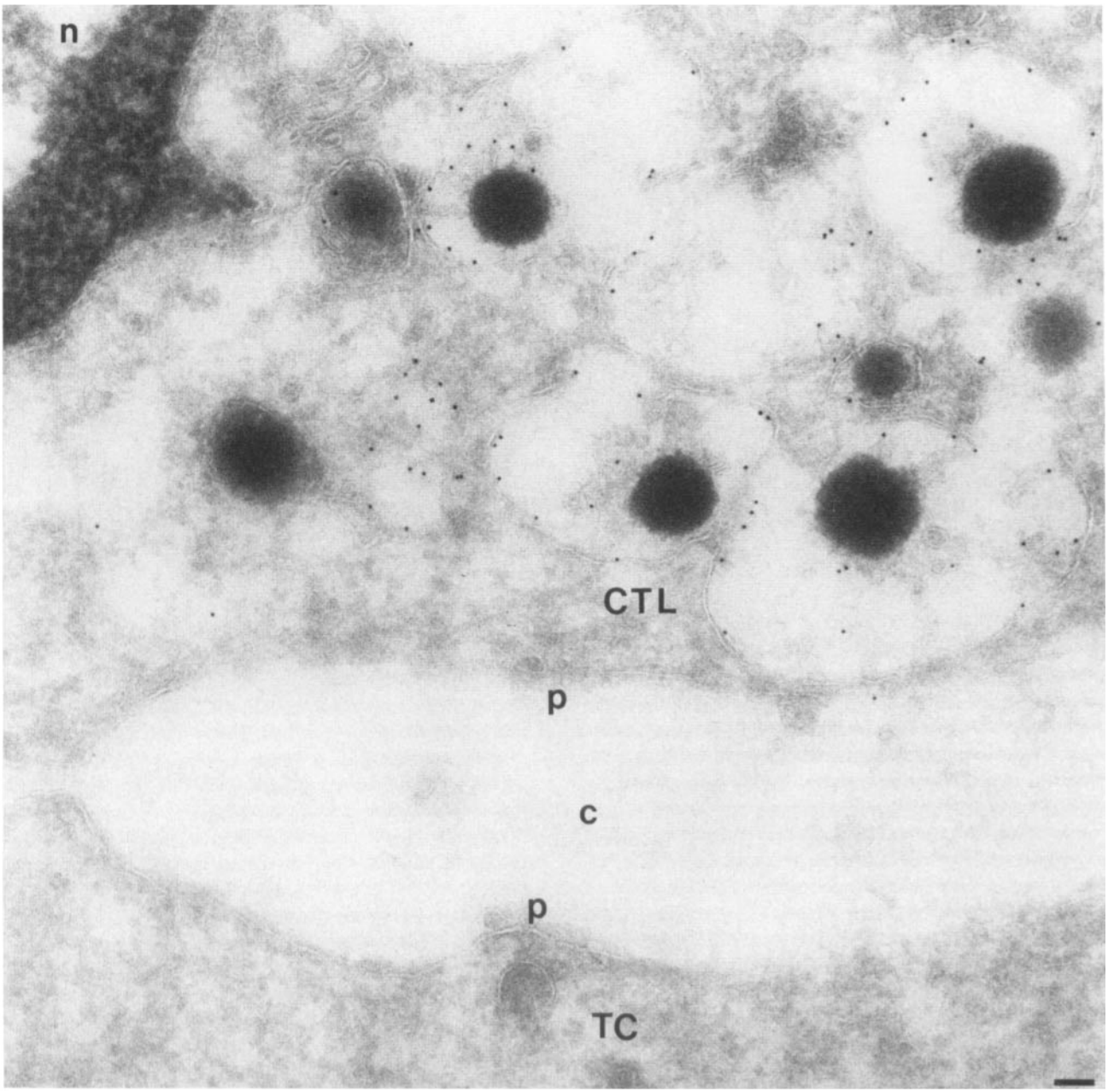


Figure 6. Section of a CTL (JS-132)/TC (JY) conjugate labeled for CD63. The gold particles are restricted to the outer membranes of the cytotoxic granules. The plasma membrane (p) bordering the intercellular cleft is devoid of label. Note that the granules are located nearby the intercellular cleft (c). Bar, 100 nm.

positive). The small proportion of granules that contains CI-MPR may represent certain developmental stages, presumably endosomes.

Discussion

CTL granules are enveloped by a lipid bilayer and contain small membrane vesicles and one or a few electron-dense cores. Lethal hit delivery by CTL most likely involves exocytosis, whereby the granule outer membrane is incorporated into the CTL plasma membrane and the granule contents are released into an intercellular cleft between CTL and TC (8, 10–12, 38, 39, 45). Not only perforin, but also the other granule constituents, granzymes, proteoglycans, and lysosomal enzymes, may play a role in TC killing. Knowledge about the exact localization of these molecules within the granule may give insight into their fate during lethal hit delivery. The subcellular distribution of the granule components and their possible routing to the granules as found in the present work are schematically shown in Fig. 7.

Perforin, granzymes, and lysosomal enzymes have been detected in subcellular fractions with similar gradient density

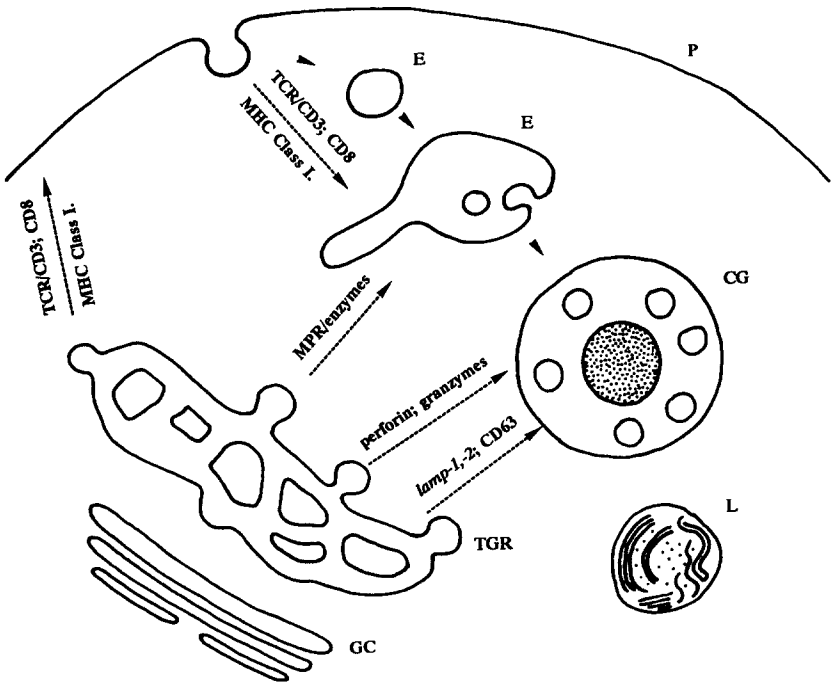


Figure 7. Schematic representation of the findings in this and a related (34) study. Symbols: CG, cytotoxic granule; E, endosome; GC, Golgi complex; L, lysosome; TGR, *trans*-Golgi reticulum; P, plasma membrane.

(8, 38). This had left unresolved whether they are present within the same granule or that different granule populations exist. In principle, they could be sorted towards specialized compartments of the biosynthetic route, since it is generally accepted that molecules traveling the regulated secretory pathway are segregated from molecules with lysosomal destination at the TGR (46, 52–55). Our double-labeling experiments show that all cytotoxic granules in CTL contain perforin and granzymes. In single-label immuno-EM studies, performed on plastic sections, perforin (33) and granzymes D/E/F (23) have been seen to be limited to the dense core. The present observation on cryo-sections reveals in addition that perforin and granzymes colocalize to the region of the granule containing the small internal vesicles. An issue that can not yet be resolved is whether perforin and granzymes reside within the small internal vesicles of the granule. It is clear that in murine CTL, perforin and granzymes are colocalized in the dense core. In human CTL, the dense core is clearly membrane enveloped and contains granzyme B. Several electron microscopic images showed association and possible fusion of the small internal vesicles with the dense core membrane. Therefore, we hypothesize that granzyme B is delivered to the dense core as a result of this fusion. It is very difficult to explain how perforin and granzymes could end up within the membrane-enveloped dense core, if they were be localized in between, rather than inside, the small vesicles. The fact that in murine CTL granules the small vesicles are very densely packed makes it very difficult to determine the exact localization of perforin. In human CTL, this is not the case, but unfortunately, anti-human perforin antibody is as yet not available. We expect that detection of perforin and granzymes in the intercellular cleft during CTL degranulation will resolve the question whether these molecules are released exclusively in membrane-enveloped and/or -associated form or also in soluble form.

So far, there has been only one study, in NK cells, regarding the biosynthetic targeting of these granule proteins (36). Burkhardt et al. (36) suggested that in NK cells, perforin, but perhaps not granzyme A, passes the TGR. Here, perforin and granzymes are shown to share the biosynthetic route from RER to TGR. Perforin and granzymes D/E/F were found in the same vesicles budding from TGR. How the vesicles transport perforin and granzyme molecules to the small internal vesicles and dense core remains to be resolved. Since the labeling intensity in TGR of lamp-1,-2 and CD63 was low, it was not possible to address the question whether they colocalized with perforin or granzymes. During lethal hit delivery, perforin and granzymes will be simultaneously exocytosed into the cleft between CTL and TC.

Apart from being a secretory organelle, the granule shares the following features with endosomes/lysosomes: (*a*) the granule incorporates extra-cellularly added protein-conjugated gold particles (34); (*b*) plasma membrane molecules, such as TCR/CD3, CD8, and MHC class I, are present in the membrane of small internal vesicles and dense core (34), probably as a result of endocytosis; (*c*) the granule is acidic (23, 32); (*d*) CI-MPR is present in only a small proportion of granules; (*e*) cathepsin D and possibly other lysosomal enzymes (acid phosphatase, arylsulphatase [8]) are contained within the lumen of the granule (34); (*f*) lysosomal integral membrane proteins, lamp-1,-2, as well as CD63 are present in the membrane enveloping the granule. Together, these findings define CTL granules as relatives of lysosomes.

Generally, lysosomes do not undergo exocytosis, except for instance in osteoclasts (56). While the cytotoxic granule is shown to have lysosomal properties, it is also a secretory or-

ganelle. Proteins destined for either the regulated secretory route or the lysosomal route are thought to be sorted at the TGR (52–55). However, within the granule, these routes are now seen to converge, since lysosomal and secretory proteins localize within one compartment.

The membranes of the dense core and small internal vesicles contain the TCR/CD3, CD8, and MHC class I molecules, facing outward (34), and also MHC class II as well as LFA-1 molecules (our unpublished results). Here, we demonstrate that perforin and granzymes are contained within the electron-dense core and over the region containing the small internal vesicles. Upon exocytosis, the membrane-enveloped dense core and small internal vesicles are released into the cleft. On the basis of these observations, we have proposed that the TCR/CD3 complex exposed on the dense core and small internal vesicles binds specifically to the relevant MHC/antigen complex on the TC membrane and not to the CTL itself, which prevents CTL damage (45). This model needs to be tested experimentally by showing killing specificity of isolated granule-dense cores and small internal vesicles.

Many recent reports on the biogenesis of lysosomes have suggested a wide variety of functions of lysosomal integral membrane proteins, such as maintenance of the acidic milieu, transport of amino acids, fatty acids, and carbohydrates, and protection from lysosomal enzymes (55). The presence of a highly glycosylated region of these molecules at the luminal side of the lysosome can possibly protect the delimiting membrane against degradation by lysosomal enzymes (57). Granule exocytosis would result in the exposure of lamp-1,-2 and CD63 at the CTL plasma membrane and, therefore, may also serve to protect the effector cell from released soluble granule proteins.

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