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Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes

An ultrastructural analysis of human cytotoxic T lymphocyte-target cell (CTL-TC) interaction has been undertaken to enable a better understanding of the killing mechanism. Attention was focused on granules in the CTL, which are known to contain lethal compounds. Within the membrane-delimited cytotoxic granule an electron-dense core as well as numerous membrane vesicles were identified. In CTL-TC conjugates, specific membrane interactions take place, allowing the formation of intercellular clefts into which the granule cores and internal vesicles are released. T cell surface membrane molecules known to be involved in CTL-TC interaction (T cell receptor, CD3 and CD8) are present on the membranes of the granule cores and internal vesicles, facing outward. An explanation for this localization of the membrane may be found in the fact that the granule is connected with an endocytotic pathway. Moreover, the lumen of the granule is rich in the enzyme cathepsin D, which indicates an association with a lysosomal compartment. Exocytosed vesicles and cores are seen to adhere to the plasma membrane of the TC. Although the exact contents of the granule vesicles and core remain to be identified, we suggest that specific interaction of CTL membrane molecules on the cytolytic granule components with molecules on the plasma membrane of the TC may ensure the unidirectional delivery of the lethal hit.

1 Introduction

CTL play a major role in the immune response against virally infected cells, allogeneic transplants and tumor cells. The actual mechanism CTL employ to destroy their target cells (TC) has been elucidated only in part. During the killing process several events take place in the following sequence: (a) binding of the cytotoxic cell to the TC, (b) reorientation of cytoskeletal elements and cytoplasmic organelles in the effector cell, (c) release of cytotoxic components towards the TC, (d) dissociation of the effector cell from the TC and (e) completion of the lytic process resulting in TC death (for reviews see [1–11]).

Binding of CTL to TC is mediated by membrane molecules and consists of two components: (a) antigen nonspecific adhesion, in which the leukocyte function-associated antigen-1 (LFA-1) and the CD2 antigen on the CTL and their respective ligands on the TC play a role [12–14], and (b) recognition of the TC by the CTL, which is mediated by the TcR on the CTL which binds to foreign antigen presented in the context of molecules of the MHC on the TC [14, 15]. The recognition step is thought to be accompanied by interaction of CD8 or CD4 antigens on the CTL with a monomorphic portion of class I or class II MHC molecules, respectively. Only when TC recognition takes place due to interaction of the TcR with the proper antigen/MHC combination does conjugate formation result in TC death. The TcR is physically associated with a complex of molecules, denoted CD3 [16]. The CD3 complex is believed to play a role in signal transduction of the cytoplasm after TcR triggering. This signal mobilizes the lytic machinery.

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Abbreviations: **TC:** Target cell **BSAG:** BSA complexed to colloidal gold

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Resting T lymphocytes do not have lytic potential. Upon activation, they may differentiate into CTL, with characteristic granules in the cytoplasm [17]. In response to CTL-TC binding the cytotoxic granules redistribute to become oriented towards the TC [18]. Several investigators have suggested that the granule contents are released into a space between CTL and TC [19, 20]. However, others failed to find any evidence for granule release after CTL-TC interaction [21, 22]. Granules of murine CTL and human CTL harbor the potent cytolytic agent perforin [23–27]. Under proper conditions, perforin can polymerize into supramolecular tubules, which insert into the TC plasma membrane resulting in tubular lesions and TC death [28]. In addition, cytotoxic granules have been shown to contain specific serine esterases with unknown function [29–33]. Purified intact granules still show potent lytic activity [4, 34, 35].

Thus, the granules in CTL contain a lytic machinery. Whether they are storage or secretory granules, lysosomes or another type of organelles is unknown. Here we present an analysis of the cell biological nature of the granules in an allospecific human CTL clone, using high resolution immuno-EM. We have followed the fate of the granules during the specific cytolytic interaction of the CTL clone with a B lymphoblastoid target cell line.

2 Materials and methods

2.1 Cells

The human CTL clone JS-132 was derived from donor JS (HLA-A3,3; B7,7; DR2,2) by mixed lymphocyte culture with cells of EBV-transformed B cell line JY (HLA-A2,2; B7,7; DR4,6) and cloning by limiting dilution [36]. The TC specificity of clone JS-132 was determined in cytotoxicity assays. The CTL recognizes the HLA-A2 molecule [37]. The CTL clone was maintained by weekly stimulation with a feeder cell mixture as described [36, 37].

2.2 Antibodies

The mAb 1C1 (IgG_1) was raised in mouse against the human T leukemic cell line HPB-ALL and recognizes an epitope on a variable region of the TcR β chain [37, 38]. RIV-9 (Sanbio, Uden, The Netherlands) is a mouse anti-human CD3 mAb (IgG_3), selected from a panel of anti-CD3 mAb on the basis of strong labeling of ultrathin cryosections of fixed CTL. Other commercially available anti-CD3 mAb were not useful for immuno-EM. OKT8 (IgG_{2a}; Ortho Pharmaceuticals, Raritan, NJ) was used to immunolabel CD8. mAb HC-10 (IgG_{2a}) was raised in mouse against purified, denatured HLA-B locus heavy chain protein [39] and HC-A2 (IgG_1) against purified, denatured and reduced HLA-A locus heavy chain protein (Stam et al., submitted for publication). All mAb were used as purified Ig. Rabbit anti-cathepsin D antibodies have been described [40].

2.3 Endocytosis of gold tracer by CTL

CTL were washed, resuspended as 10×10^6 cells/100 µl and incubated at 37 °C with BSA complexed to 5-nm colloidal gold (BSAG; [41]), at a concentration of about 50 gold particles/μm^3 medium. Samples were drawn after 10 min, 30 min or 2 h of incubation. Endocytosis of tracer was stopped by adding excess of ice-cold gold-free medium and subsequent centrifugation of cells (for 5 min at 4 °C). The samples were either directly fixed for immuno-EM or used for conjugate-formation experiments.

2.4 CTL-TC conjugate formation

Cells of CTL clone JS-132 and JY cells were harvested from culture 6 days after stimulation, washed in serum-free medium which includes 0.25% BSA [42] and resuspended in this medium at 10×10^6–20×10^6 cells/50 µl. CTL and TC were incubated separately at 37 °C for 10 min and then added together at a CTL/TC ratio of 2/1. Incubation took place for 1, 5, 10, 20, 30 or 60 min at 37 °C. At each time point a sample was drawn and suspended directly into 1 ml of fixative.

2.5 Morphology of CTL-TC conjugates

Cell samples were fixed at the indicated times after mixing of CTL and TC in a mixture of 2% formaldehyde and 2% acroleine in 0.1 M phosphate buffer, pH 7.4, for 2 days. They were postfixed in a 1% OsO_4 and 1% potassium ruthenium cyanide mixture in distilled water at room temperature for 1 h. After thorough washing in distilled water, cells were embedded in 2% agar and further processed for Epon embedding. For EM, sections were contrasted with 3% magnesium uranyl acetate at 63 °C for 30 min and Reynolds solution at 20 °C for 2 min. The number of CTL-TC conjugates relative to the number of CTL were evaluated by light microscopy to find the optimum incubation time for all the EM studies.

2.6 Immuno-EM

Concentrated cell samples were fixed in 2% formaldehyde and 2% acroleine in 0.1 M phosphate buffer, pH 7.4, for 2 h at 37 °C and 2 days at 4 °C. After washing and reconcentration, cells were embedded in 10% gelatin [43]. Gelatin blocks with cells were postfixed in the same fixative for 2 h and stored in 0.1 M phosphate buffer. The ultrathin cryosectioning and immunolabeling procedures have been described [44]. Briefly, cryosections of about 80 nm were immunolabeled with 6- or 9-nm colloidal gold particles complexed to protein A [41]. In the case of mouse mAb, a rabbit anti-mouse Ig was used as a bridging antibody between the primary mAb and the protein A-gold complex. When a rabbit polyvalent antibody was used first, an intermediate swine anti-rabbit IgG was used to enhance the immunolabeling. At the proper concentrations, background labeling over nuclei and mitochondria was < 1%. As controls, irrelevant primary antibodies were used at the same known concentrations (rabbit anti-rat pancreatic amylase and mouse anti-human mAb), or the antibodies were omitted. All controls were negative.

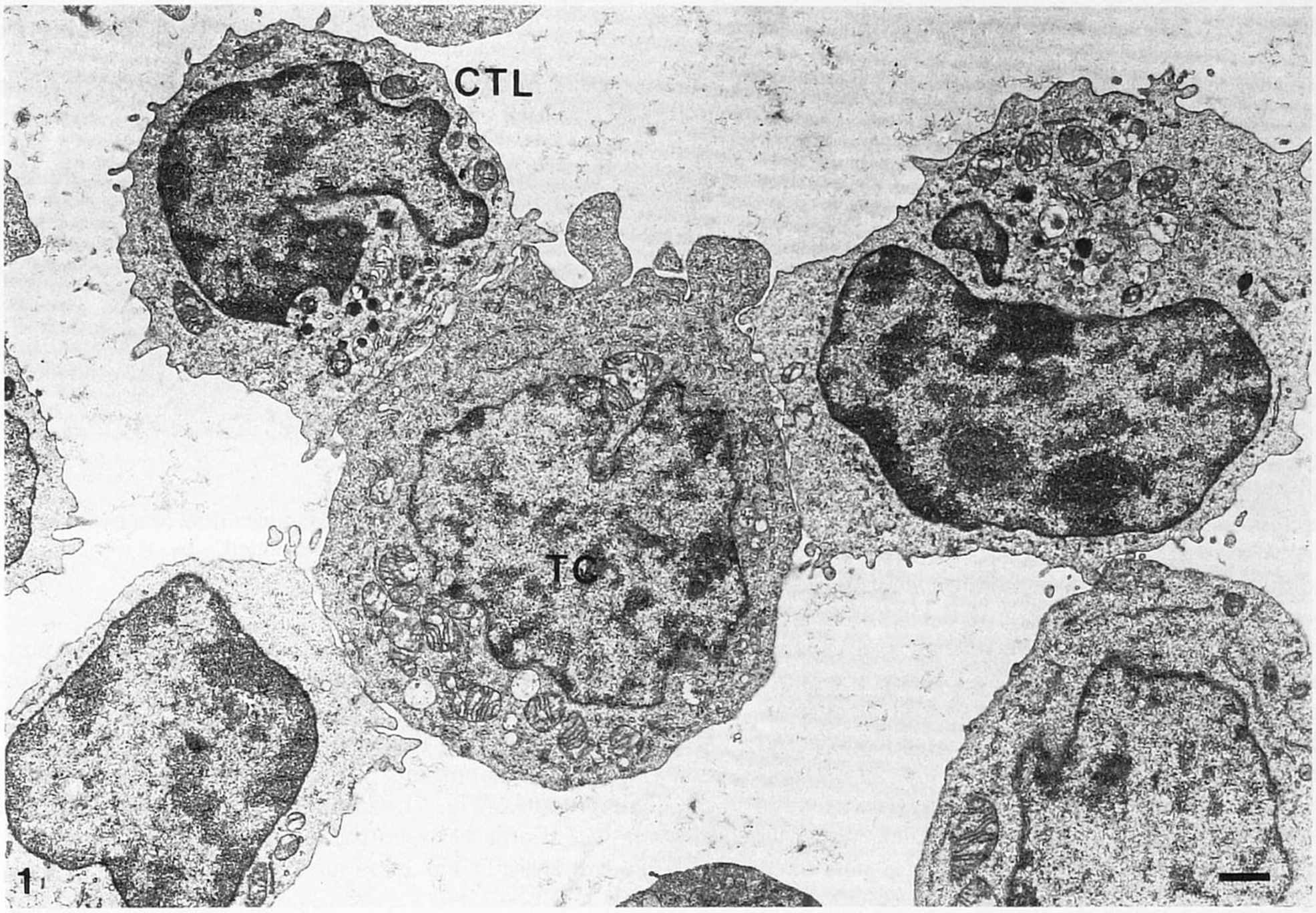
3 Results

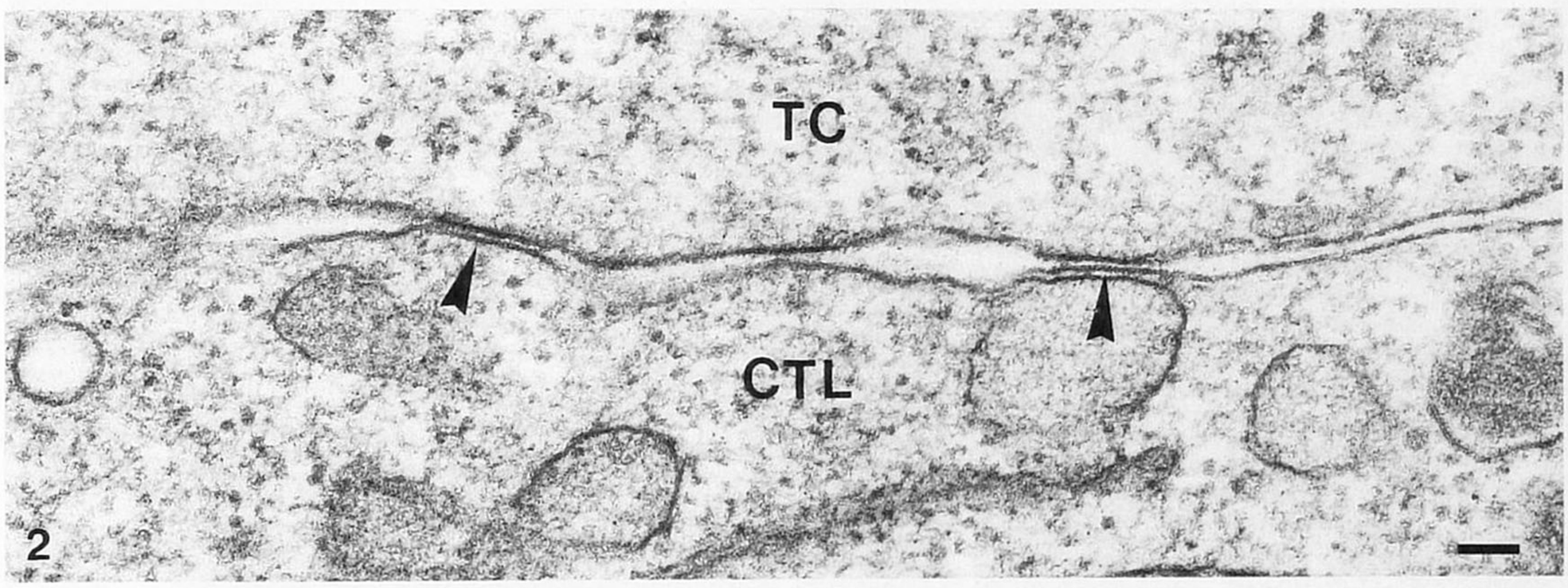
An impression of the ultrastructure of CTL-TC conjugate formation and the subsequent granule release is given in Fig. 1*. Conjugate formation between CTL and TC took place within seconds after cell mixing in our experimental set up. CTL could readily be recognized by the numerous cytotoxic granules containing electron-dense material and by nuclei with abundant heterochromatin. Nuclei of TC were bigger than those of CTL and were more electron lucent. Already 1 min after cell mixing, several CTL had directed their Golgi complexes and cytotoxic granules towards the TC (Fig. 1). We chose to concentrate on cells fixed at 5 min after cell mixing, when CTL were releasing granules. Narrow clefts occurred between CTL and TC, which seemed to be delimited at their lateral borders by membrane specializations (Fig. 2). The plasma membranes were locally thickened, but no tight junctions were found. Screening of numerous conjugates revealed several images of granules that were in a process of exocytosis, whereby their contents were released into intercellular clefts (marked C in Fig. 3).

The cryosectioning technique employed ensures excellent preservation of membrane detail. This was of particular value in resolving the substructure of the cytotoxic granules. The granules were membrane delimited and contained numerous small membrane vesicles and a membrane-delimited electron-dense core (Figs. 4, 9–12)**. Not in all core profiles could an enveloping membrane be seen and not all of the cytotoxic granules showed internal membrane vesicles. Furthermore, the cores are often surrounded by small vesicles which seem to fuse with the limiting membrane of the core (Fig. 12). The intercellular clefts contained small membrane vesicles and electron-dense membrane-delimited cores (Figs. 6–8). Both structures apparently originated from exocytosed cytotoxic granules; the process of exocytosis is seen in Fig. 5. The cores were often in close contact with the plasma membrane of the TC. Images as shown in Figs. 6–8 suggest binding of internal vesicles and cores to the TC plasma membrane. Binding of cores and vesicles to the CTL plasma membrane was not seen. The fate of the bound cores remained unclear. We have not

* Figs. 1–3 are electron micrographs of plastic section for routine morphology.

** Figs. 4–12 are electron micrographs of ultrathin cryosections, labeled with immunogold for detection of the antigens indicated. Unless indicated otherwise, fixation of CTL-TC clusters took place 5 min after cell mixing.





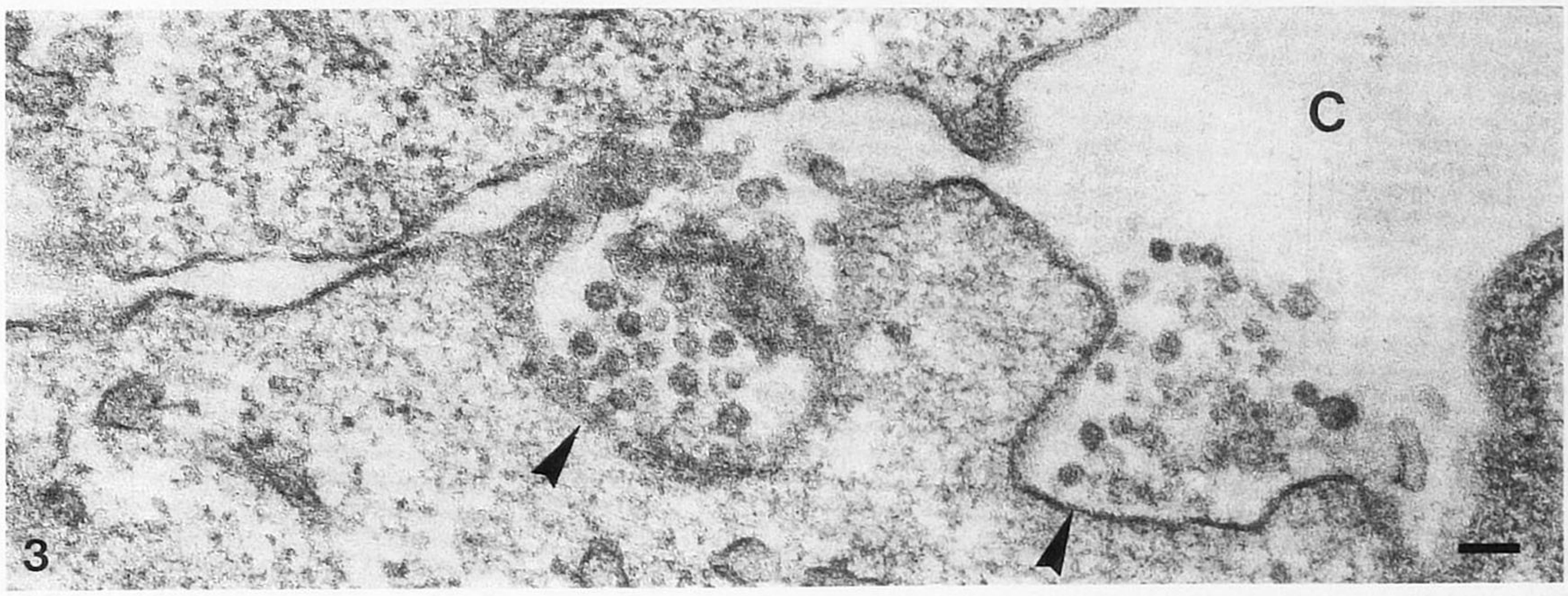


Figure 1. Cluster of CTL and TC, fixed 1 min after cell mixing. Three CTL, with characteristic dark nuclei, adhere to one TC (center). The cytotoxic granules in the upper left CTL are oriented towards the TC. The flattened contact zones between TC and upper CTL show narrow intercellular clefts. × 6200; Bar, 1 μm.

Figure 2. CTL-TC interface 1 min after cell mixing. A narrow cleft is present between CTL and TC. The arrowheads mark two sites of close apposition of the CTL and TC plasma membranes. × 61 000; Bar, 0.1 μm.

Figure 3. CTL-TC interaction 10 min after cell mixing. The CTL (lower) shows two exocytotic profiles of cytotoxic granules (arrowheads) with numerous membrane vesicles. C, cleft between CTL and TC. × 61 000; Bar, 0.1 μm. The dark core is not present in this particular ultrathin section.

found recognizable electron-dense material reminiscent of cores within the TC.

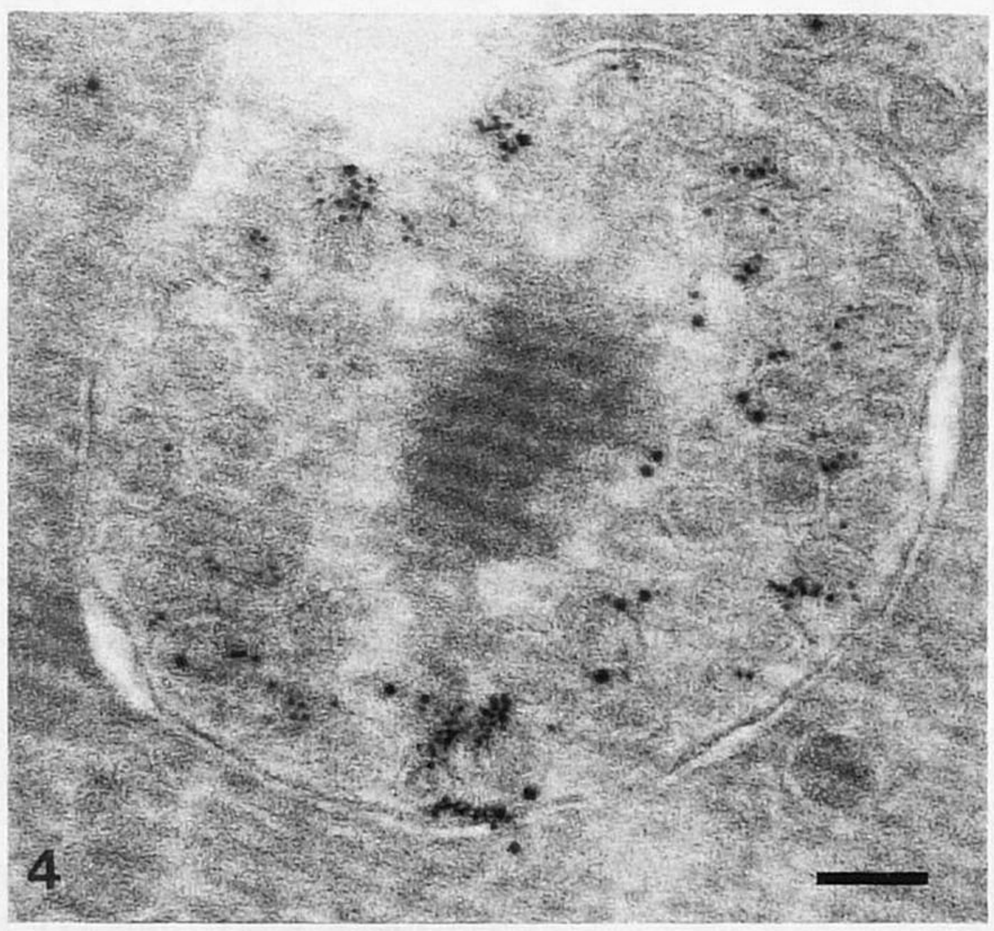
We were interested to know the localization of membrane molecules relevant for CTL-TC interaction. Therefore, TcR, CD3, CD8 and MHC membrane proteins were identified by immunogold labeling. Surprisingly, these molecules were not only present at the expected sites at the cell surface and in the biosynthetic route (rough endoplasmic reticulum, Golgi, trans-Golgi vesicles; not shown), but also in the cytotoxic granules. As shown in Figs. 9–12, the proteins occurred on the membranes of the cytotoxic granule, the dense core and the internal vesicles. The gold label on the membranes was oriented towards the lumen of the cytotoxic granule (quantitated in Table 1).

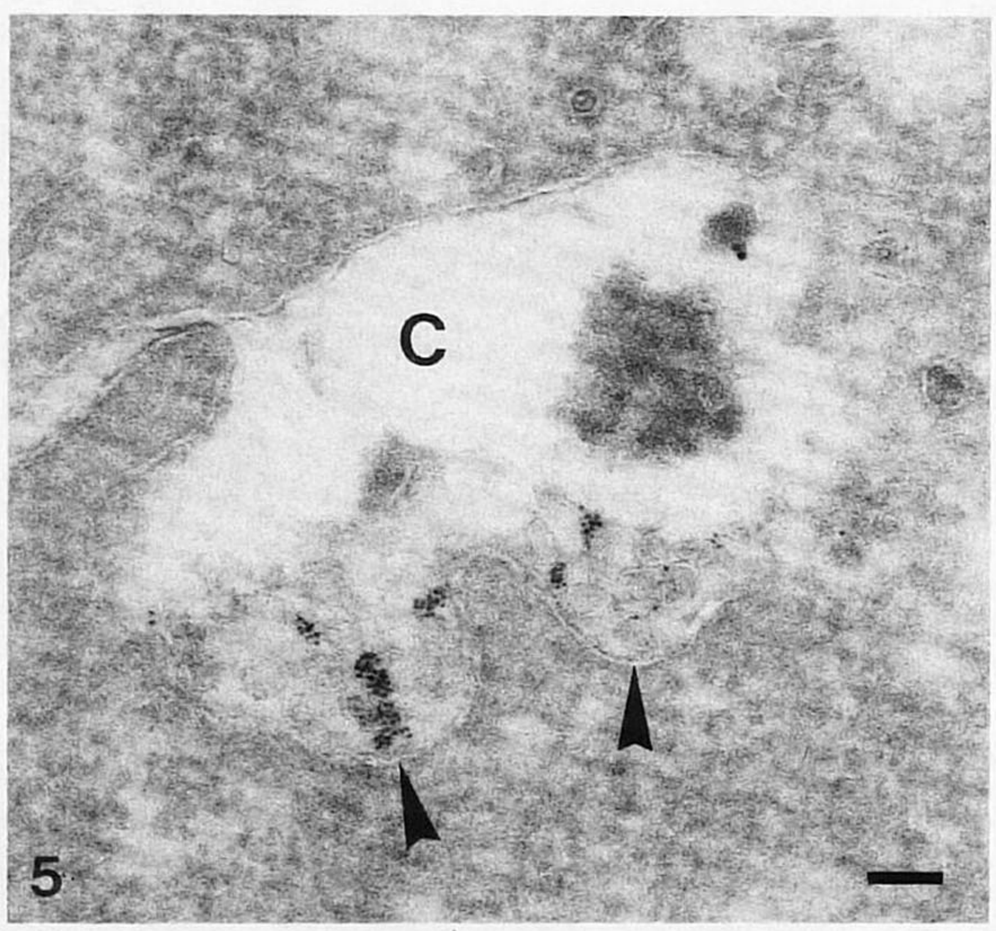
Table 1. Relative percentual distribution of gold particles labeled for TcR, CD3 and MHC class I[a)]

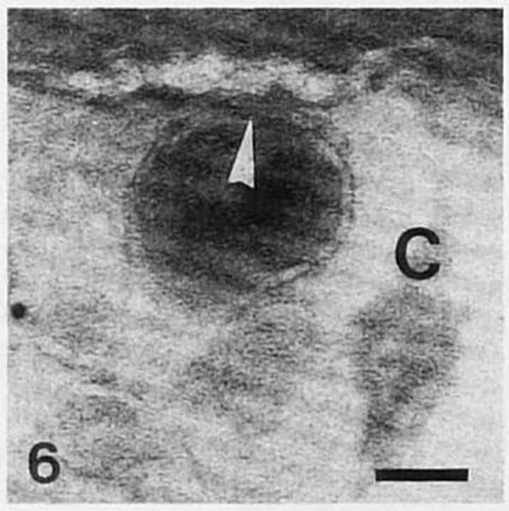
	TcR	CD3	MHC class I
On the outer surface of vesicles and dark core	58	67	68
Inside the vesicles and dark core	6	9	6
Over nondefined structures; probably tangentially cut vesicles	36	24	26

a) About 60 cytotoxic granules were examined from four experiments and about 1000 gold particles were counted.

To understand the presence of surface membrane molecules in the cytotoxic granules, the relationship of the granules with the plasma membrane was studied. We investigated to what extent cytotoxic granules are part of the endocytotic route when receiving BSAG as a tracer applied to CTL in suspension. Fig. 4 shows that BSAG was internalized and recovered in the cytotoxic granules amongst the internal membrane vesicles. BSAG was not found in the dense cores. Ten minutes after BSAG application, only occasional granules contained BSAG, while at 60 min, >20% of the granules contained BSAG. When CTL were first fed with BSAG for 30 min, incubated without tracer for another 30 min and next mixed with TC for 5 min, clefts and exocytotic profiles occasionally showed BSAG (Fig. 5). Immunogold labeling of cathepsin D, a major lysosomal enzyme, showed intense labeling of the cytotoxic granules (Fig. 4). Cathepsin D was confined to the electron-lucent area between the internal membrane vesicles. Labeled cathepsin D was also found in the clefts (not shown).







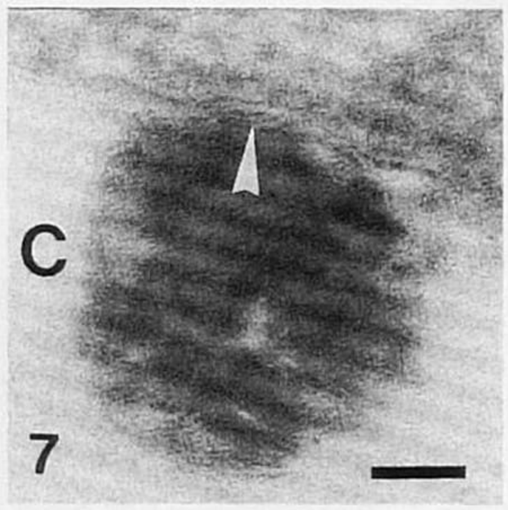
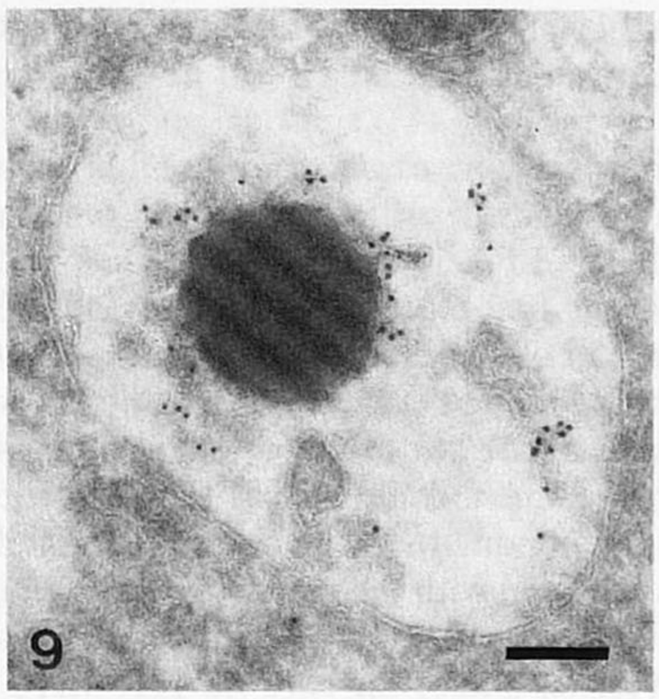
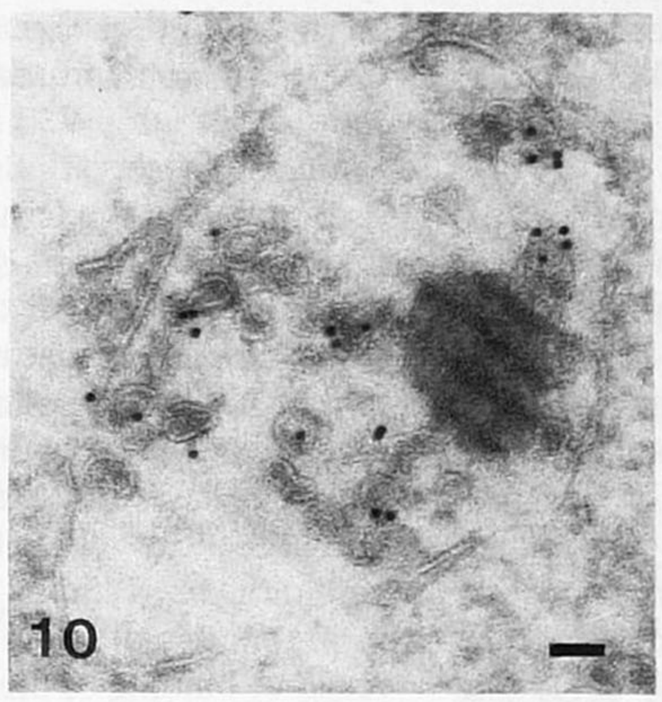


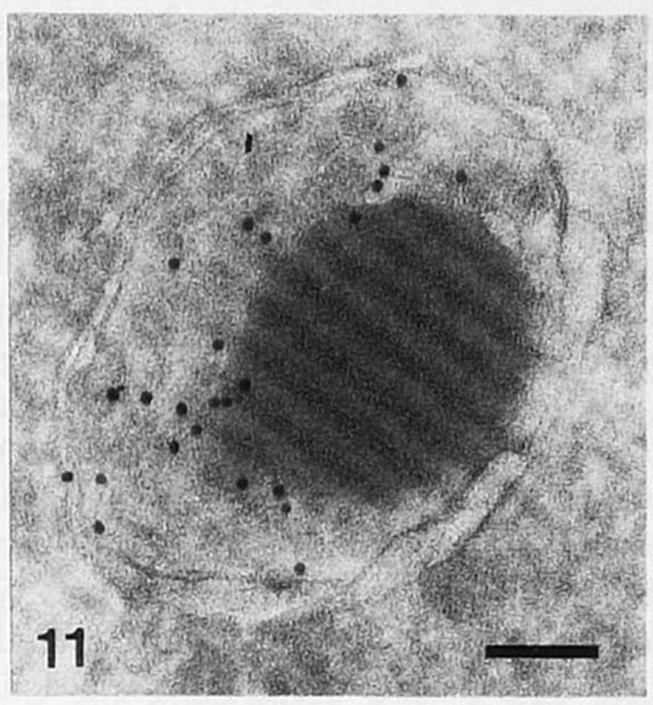
Figure 4. Cytotoxic granule with obliquely sectioned core and numerous internal membrane vesicles. Both cathepsin D (large particles) and BSAG (small particles) occur between the vesicles. × 90 000; Bar, 0.1 μm.

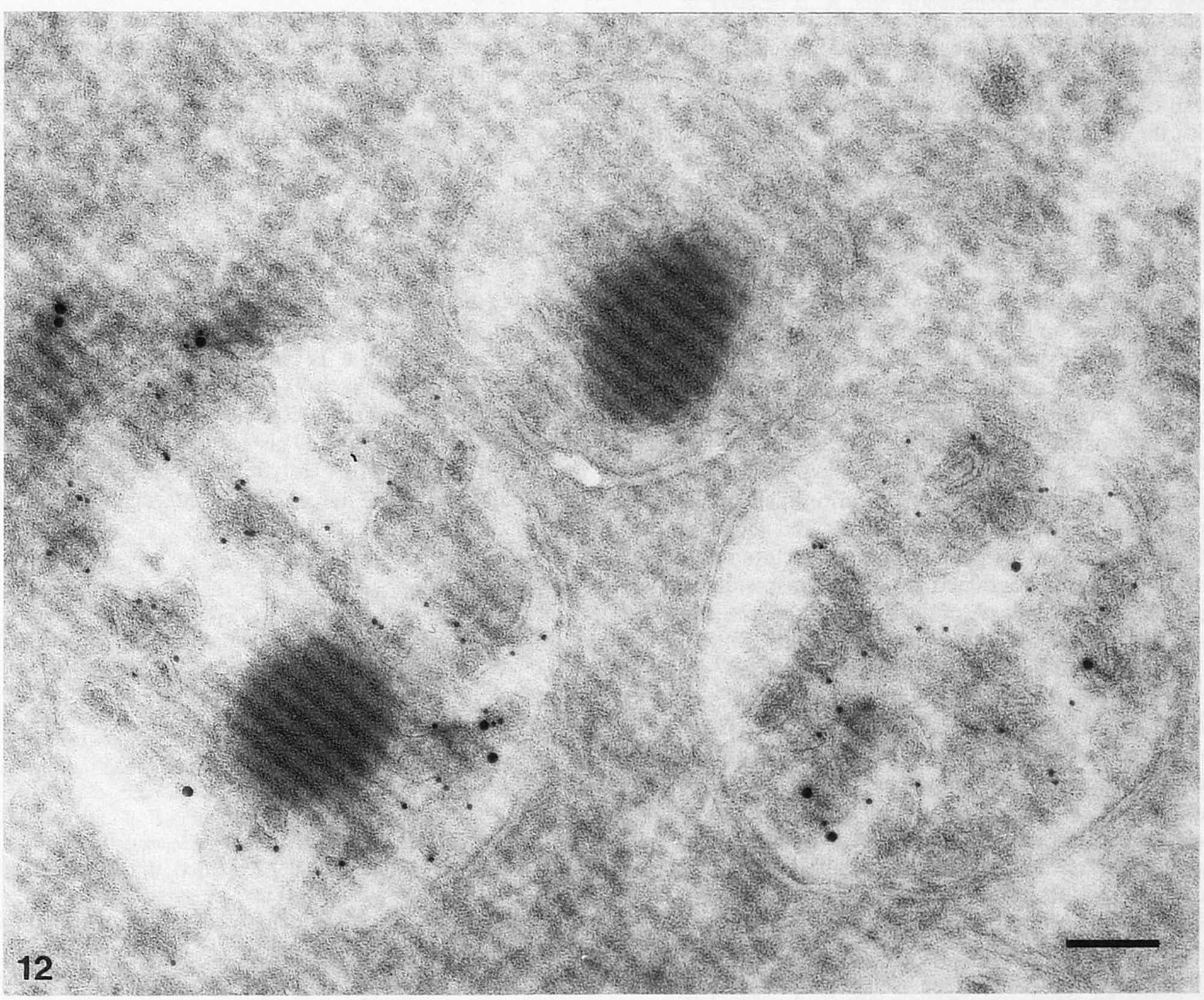
Figure 5. The two exocytotic profiles (arrowheads) show internal granule vesicles and BSAG internalized 2 h before mixing of the cells as described in Sect. 2.3. C, cleft. × 53 000; Bar, 0.1 μm.

Figures 6–8. Clefts (C) showing electron-dense cores of which the membranes are in intimate contact with the plasma membrane of the TC (arrowheads). The few gold particles represent cathepsin D labeling. Figs. 6 and 7, × 76 000; Fig. 8, × 74 000; Bars, 0.1 μm.









Figures 9–11. Labeling of TcR, CD3 and CD8, respectively, on the membranes of internal vesicles and electron-dense cores of cytotoxic granules. Fig., 9, × 80 000; Fig. 10, × 45 000; Fig. 11, × 92 000, Bars, 0.1 μm.

Figure 12. Double-labeling with anti-MHC HC-10C (large particles) and HC-A2 (small particles) mAb. Labels are present on the membranes of internal vesicles of two cytotoxic granules. One granule is completely negative. The core in the lower left granules shows a scalloped membrane that seems to be formed by fusion of internal vesicles. × 128 000; Bar, 0.1 μm.

4 Discussion

Ultrastructural features of the cytotoxic granules in murine and human CTL and NK cells have been described in detail [20, 35, 45–52]. The granules of both cell types are membrane delimited and contain an electron-dense core in an electron-lucent area with small membrane vesicles or tubules [35, 47, 50, 51, 53]. Our present observations in a human CTL clone confirm this morphology and add that electron-dense cores in CTL granules are themselves enveloped by a membrane. The origin of the core membrane is uncertain, but the morphology suggests that it is formed by fusion with the internal vesicles.

No parallel tubular structures as have been described for human NK cells [52] were detected in the CTL granules studied here.

Cinematography, videomicroscopy and conventional microscopy have shown that cytotoxic granules redistribute towards the TC within a few minutes after cell-cell contact. This is followed by degranulation [18, 45, 54, 56]. Our observation that many CTL had lost a considerable fraction of their granules within 10 min after initiation of cell-cell contact (data not shown) is in agreement with a rapid release of granules. The precise mechanism of granule release into the cleft has not previously been documented with micrographs. We show that release occurs by exocytosis, whereby the granule membrane fuses with the CTL plasma membrane and dense cores and internal vesicles are externalized into the clefts. The presence of membraneous materials and dense material in clefts has been noticed before [20, 53, 55]. In addition, we have observed that internal vesicles as well as dense cores can associate with the target cell membrane.

Recently, discussions have arisen about the necessity of cytotoxic granules for target cell destruction [57]. It has been demonstrated that granule-independent mechanisms of lysis may play a role as well [58, 59]. However, it is also beyond doubt that the granule component perforin can be instrumental in target cell destruction [29]. The granules contain in addition a family of serine esterases [33]. Knowledge about the exact location of perforin and the serine esterases within the cytotoxic granules would aid in the understanding of the mechanism of cytotoxicity. Particularly, it must be investigated whether these components are contained within the internal membrane vesicles and/or dense core, or like cathepsin D, are present in between these structures. This localization will determine whether the components are secreted into the cleft within membrane-contained structures or in soluble form. In the first case, the delivery of the cytolytic components to the target cell would require a specific mechanism. Of interest, preliminary observation showed the presence of perforin both in the dark core and associated with internal vesicles of the cytotoxic granules in murine CTL (manuscript in preparation). Along which pathway biosynthetic products are delivered to the granules is entirely unknown.

We have shown that the granule membranes are most likely, at least in part, derived from the plasma membrane through endocytosis. Fig. 13 illustrates the possible relationship between the plasma membrane and the granule membranes. The transmembrane orientation of membrane molecules and the localization of soluble components is also indicated in this figure. Endocytotic vesicles could fuse with the granule outer membrane. From this membrane the internal vesicles could derive by budding, which is also thought to occur in the case of the formation of internal vesicles of multivesicular endosomes and lysosomes [60]. In this way, cytoplasmic components could gain access to the interior of the vesicles within the granule. These vesicles could fuse with, or form the membrane of the dense core. Molecules originally present on the plasma membrane could in this way become localized on the membranes of the internal vesicles and dense core, with the original extracellular portion of the molecule facing outward (this has been substantiated by quantitating the gold labeling in Table 1). Upon granule exocytosis, the outer membrane of the granules fuses with the plasma membrane and the granule contents are released into the cleft. The granule vesicles and cores would thus expose plasma membrane molecules in their original transmembrane orientation.

From this observation we derive an explanation for the unidirectionality of the lethal hit. The T cell molecules involved in the cytolytic process, such as LFA-1, CD2, CD4 or CD8 and the TcR/CD3 complex in the first place play a role on the cell membrane by mediating CTL-TC contact and signal transduction resulting in the mobilization of the lytic machinery [14]. A second role for membrane molecules in the cytolytic process would be as constituent of the membranes of the cytolytic granules. The cytolytic components which are contained within the core and/or associated with the vesicles (manuscript in preparation) would be specifically delivered to the TC as result of binding of the vesicles and/or the core via TcR-MHC/antigen interaction, followed by membrane fusion. The orientation of the proteins in the membrane is consistent with this model. To explain why only interaction with a specific target cell occurs, we must assume that binding mediated only by molecules other than the TcR, such as LFA-1 is too weak or absent.

This model explains not only why a CTL does not kill itself, but also why so-called bystander cells, which are in close proximity, but do not bear the proper antigen, are not killed. Lanzavecchia [61] has shown that so-called backward killing can take place. A CTL clone was presented with a specific TC and at the same time with a T_h clone which recognized allo-MHC on the CTL clone. Both TC and T_h clone were killed, despite the fact that the helper cell did not bear the antigen recognized by the CTL clone. This result can be explained with the current model. The cytolytic components could specifically be delivered to the helper cell via interaction between

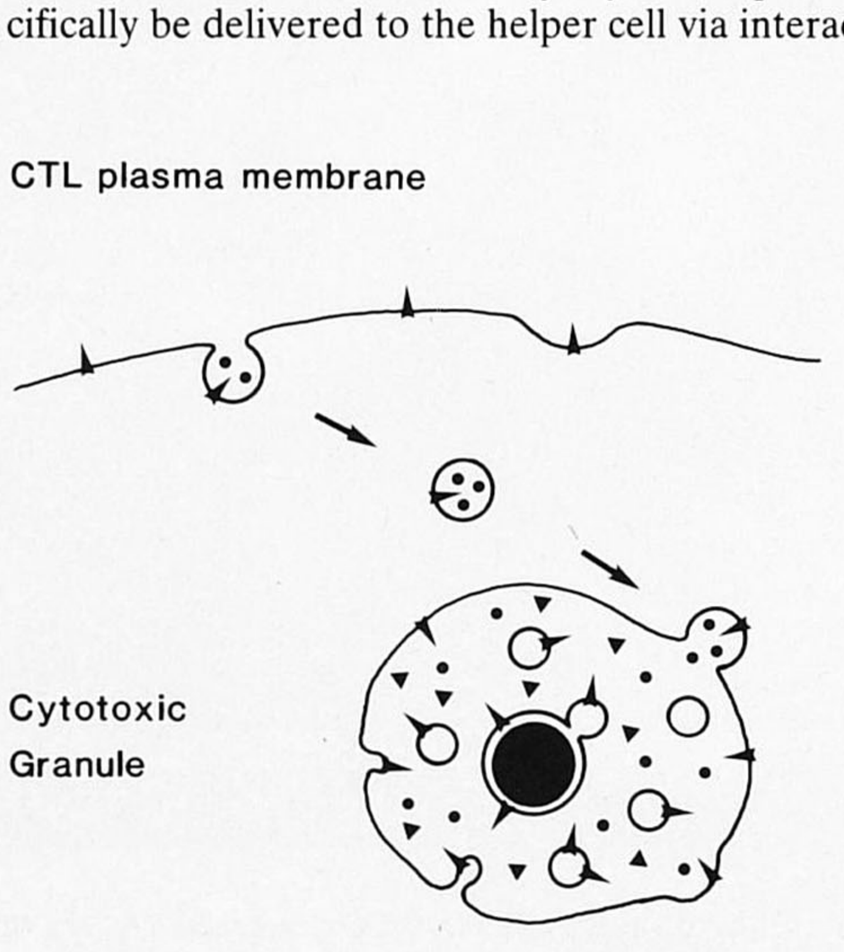


Figure 13. Schematic representation of a cytotoxic granule and the proposed origin of some granule components. Human cytotoxic granules contain an electron-dense core and numerous internal membrane vesicles. The limiting membrane of the cytotoxic granule gives rise to the internal vesicles by budding and fusion. The core is itself surrounded by a membrane which probably is formed by fusion of internal vesicles. Gold particles (dots) internalized by the cells are delivered to the granule content by fusion of endocytotic vesicles (arrows). The multiple fusion and fusion processes result in a proper orientation of TcR, CD3, CD8 and MHC molecules (arrowheads) in the membranes of internal vesicles and core to interact with the TC plasma membrane after exocytosis of the granule content. The lysosomal enzyme cathepsin D (triangles) is present in the granule content in between core and internal vesicles.

the TcR on the helper cell membrane and the proper MHC molecule on the granule membrane. This interaction is of the same nature as the CTL-TC interaction. We are aware that this model needs further work to be verified both by biochemical and ultrastructural studies.

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