Highly lytic in vivo primed cytolytic T lymphocytes devoid of lytic granules and BLT-esterase activity acquire these constituents in the presence of T cell growth factors upon blast transformation in vitro.

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HIGHLY LYTIC IN VIVO PRIMED CYTOLYTIC T LYMPHOCYTES DEVOID OF LYTIC GRANULES AND BLT-ESTERASE ACTIVITY ACQUIRE THESE CONSTITUENTS IN THE PRESENCE OF T CELL GROWTH FACTORS UPON BLAST TRANSFORMATION IN VITRO

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Demonstration of C-like "rings," lytic granules, and the Ca\textsuperscript{2+}-dependent lytic proteins—perforin/cytolysin—thereof, in certain cytoidal lymphocytes has led to the hypothesis of a mechanism of lytic granule-exocytosis and a common terminal lytic step in lymphocyte and C-induced lysis. However, neither cytoidal granules, nor formation of C-like rings during lysis have been detected in mature, highly potent, peritoneal exudate CTL (PEL) derived directly from the site of allograft rejection or in cytoidal hybridomas derived from them (PEL hybridomas). We now report that when stimulated in vitro in the presence of Con A supernatant, as a source of T cell growth factors (TCGF) or rIL-2, small in vivo primed PEL transform into large, dividing cytoidal T cells (PEL blasts) that express the same lytic specificity of the original PEL in short term lytic assays. The PEL blasts, in contrast to PEL, possess massive quantities of lytic granules, and protease (N-\textalpha-benzyloxycarbonyl-L-lysine thiobenzyl ester esterase) (BLT-esterase) activity as well as non-specific, cell-mediated cytolytic activity in a long term (4-h) assay. These results suggest that the proposed lytic mechanism involving exocytosis of lytic granules, perforin, and BLT-esterases and the formation of 10 to 20-nm lesions may apply to lysis induced by granule-containing effectors such as large granular lymphocytes and TCGF-dependent CTL lines, such as PEL blasts. However, killing by mature, in vivo primed CTL, such as PEL or their hybridomas, appears to be effected through an alternative, contact-induced, self-destruction process(es) of the target not involving secretory lytic granules or the above lesions. Hence, although the expression of lytic granules and BLT-esterase activities in cytoidal lymphocytes devoid of these components is induced by TCGF, these cellular constituents are not necessary for the expression of CTL-mediated target cell lysis by mature effector cells.

A unified concept for the lethal phase of immune cytolysis, lymphocyte-mediated (CTL/NK) or Ab+C-induced, has been proposed (1–3); namely, lysis caused by perforation of the target cell membrane by protein-lined membrane lesions (ID, 10 to 20 nm) believed to be structurally and functionally analogous to lesions produced by the membrane attack complex of the C system. Ca\textsuperscript{2+}-dependent secretory lytic granules containing the Ca\textsuperscript{2+}-dependent lytic proteins perforin/cytolysin and BLT-esterase activity have been proposed to be the origin of the lesion-forming material(s) (4–7). On the other hand, certain pre-lytic events inside the affected target cells led to the suggestion of an inductive mode of lysis initiated by submicroscopic target membrane rearrangements rather than perforation by the infliction of 10 to 20-nm pores (8–10). These include such events as the sudden elevation of cytosolic Ca\textsuperscript{2+} (9), and DNA disintegration before \textsuperscript{11}Cr-release from affected target cells (10). Also effective lysis induced by in vivo primed CTL devoid of lytic granules and in the absence of "ring" formation in the target has strengthened this notion (11, 12).

It is noteworthy that most, if not all, recent studies proposing a granule-exocytosis mechanism have utilized cytoidal effector cells growing in vitro in TCGF, Con A or antibody-activated lymphocytes, or LGL or LGL tumor cells (1–3, 13, 14). In the present study we explored the presence of lytic granules and BLT-esterase activity in mature in vivo primed, specifically sensitized CTL before and after cultivation in vitro in the presence of TCGF. To this end we exploited PEL, derived from the rejection site of second set tumor allografts that had been shown previously to be a rich source of highly potent, specific, small-to-medium sized CTL (15–17). In preliminary experiments (8, 11, 18) only background levels of lytic granules and BLT-esterase activity were detected in these highly potent PEL or in PEL hybridomas thereof. In the present study we show that lytic granules and BLT-esterase activity, as well as non-specific cytoidal activity, are acquired by PEL upon cultivation in vitro in

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\textsuperscript{5}Abbreviations used in this paper: ID, internal diameter; PEL, peritoneal exudate CTL, BLT, N-\textalpha-benzyloxycarbonyl-L-lysine thiobenzyl esterase; LGL, large granular lymphocytes; TCGF, T cell growth factors in Con A supernatants; Con A Sup, Con A Supernatant.

\textsuperscript{6}In a recent study Dennert et al. (19) reported the lack of lytic granules in PEL in line with our own observations on PEL reported earlier (11, 12, 20) and here. The origin of materials responsible for the C"rings" induced by PEL documented in an earlier study by Dennert and Podack (J. Exp. Med. 157: 1483, 1983) thus remains to be defined.}
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TCGF.  The acquisition of lytic granules and BLT-esterase activity by PEL that are already fully potent cytotoxic cells suggests additional functions of lytic granules and their constituents (21, 22) and has important implications regarding the mechanisms whereby granule-containing and granule-deficient effectors lyse target cells.

MATERIALS AND METHODS

Animals and Target Cells

BALB/c, C57BL/6, and DBA/2 mice and Lewis rats of both sexes were used. Leukemias EL4 of C57BL/6, L1210 and P815 of DBA/2, YAC-1 of A/Sn, and BW of AKR/J mice were either carried as ascites in syngeneic hosts or grown in vitro. SRBC were used within a month after collection of blood.

Chemicals, Isotopes, Culture Media, and Antibodies

The following reagents were employed: DNase I, BLT, dithiobis-2-nitrobenzoic acid, and NP-40 (Sigma Chemical Co., St. Louis, MO); uranyl acetate, Poly/bed 812. Na-cacodylate, glutaraldehyde, os-

Electron Microscopy

Cells and granules were fixed in 1% osmium tetroxide in the same buffer for 1 h. After washing in distilled water, the material was stained for 20 min in uranyl acetate and lead citrate and examined in a Philips EM 1430.

RESULTS

PEL and the generation of TCGF-dependent PEL-blasts. In preliminary studies, neither cytotoxic granules nor the formation of C-like ring structures were observed with potent, in vivo primed CTL such as PEL (11, 12).

Isolation and Testing the Lytic Activity of Granules and Post-Nuclear Extracts

The methods of Millard et al. (24) and Podack and Kungiebs (25) were followed. Washed cells (in Ca²⁺-free PBS, usually 200 to 500 × 10⁶ cells), suspended in disruption buffer containing 0.25 M sucrose, 4 mM EGTA, and 10 mM HEPES, pH 7.5, were subjected to N₂- cavitation (10 min, 450 psi at 0°C using a Yeda Press, a N₂-bomb equivalent). Alternatively, cells were subjected to glass/glass or Tef-lon/glass homogenization. N₂-pressed cells were ejected slowly to allow cavitation, brought to room temperature, treated with DNase I, and then centrifuged at 170 × g to remove whole cells, nuclei, broken cells, and large fragments. The supernatant (usually 5 ml) was overlayed on a Percoll gradient composed of a mixture containing 9.6 ml stock Percoll (Percoll suspension in 0.25 M sucrose, 10.4 ml disruption buffer and centrifuged at 29,000 rpm for 30 min in a Beckman Ti60 rotor at 5°C. The gradient was fractionated into 0.8-ml aliquots that were tested for cytolytic activity against ¹¹¹Cr-labeled SRBC in the presence of Ca²⁺, and in its absence (see below). Percoll particles were removed by high speed centrifugation (Beckman Ti60 rotor, 45,000 rpm, 2.5 h, at 4°C). Percoll-fractionated cytotoxic granules or crude post-nuclear extracts were serially diluted in 10 mM HEPES, 150 mM NaCl, pH 7.4, to test their lytic activity. Twenty microliters of the diluted granules were added in round bottom microtiter plate wells with 30 μl ¹¹¹Cr-labeled SRBC suspended in the above buffered saline supplemented with 7 mM CaCl₂. After a 30 to 60-min incubation at 37°C, the reaction was stopped by adding 150 μl 2% FCS in PBS, and the remaining cells were pelleted at 2000 rpm, 4°C, for 10 min. One hundred microliters of the supernatant were counted in a gamma counter to determine the percentage of ¹¹¹Cr released and the extent of lysis using the aforementioned formula.

Protease (BLT-Esterase) Activity

Serine esterase activity was determined essentially as described (6). Briefly, cells were washed twice in Ca²⁺-free PBS and lysed on ice for 30 min in 0.5% NP-40 in PBS-(Ca⁺²)(10 × 10⁶ cells/ml). One hundred microliters of diluted cell lysates, granules, or post-nuclear preparations were mixed with 900 μl of the reaction mixture (0.2 M Tris- HCL, pH 8.1, 0.2 mM BLT (Sigma Chemical Co., St. Louis, MO), 0.2 mM dithiobis-2-nitrobenzoic acid (Sigma). After 15 to 30 min at room temperature, light absorbance at 412 nm was measured using 100 μl PBS-(Ca⁺²) + 900 μl reaction mixture as blank. Results were expressed as OD units recorded at 412 nm/1 × 10⁶ cells/15 min at room temperature.

Electron Microscopy

Cells and granules were fixed in 0.1 M cacodylate buffer, pH 7.4, containing 5 mM CaCl₂ at room temperature for 2 h. Fixed material was re-pelleted, wrapped in agar (3.4% Bacto agar in H₂O), washed overnight with the cacodylate buffer, and postfixed in 1% osmium tetroxide in the same buffer for 1 h. After washing in distilled water, the material was stained for 20 min in the dark in block with 2% aqueous uranyl acetate, dehydrated in ethanol, and embedded in Poly/bed 812. Thin sections were stained with uranyl acetate and lead citrate and examined in a Phillips EM 410 electron microscope operated at 80 kV.
PEL consist of mostly small-to-medium-sized Thy-1⁺, Ly-2⁺, L3T4⁻ TcR⁻ non-dividing lymphocytes of which 35 to 50% specifically bind to and lyse target cells (15-17, 26). In the present studies, we wished to ascertain whether PEL might acquire cytocidal granules upon in vitro stimulation with TCGF and Ag. BALB/c anti-EL4 (H-2b anti-H-2d) or C57BL/6 anti-L1210 (H-2b anti-H-2d) secondary PEL were prepared and purified as described earlier (16). Cultivation of PEL in vitro for several days with or without mitogens (e.g., Con A), resulted in loss of viability and cytocidal activity. However, when cultured in the presence of TCGF-containing Con A sup (20%) or rIL-2 (50 U/ml), with or without x-irradiated spleen cells of the immunizing genotype, a certain fraction of the small, non-dividing PEL transformed into large, dividing granular cells now called PEL blasts. One line, designated E, has been cultured in vitro with TCGF (Con A sups or rIL-2) for more than 1 year, and is still growing. Table I shows the cytocidal activity and specificity of BALB/c anti-EL4 PEL blasts (line E) as defined in Materials and Methods. The growth and cytocidal activity of PEL blasts and the presence of cytocidal granules thereof were obligatorily dependent on the supply of external TCGF (Con A sup). Omitting TCGF from the medium resulted in a marked reduction in cell number, viability, and anti-target activity by both intact PEL blasts and their lysates when tested against SRBC (Table III).

BLT-esterase, lytic granules, and lymphocytotoxicity in the course of transformation of PEL into PEL blasts. As with cytocidal granules, high levels of protease (BLT) esterase activity have been detected in a wide range of cytocidal lymphocytes, for the most part growing in vitro in IL-2, and in NK cells (6, 28, 29). This observation, combined with the demonstration of enzyme release during lymphocyte-induced killing (6, 30) led to the suggestion of its possible involvement in the cytocidal process (6, 28-32), although lysis in the absence of secretion has been shown (33, 34). As Table IV illustrates, highly potent...
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Figure 1. Electron micrographs of BALB/c anti-EL4 PEL (A) BALB/c anti-EL4 PEL blast (B), and CTLL-2 (C) with their corresponding isolated cytoplasmic granules (a, b, and c, respectively). The single PEL granule (a) is the only granule structure observed in PEL preparations fractionated on Percoll and is lacking the dense inner structure observed in bands b and c. Bar represents A) 483 nm; a) 79 nm; B) 2100 nm; a) 610 nm; C) 505 nm; c) 555 nm.

PEL and PEL hybridomas possessed only background levels of BLT-esterase activity, comparable to non-cytocidal cells, such as EL4, and P815 cultured with and without TCGF. In contrast, the PEL blasts cultured either in rIL-2 or in TCGF exhibited massive quantities of BLT-esterase activity—most, if not all, of which were associated with the lytic granule fraction (not shown). It is noteworthy that BLT-esterase activity increased with the age of the culture.

Figure 3 shows the time course (over a 12-mo period)
lytic granules and BLT-esterase were abundant. These results suggest that neither BLT-esterase nor lytic granules are involved in lysis by in vivo primed CTL (such as PEL) and that production of the enzyme and lytic granules is influenced by TCGF.

The most important finding reported in this paper is that highly potent CTL primed in vivo, devoid of lytic granules and BLT-esterase activity, acquire these constituents upon blast transformation induced in vitro in the presence of TCGF. This finding has implications with regard to the mechanism of lymphocytotoxicity by in vivo primed cells devoid of lytic granules before and after in vitro cultivation with TCGF as discussed below. In many previous studies, cytocidal granules were isolated from lymphoblastoid cell lines growing in IL-2, from MLR-activated lymphoblasts, or from LGL or NK-like tumor cells growing in vivo, resulting in the proposal of a causal relationship between cytocidal granules and cellular cytotoxicity (1-3). In a few cases, however, granules could not be extracted from certain cytocidal cells whereas in others, paradoxically, lytic granules were derived from non-lytic cells (4). We have exploited highly potent cell-mediated cytolytic activity in the absence of BLT-esterase and lytic granules at the time of commencement, later the PEL gradually transformed into PEL blasts (first detected after 3 days in culture) and acquired both the enzyme and lytic granules. After 2 mo in culture lymphocytotoxicity began to decline, whereas BLT-esterase and lytic granules were abundant. These results suggest that neither BLT-esterase nor lytic granules are involved in lysis by in vivo primed CTL (such as PEL) and that production of the enzyme and lytic granules is influenced by TCGF.
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cated by the lack of C-like ring structures on target cell membranes after PEL-induced lysis (12). Although lysosomal granules and a giant Golgi apparatus were observed by Zagury et al. in PEL conjugated to target cells, in our own laboratory (27) typical lytic granules with a dense inner mass (5) were not seen in PEL in that study (27) and in the present study (Fig. 1Aa). In accordance with this observation, only background hemolytic activity against SRBC was detected with isolated granule fractions and crude lysates derived from several batches of up to 500 x 10^6 secondary PEL (containing about 200 to 250 x 10^6 CTL) (Fig. 3) (8, 11, 12, 18, 21). This finding was confirmed by Dennert et al. (19) and more recently by Nagler-Anderson et al. (35). On the other hand, Munger et al. (36) reported a total of 12 Ca^{2+}-dependent LU per 300 million PEL, concluding that these CTL also utilize lytic granule exocytosis to kill. However, in earlier studies (4), that group reported that various non-lytic control cells possess 10 or fewer LU/10^6 cells, whereas cytoidal cells contain hundreds and up to 4200 U/10^6 cells. The near background cytolysin activity found in PEL by Munger et al. may be accounted for by a 1/1000 contamination with peripheral blood LGL during the collection of PEL from the peritoneum or with a few PEL precursors (26). Granules exhibiting both the typical ultrastructure (Fig. 1) and Ca^{2+}-dependent anti-SRBC activity (Fig. 2) were induced in PEL upon cultivation in vitro in TCGF and transformation from small, non-divided PEL into large, granular PEL-blasts (Fig. 3).

Structural studies of CTL/target conjugates revealed distinct effector cell projections at the contact area, containing a network of fine fibrillar materials devoid of ribosomes, granules, and other organelles (37). Close proximity between the effectors’ Golgi zone (27, 38), microtubules organizing center as well as other cytoskeletal elements (39-41) and the contact zone has been shown. Importantly, however, no granules originating in the effectors were convincingly detected at, or in the intercellular space, or in the process of fusion with the plasmalemma of the effector or the target. Furthermore, of the typical cytoidal granules observed by electron microscopy in PEL blasts (Fig. 1Bb), none was observed in effector cell projections penetrating conjugated target cells or in the process of fusion (8). Although lysosomal enzymes were reported at the CTL/target cell (TC) contact area (42), neither their cellular source (effector or the affected target) nor their possible role in lysis was determined.

Both BLT-esterase and lytic activities are packaged within lymphocyte granules (5, 6) although there is no correlation between the expression of BLT-esterase and cytotoxic activity (31). Testing for BLT-esterase in PEL and in PEL blasts (Table IV, Fig. 3) was of particular interest in view of the absence of lytic granule activity in PEL and the abundance of lytic granules in PEL blasts (Fig. 2). Only background levels of BLT-esterase activity were detected in PEL and PEL hybridomas; on the other hand, certain lines of PEL blasts revealed up to 100-fold higher enzyme activity as compared with PEL (Table III). These results correlate well with the acquisition of lytic granules upon TCGF-induced transformation of PEL into PEL blasts (Figs. 2 and 3). We have recently obtained evidence for the acquisition of transient BLT-esterase activity in early stages of primary, but not secondary, CTL response in vivo, in accord with the proposal (21) that granules are induced under the influence of TCGF. Mature PEL are small, non-dividing cells that exhibit potent, specific cytocidal activity (16) but no BLT-esterase or lytic granules. On the other hand, the cell population that contains PEL precursors is comprised of dividing lymphoblastoid cells (26) that express BLT-esterase activity in vivo, possibly as differentiating factors. Hence a small proportion of such not fully differentiated lymphoblastoid cells generated in vivo in the course of primary i.p. alloimmunization may account for the near background BLT-esterase observed by Munger et al. (36).

No BLT-esterase or lytic granules have been detected in early or later stages of secondary CTL employed in the present study. Enzyme secretion during lysis induced by PEL blasts but not by PEL, has been observed (G. Berke and D. Rosen, unpublished observations).

Obvious secretion of esterases during lysis induced by certain CTL/NK has been demonstrated before (6, 30), but no clear role for the enzyme in lysis induction was proposed or has been shown to date. Finally, based on the finding that certain target cells can undergo CTL-mediated lysis in the absence of Ca^{2+} in the medium (9), two recent studies show effective CTL-mediated lysis in the absence of enzyme (BLT-esterase) secretion (33, 34). We have proposed (21) that the enzyme-containing lytic granules are generated as part and parcel of TCGF-dependent Ag/mitogen/lymphokine-induced lymphocyte activation in vivo or in vitro. These granules and their enzymes may play only an indirect function in the cytoidal process exhibited by activated lymphocytes, and their primary role may be in the extravasation of responding lymphocytes and secretion of cytokines (22, 31), and perhaps even in the self-annihilation of responding lymphoblasts when the supply of TCGF and other growth-promoting factors become limiting (8, 21).

Precisely how lymphocytes kill other cells has been the subject of numerous investigations since Govaerts’ original observation of cytoidal lymphocytes in alloractivity. Recently, the insertion of protein-lined, cylinder-shaped lesions (ID, 10 to 20 nm) in the target cell membrane has been proposed as a unified mechanism in lymphocytotoxicity (1-3, 32). The proposed protein-lined lesion has been attributed to a polymerized form of a Ca^{2+}-dependent lytic protein (perforin/cytolysin), originating in CTL/NK cytoidal granules and secreted in a Ca^{2+}-dependent manner upon effector/target interaction (2-4). However, several lines of evidence do not support a unified mechanism in lymphocytotoxicity. These include 1) the finding of distinct non-perforin 1 lytic molecules such as lymphotoxin, TNF, NK cytotoxic factor, and others (10, 43-46) shown to act differently; 2) effective CTL-mediated lysis in the absence of external Ca^{2+} (9) and without exocytosis, even with granule-containing effectors (33, 34) has been reported; 3) lysis by in vivo primed PEL devoid of lytic granules and in the absence of C-like lesions (ID, 10 to 20 nm) (12); and 4) prelytic events that are difficult to explain with the above lesions as first events in lysis induction (see below and Reference 10).

Studies on the mechanism of CTL-mediated lysis indicate that lysis is initiated by sub-microscopic perturbations in membrane structure (“membrane derangements”) resulting in ion (K+) leakage and membrane de-
polarization (8, 9, 47, 48) rather than by perforation of the membrane by 10 to 20 nm holes (8). Perturbations of the target cell membrane may be effected by binding of the intact CTL itself, through its T-T₃ complex or by a secreted effector molecule. Subsequent permeability changes observed in the affected target (49) probably reflect progressively falling ion pumps that are only initially capable of controlling K⁺ efflux (depolarization) and Na⁺ and Ca²⁺ influx through the deranged membrane. An important early event in CTL-induced lysis is the sudden prelytic increase in cytosolic Ca²⁺ in the target, initially observed in this laboratory (S), and more recently by Poenie et al. (50) and by Albrighton et al. (51). A sudden prelytic influx of Ca²⁺ into cells under C attack has been reported earlier (52). Cytosolic Ca²⁺ can rise through the deranged target cell membrane or through voltage-dependent gates of the depolarized membrane down a 10,000-fold Ca²⁺ concentration gradient, or by a (pH- or IP₃-mediated) release mechanism from internal stores (47, 52). Intracellular Ca²⁺ concentration is a well appreciated factor involved in cell injury induced by a wide range of molecularly unrelated toxic agents (53, 54). Lysis induced by the Ca²⁺-ionophore A23187 provides a good example for the primary relationship between increased cytosolic Ca²⁺, temperature, and cell damage. In the absence of Ca²⁺, certain cells tolerate up to 100 μM A23187; the inclusion of Ca²⁺, but not Mg²⁺, brings about sudden cell death. Hence A23187 in itself is not toxic to the cell; its toxicity is Ca²⁺ mediated (8). On the other hand, controlled Ca²⁺ influx or its release from internal stores is a well recognized signal, including the induction of repair mechanisms after low level Ab+C attack (53.54). Lysis under CTL attack, when derangement of the membrane is considerable, prelytic elevation of cytosolic (and probably nuclear) Ca²⁺ may account for distinguishable prelytic effects such as DNA disintegration, mitochondrial damage, blebbing, cytoplasmic streams, membrane damage, and zeliosis (8, 48). The mechanisms whereby a persistent elevation of cytosolic Ca²⁺ brings about cytolysis is probably many faceted (9, 53, 54). The effects of Ca²⁺ on phospholipase A₂, resulting in production of lyso-derivatives, may account for terminal damage to the membrane. Ca²⁺-dependent proteases may be responsible for damage to the cytoskeleton (55). Direct Ca²⁺ effects on mitochondria can account for early damage to these organelles, in the target observed during CTL-mediated lysis (56). This in itself can suppress energy production, hence exacerbating the function of ATP-dependent ion pumps. Ca²⁺ activation of a major cellular ATPase such as actomyosin could lead to major ATP depletion, and consequently to the additional suppression of ATP-fueled Na⁺/K⁺-ATPase (pump) activity (9), already compromised by the depolarization of the membrane.

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