CYTOLYTIC T CELL GRANULES

Isolation, Structural, Biochemical, and Functional Characterization

By ECKHARD R. PODACK and PAULA J. KONIGSBERG

*From the Department of Immunology, Research Institute of Scripps Clinic,
La Jolla, California 92037*

Previous studies (1–6) on the mechanism of cell-mediated cytotoxicity have suggested, largely on the basis of morphological evidence, that the dense, cytoplasmic granules of killer lymphocytes of NK[1] and T lineage contain cytolytic proteins (perforins) that may be responsible for target cell lysis by a mechanism involving protein polymerization and insertion of transmembrane channels (poly perforins) into target membranes. As a critical test for this postulate, granules were isolated from cloned T cell lines and tested for their cytolytic activity. It will be shown in this study that isolated T cell granules are highly cytolytic for tumor cells and that they contain a distinct set of proteins capable of assembling poly perforin tubules that may be responsible for target cell lysis. A preliminary account of this work has been published (6).

Materials and Methods

Cell Lines. El4, S194, P815, P815 RW, and BW5147 were maintained in culture by standard tissue culture procedures. P815 and P815 RW are cells sensitive to macrophage- or NK cell–mediated lysis, respectively. P815 RW was isolated by Dr. R. Welsh and provided by Dr. R. Schreiber of this Institute. CTLL-2 (obtained from Drs. Amnon Altman and Dennis Carson of this Institute) were maintained in antibiotic-free Iscove's modified Dulbecco's minimum essential medium (7, 8) supplemented with 10% rat concanavalin A (Con A) supernatant, 5% fetal calf serum, 10 mg/l insulin (Boehringer, Indianapolis, IN), 10 mg/l transferrin (Boehringer), 30 mg/l soybean lipids (Boehringer), and 10^{-5} M β-mercaptoethanol.

A murine, long-term, cytolytic T cell line, B6, was generated from a mixed lymphocyte culture of C57 B16 (H2^b) spleen cells against BALB/c (H2^d) irradiated spleen cells in the same medium. Initial stimulation was without Con A supernatant at 10^6/ml responder cells and 4–8×10^6/ml irradiated stimulator cells for 5 d. Subsequently the cultures were maintained in 9% rat Con A supernatant and stimulated with 10^6/ml irradiated stimulator cells. After three months in culture, effector cells were stimulated with the irradiated T cell lymphoma line S194 (H2^d) at 10^5–10^6 cells/ml instead of BALB/c spleen cells. Effector cells continued to proliferate and were used for preparation of granules after a total of

---

[1] *Abbreviations used in this paper:* Con A, concanavalin A; DOC, deoxycholate; EGTA, ethylene glycol-bis-(β-amino-ethyl ether)N,N'tetra-acetic acid; NK, natural killer; PMA, phorbol myristate acetate; Poly P1, poly perforin 1, the $\sim$160-Å wide tubular complex assembled during cytolysis by NK and cytolytic T cells; Poly P2, poly perforin 2, tubular complex of 50–90 Å width; SDS, sodium dodecyl sulfate.

five months in culture. At this time the effector cell population was exclusively specific for $H2^d$ targets and showed no NK-like specificity.

For bulk cultures of CTLL-2 and B6, the cells were grown in 15-cm petri dishes containing 150 ml of the above medium. The cells were seeded at a density of 10^4/ml (CTLL-2) or 5×10^4/ml (B6) and harvested when they reached 6×10^5 to 10^6/ml.

Rat Con A supernatant was produced by incubating 2×10^6/ml rat (any strain) spleen cells in 2% fetal calf serum, 10^{-5} M mercaptoethanol containing RPMI 1640 with 5 μg/ml Con A for 36 h at 37°C, 5% CO_2, in 15-cm petri dishes. The supernatant was harvested, sterile filtered through 0.22-μm membranes and frozen at -20°C.

Preparation of Granules from CTLL-2, from B6 and from El 4. 5×10^8–1.5×10^9 cells were harvested by centrifugation, washed twice with 50 ml Hank's balanced salt solution, and resuspended in 20 ml ice cold relaxation buffer (100 mM KCl, 3.5 mM $MgCl_2$, 1 mM ATP, 10 mM piperazine-N,N'bis (2-ethane-sulfonic acid)) (pH 6.8) (9) containing 1.25 mM ethylene glycol-bis-(β-amino-ethyl ether)N,N'tetra-acetic acid (EGTA). The plasma membranes were disrupted by N_2 cavitation after equilibration in N_2 for 30 min at 4°C at 29.4 kg/cm^2 and the lysate collected. Nuclei were removed by centrifugation at 1,500 rpm at 4°C in the SS34 rotor of the Sorvall centrifuge. The pellet (nuclei) was washed twice at the same speed with 4 ml relaxation buffer and the supernatants combined with the first supernatant (postnuclear supernatant). The postnuclear supernatant was applied in two equal aliquots to Percoll gradients that were made and centrifuged exactly as described by Borregaard et al. (9). After centrifugation, the gradient was collected in 15–20 2-ml fractions that were analyzed for cytolytic activity, for protein, and by electron microscopy. Percoll was removed from subcellular fractions by centrifuging pools at 4°C for 2.5 h at 45,000 rpm in a 60 Ti rotor (Beckman). Under these conditions, Percoll forms a tight pellet on top of which the subcellular organelles form a sediment that can be easily recovered with minimal Percoll contamination.

Antisera to CTLL-2 granules were raised in rabbits by biweekly injections of 50–100 μg purified granule protein boiled in 1% sodium dodecyl sulfate (SDS) and cross-linked with 0.1% glutaraldehyde. After several boosts, a high titer polyvalent antiserum was obtained, as detected by immunoblotting of granules separated on SDS polyacrylamide slabs. The IgG fraction was prepared by standard techniques and used for fluorescent staining of granules of CTLL-2, of B6 (see above) and of a number of T-cell lymphoma lines by the following protocol: T cells were resuspended at 2×10^6 cells/ml and centrifuged in a cytocentrifuge onto microscope slides for 2 min at ~300 rpm. The cells were air-dried and then exposed to the following solutions at room temperature: 40 min 4% paraformaldehyde in phosphate (10 mM)-buffered saline (0.15 M), pH 7.2; 2×5 min 20 mM NH_4Cl in Tris (10 mM)-buffered saline (0.15 M), pH 7.4; 5 min Tris-buffered saline, pH 8.0, containing 1 mg/ml ovalbumin; 10 min 0.1% NP40 in Tris-buffered saline, pH 7.4, containing 1 mg/ml ovalbumin; 2×5 min Tris-buffered saline, pH 7.4, with ovalbumin; 20 min 1:100 dilution of anti-granule IgG (6 mg/ml) or control antibody (preimmune serum and anti-C5 IgG) in Tris-buffered saline; 4×5 min Tris-buffered saline, 1 mg/ml ovalbumin; 20 min 1:200 fluorescein-labeled goat anti-rabbit IgG (Miles-Yeda) in Tris-buffered saline; and finally 4×5 min Tris-buffered saline ovalbumin. After blotting, 10 μl glycerol was added and the slide examined in a Zeiss III RS fluorescent microscope at 630-fold direct magnification.

Cytolyic Assays. Sheep erythrocytes were washed and resuspended in veronal (5 mM)-buffered saline (0.15 M), pH 7.4, containing 5 mM $CaCl_2$ to a density of 12.5 hematocrit. 100-μl aliquots of these cells were incubated with 1–20 μl of Percoll gradient fractions or with purified granules for 30 min at 37°C and hemolysis determined after dilution to 2 ml and pelleting unlysed cells by centrifugation and measuring the release of hemoglobin at 541 nm in the supernatant. Results are expressed in Z by using the Poisson analysis $Z = -\ln(1-y)$ where y is the fraction of cells lysed. Tumor cells were labeled with ^{51}Cr and resuspended in 10 mM Hepes, 0.15 M saline, pH 7.4, at 2×10^6 cells/ml. 20-μl aliquots (4×10^4 cells) were incubated with 1–20 μl purified granules in a total volume of 50 μl in suspension for 30 min at 37°C. $CaCl_2$ was added to give a final concentration of 5 mM in the assay. Controls received the same amount of relaxation buffer. After

centrifugation, chromium release was determined in the supernatant by counting a 25-μl aliquot. Nonspecific release was <5% under these conditions. Total release was determined by adding 1% SDS to labeled targets.

Poly Perforin Purification. 10^9 CTLL-2 were resuspended in 180 ml serum-free RPMI 1640 containing 10 μg/ml Con A and 10 μg/ml phorbol myristate acetate (PMA) and sedimented in six 30-ml aliquots in sterile round-bottom glass tubes. After incubation for 16 h at 37°C in 5% CO_2, the supernatant was aspirated and discarded and the cell pellets combined in 5 ml 10 mM Tris Cl, pH 8.1. After vortexing, 5% Na deoxycholate (final concentration) was added followed by 100 μg/ml DNAse I and II (Sigma). The mixture was incubated for 1 h at 37°C centrifuged for 10 min at 3,000 rpm and then applied to a 2.5 × 80 cm column of Sepharose CL4B equilibrated with 1% deoxycholate in 20 mM Tris acetate, 90 mM NaCl, 0.2 mM EDTA, 0.02% NaN_3, pH 8.1. Gel filtration was carried out at 15 ml/h, 5-ml fractions being collected.

SDS polyacrylamide slab gel electrophoresis was done using previously described methods (10). Slabs were scanned at 630 nm using a soft laser scanning densitometer. Thin section and negative staining electron microscopy was performed by standard techniques.

Results

Isolation and Biochemical Characterization of T Cell Granules.   CTLL-2 is an originally cytolytic, murine T cell line that was cloned in 1979 (11, 12). Although still Il-2 dependent, CTLL-2 has lost to a large extent its cytolytic activity. However, upon stimulation with Con A, CTLL-2 assembled poly perforins (3) and it contained cytoplasmic granules of the same morphology as those of cytolytic NK or T cell clones (2, 3). Since CTLL-2 can be expanded relatively easily in culture and since it is derived from a cytolytic T cell clone, it was chosen for a study of the biochemical properties of T cell granules. The ultrastructural examination of CTLL-2 in Fig. 1 (panel *A* and *B*) shows its content of numerous granules of characteristic structure. A dense, homogeneous core is surrounded by small vesicular bodies which, in turn, are enclosed by a limiting bilayer membrane (arrows in Fig. 1, *B*, *D*, and *E*). The granules (*G*) are usually located towards the concave face of the reniform nucleus (*N*) in that area of the cell also containing the Golgi apparatus (*Go*). A well-developed endoplasmatic reticulum in the CTLL-2 suggests a high level of synthetic activity.

For the isolation of granules, CTLL-2 are expanded to approximately 10^9 cells by culture in 15-cm petri dishes containing Iscove's modified Dulbecco's medium supplemented with 10% rat Con A supernatant, insulin, transferrin, and lipids as described in Materials and Methods. The isolation procedure, after disruption of the cells by nitrogen cavitation in isotonic buffer containing EGTA and ATP (relaxation buffer) (9), followed the method of Borregaard et al. (9), using self-generated Percoll density gradients. Fig. 2 (upper panel) shows the protein profile and cytolytic activity after Percoll density gradient fractionation of the postnuclear supernatant of CTLL-2. The middle and lower panels of Fig. 2 show for comparison the fractionation of the cytolytic T cell line B6 and the noncytolytic lymphoma El4, respectively. Soluble proteins are located above the Percoll gradient (fraction 15–20) followed by a sharp protein peak in the beginning of the gradient (ρ ~1.049) and a second, broader and shallower protein peak of higher density (ρ = 1.076–1.09). This peak consists of pure granules as shown by ultrastructural analysis of Percoll fractions (Fig. 1, *D* and *E*). No other cellular organelles are detectable in this peak by electron microscopy. In contrast, the
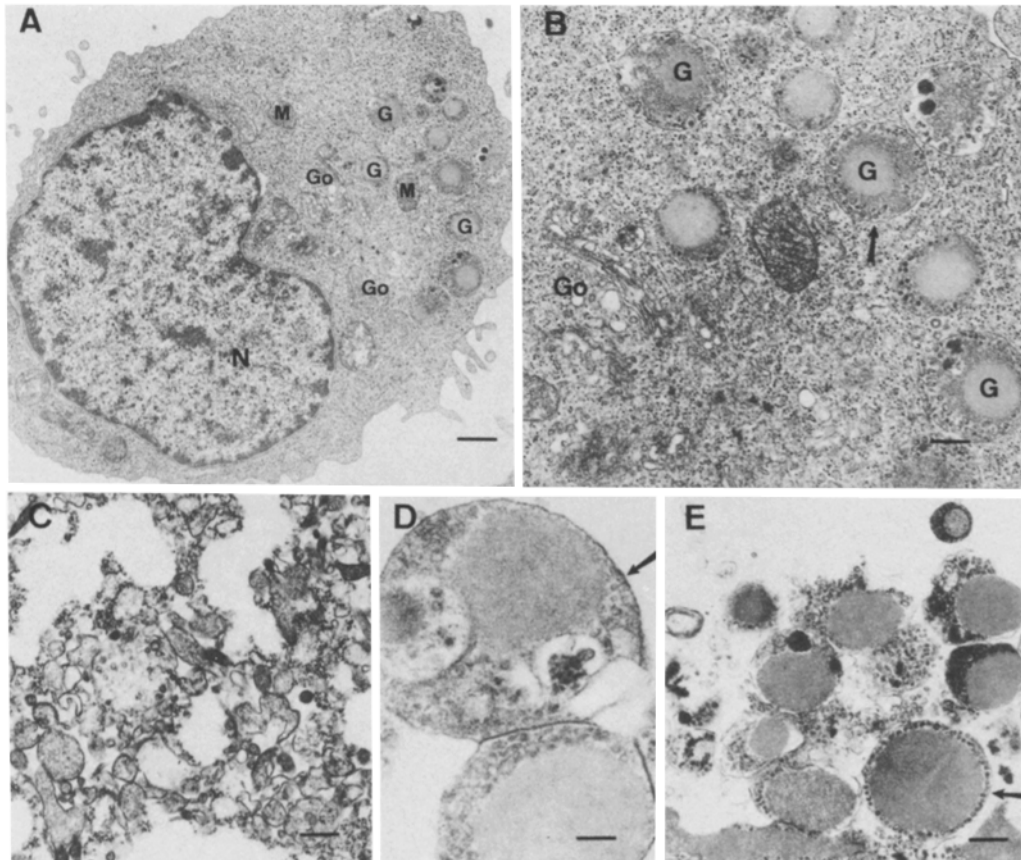
FIGURE 1. Ultrastructure of CTLL-2 and of subcellular fractions. (A) Thin section of typical CTLL-2. N, nucleus; M, mitochondria; Go, Golgi; G, granule. Bar, 0.66 μm. (B) Golgi and granule containing area of CTLL-2 at higher magnification. Arrow points to the granule membrane. Scale bar in this panel and in panels C and E-0.33 μm. (C) Subcellular fraction sedimenting in the low density area (ρ − 1.049) of Percoll gradients (see Fig. 2) showing various cellular organelles. (D and E) Ultrastructure and homogeneity of isolated granules at two magnifications sedimenting in the high density area of Percoll gradients (ρ = 1.076 − 1.09) (see Fig. 2). The arrows point to the limiting membrane enclosing the granules. Scale bar in D-130 nm.

low densii v peak (ρ = 1.049) contains a variety of cellular organelles identifiable as rough endoplasmic reticulum, microfilament-rich bodies (plasma membrane processes), mitochondria, and Golgi structures as well as fragmented granules (Fig. 1C). The Percoll gradient fractions were also assayed for cytolytic activity in the presence and absence of Ca ions using unsensitized sheep or rabbit erythrocytes (E in Fig. 2) as targets. As seen in Fig. 2, cytolytic activity of fractionated CTLL-2 and B6 is associated with the high density, broad protein peak containing the granules. Fractionated E14 (lower panel) show no cytolytic activity in any area of the gradient. Activity is dependent on the presence of Ca, no lysis was observed in the presence of EDTA or Mg-EGTA (not shown). A variable, second peak of activity was detected occasionally in the low density
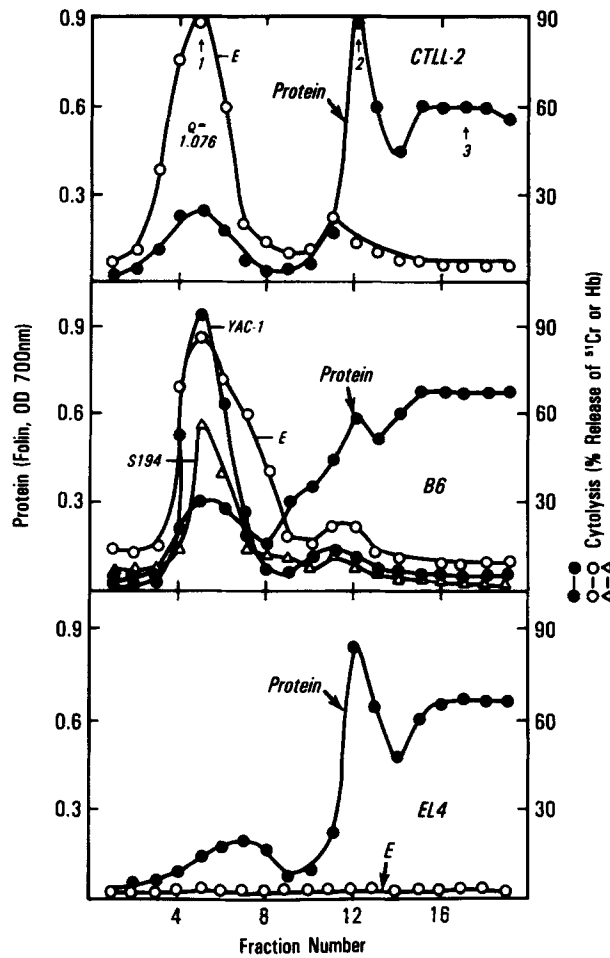
FIGURE 2. Isolation of cytolytic granules by Percoll density gradient centrifugation. A solution of Percoll in relaxation buffer of density 1.08 g/ml was precentrifuged in the Sorvall SS34 rotor for 10 min at 19,500 rpm. The postnuclear supernatant was then loaded onto the Percoll gradient and centrifuged for an additional 35 min at 19,500 rpm. 2-ml fractions were collected and assayed by electron microscopy (Fig. 1), for protein, for hemolytic activity in the presence of 5 mM CaCl₂, and by SDS polyacrylamide slab gel analysis (Fig. 3). The numbered arrows of the upper panel depict fractions used for SDS-slab gel analysis. Upper panel: Fractionation of CTLL-2 and analysis for protein and hemolytic activity. Middle panel: Fractionation of alloreactive cytolytic T cell line B6 and analysis for protein and cytolytic activity towards erythrocytes (E) and the tumor targets YAC-1 and S194. Lower panel: Fractionation of El4 and analysis for protein and hemolytic activity (E).

protein peak. The increase of activity in this peak after N₂-cavitation at higher pressure suggested that this activity may arise from its content of ruptured granules. SDS-polyacrylamide slab gel analysis under reducing and nonreducing conditions of gradient fractions of CTLL-2 shows that the high density granule fractions ($p = 1.076$–1.09) containing the cytolytic activity (Fig. 3, lane *1*, Fig. 2, upper panel arrow *1*) comprised a set of unique protein bands. A different pattern of protein bands is seen in lanes *2* and *3* of Fig. 3 corresponding to the
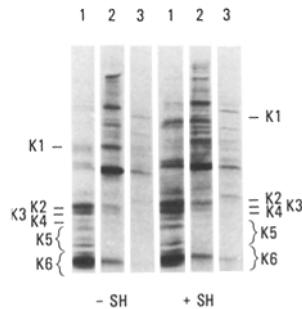
low density protein peak and the soluble proteins (Fig. 2, upper panel arrows *2* and *3*). A group of six major protein bands (*K1–K6*) is characteristic for CTLL-2 granules (Fig. 3, lane *1*, −*SH*, +*SH*). Several minor protein bands are also detectable; however, they are somewhat variable from preparation to preparation and their precise migration requires further analysis. The major bands migrate with the following molecular weights by comparison with marker proteins: K1 57,000 (unreduced), 75,000 (reduced); K2, 3, 4, 38,000, 35,000, 33,000 both reduced and unreduced; K5 in the range of 22,000 to 30,000; and K6 between 10,000 and 20,000. A doublet of protein bands (lane *1*, Fig. 3) migrating between K1 and K2 with molecular weight of 46,000 and 50,000 probably also is granule specific. Although the protein banding pattern did not change when serine esterase inhibitors were included during granule separation, it cannot be excluded that some bands may have arisen through proteolytic degradation. The characteristic pattern of K1 to K6 was also obtained with granules from other T cell clones (manuscript in preparation). Fig. 4 shows the densitometric scanning of the granule banding pattern and the low density peak pattern in comparison to the position of marker proteins. Granule proteins K1 through K5 are detectable in the low density peak, presumably due to partial granule fragmentation during N₂-cavitation. Conversely, proteins characteristic for the low density peak, e.g. the 45,000 mol wt protein, are detected in only trace amounts in the granule fraction, indicating an estimated purity of granules of ~90%.

Table I summarizes the purification procedure of CTLL-2 granules and the recovery of cytolytic activity and of protein. Removal of nuclei from the cavitate by differential centrifugation results in a loss of 70% of the activity, presumably by loss and/or inactivation of granules. Gradient centrifugation results in a substantial purification (~140-fold) and increase of total activity probably due to the removal of soluble inhibitors present in cytoplasm.

Specificity of Cytolytic Granules for Cytolytic Cells. To address the question as to
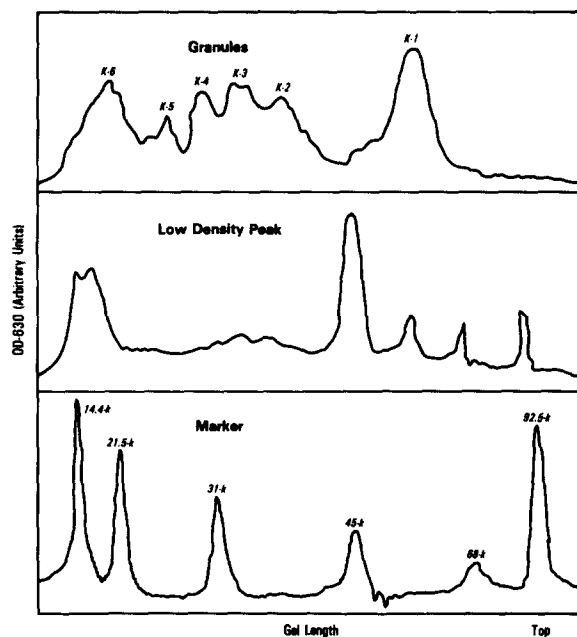
FIGURE 4. Densitometric scan of granules (upper panel) of the low density material (middle panel) and of molecular weight markers separated by SDS polyacrylamide gradient slab gel electrophoresis. K1 to K6 are labeled as in Fig. 3. The molecular weights are indicated on the peaks of the marker proteins. The scanned samples were separated under nonreducing conditions.

TABLE I

Purification of Cytolytic Granules*

Fraction	Volume	Protein	Total protein	Activ-ity‡	Specific activity‡	Total activity	Purifi-cation factor
	ml	*mg/ml*	*mg*	*Z/ml*	*Z/mg*	*Z*	
Cavitate	23	27.9	643	107	3.8	2,460	1
Nuclei	2.5	156	390	25	0.16	62.5	—
Postnuclear supernatant	23.5	8.2	192.7	29	3.5	681.5	1
Granules	15	0.43	6.45	208	483	3,120	138

* Values derived from preparation #18 starting with 1.5×10^9 CTLL-2.

‡ Activity is expressed in Z by Poisson analysis: $Z = -\ln(1 - y)$ where y is the fraction of cells lysed. 1 Z corresponds to 1.25×10^9 effective lytic events.

whether CTLL-2 granules are representative for granules of cytolytic T cells or whether these granules occur in all T cells and are not related to cytolytic functions, the following two approaches were taken. (a) Granules were isolated from a long-term alloreactive cytolytic T cell line (B6) and from the T cell lymphoma E14 and analyzed for cytolytic function. (b) Antiserum raised against CTLL-2 granules was used to determine staining of various cytolytic and non-cytolytic T cells.

Table II shows the cytolytic activity and specificity of B6 as intact cell prior to

TABLE II

Lack of NK-like Specificity of B6

Killer/target ratio	^{51}Cr-Release	
	S194	YAC-1
0	7	5.2
1:1	54	4.1
10:1	100	6
30:1	100	11
SDS	100	100

2×10^4 labeled target cells were incubated for 3.5 h at 37°C with B6 cells at the appropriate ratio in a total volume of 200 μl in conical centrifuge tubes and ^{51}Cr-release determined in the supernatant.

N_2 cavitation. B6 is $H2^d$ restricted and causes 54% specific ^{51}Cr release at 1:1 killer/target ratio within 3 h from $H2^d$ targets. The NK-sensitive target YAC-1 is not lysed by intact B6 cells under the same conditions. In contrast, isolated B6 granules in the presence of Ca efficiently lyse YAC-1 as well as S194 and sheep erythrocytes (Fig. 2, middle panel). El4 is a T cell lymphoma line producing Il2 with a similar gross morphology as cytolytic T cells. 10^9 El4 cells were disrupted by nitrogen cavitation and the postnuclear supernatant applied to Percoll gradients as described above. A protein peak of somewhat lower density than that of CTLL-2 or B6 granules was detectable which, however, showed no cytolytic activity to any target tested. Granule-mediated cytotoxicity thus is only expressed in granules isolated from cytolytic T cells, suggesting the presence of a unique set of cytolytic granule proteins.

This conclusion was confirmed by antiserum raised to CTLL-2 granules and used for immunofluorescence studies. Fig. 5 shows immunofluorescence and phase contrast images of paraformaldehyde-fixed and NP40-permeabilized CTLL-2, B6, and S194 after incubation with rabbit anti-granule antibodies (detecting in Western blots K1 through K6) and fluorescein-conjugated second antibody directed to rabbit IgG. Both CTLL-2 and B6 show strong staining of the cytoplasmic granules with anti–CTLL-2 granule IgG. No staining was observed when preimmune rabbit IgG or anti-human C5 rabbit IgG was used as first antibody (not shown), excluding unspecific binding of rabbit IgG to cytolytic granules. The left panel (Fig. 5) shows the lack of staining of S194 granules with the anti–CTLL-2 granule antiserum. This result indicates the presence of specific antigens in cytolytic T cell granules not expressed in the granules of other T cells.

Functional Characterization of CTLL-2 Granules. Isolated granules display strong Ca-dependent tumoricidal and hemolytic activity. Fig. 6A shows the hemolytic titration of granules with sheep erythrocytes in the presence of 5 mM $CaCl_2$. The dose response curve shows an initial sigmoidal shape followed by a linear increase of activity. 1.4 μg granule protein corresponds to 1.25×10^9 effective molecules. Cytolysis is extremely rapid (<2 min at 37°C) and strongly temperature dependent (Fig. 6B). A substantial reduction of activity is observed already at 30°C and even more so at 22°C. No activity was observed at 10°C and 4°C under the same conditions. The Ca dependence of cytolytic activity is
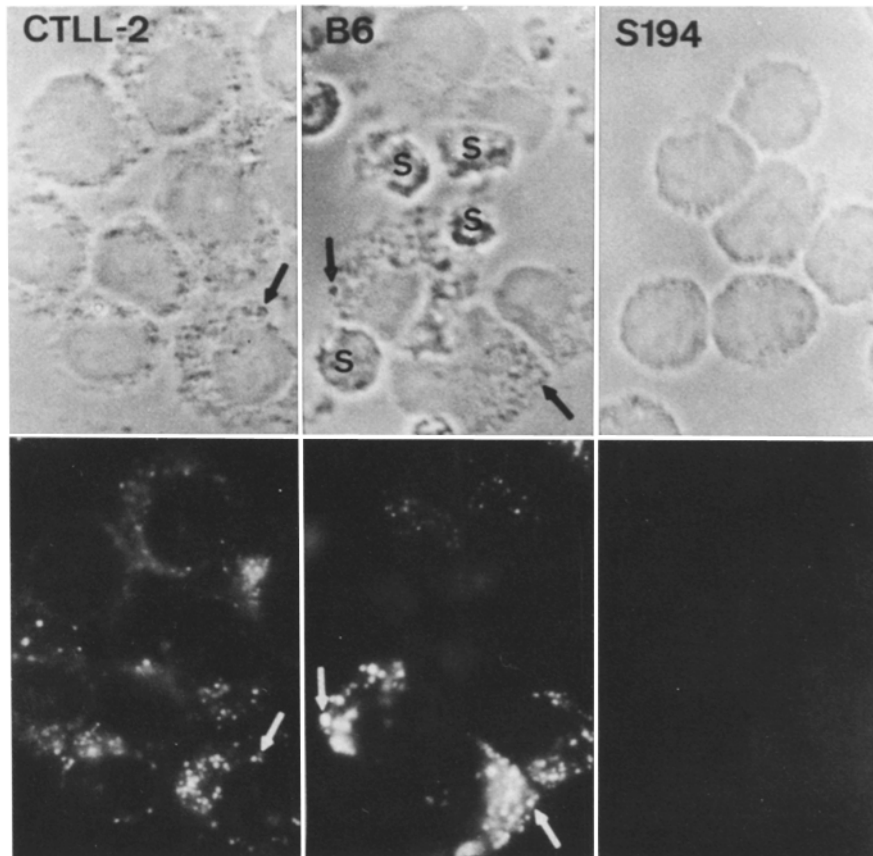
FIGURE 5. Immunofluorescence staining of cytolytic granules. Cells were sedimented in the cytocentrifuge, fixed with paraformaldehyde, and permeabilized with NP40. The IgG fraction of rabbit anti–CTLL-2 granule antiserum was used as first antibody and fluorescein-conjugated goat anti rabbit IgG as second antibody. Left panels: CTLL-2, middle panels: B6, right panels S194. Upper panels: phase contrast; lower panels: fluorescence. Arrows point to granules seen in phase contrast as dark spot and in fluorescence as bright spots. The middle panels contain pycnotic structures of dead S194 (S) used for antigen stmiulation of B6 during culture.

shown in Fig. 6C. Cytolysis is absolutely Ca dependent with a relatively broad optimum at 4–6 mM free Ca. Zinc ions are strongly inhibitory at 0.1 mM, whereas Mg, in the presence of low (2 mM) Ca, slightly enhances the lytic activity (not shown). However, Mg alone, in the absence of Ca, does not support cytolysis.

Ultrastructural analysis of erythrocyte membranes lysed by isolated granules shows characteristic 160-Å (arrowheads, Fig. 7, A and B) and 50–90-Å (open arrows, Fig. 7B) wide and somewhat irregular membrane lesions (poly P1 and poly P2). When granules are incubated with erythrocytes in the absence of Ca, no lysis occurs and no membrane lesions are detectable. These studies, therefore, indicate that the precursor proteins (perforins) for lesion formation are located in the granules and assemble to poly perforins during cytolysis in a Ca-dependent reaction.

The tumoricidal activity of isolated granules was titrated by incubating various
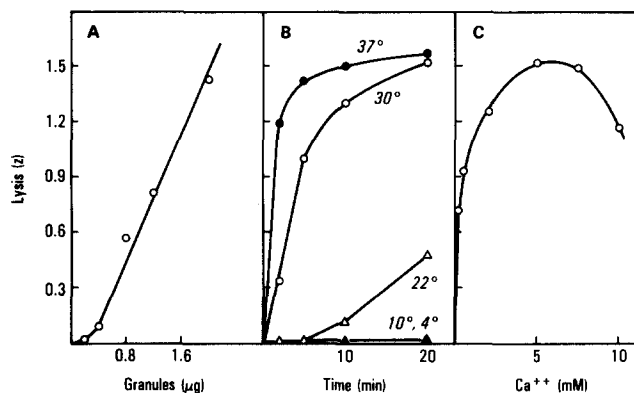
FIGURE 6. Cytolytic properties of isolated granules. (A) Dose response of sheep erythrocyte lysis by isolated granules. The reaction was carried out for 30 min at 37°C in the presence of 5 mM $CaCl_2$. Note the initial sigmoidal dose response. (B) Time and temperature dependence of sheep erythrocyte lysis by isolated granules in the presence of 5 mM $CaCl_2$. Granule protein concentration in the assay was adjusted to give 1.5 Z at 37°C. The reaction was stopped by chilling samples on ice and centrifugation in the cold. (C) Ca dependence of sheep erythrocyte lysis by isolated granules.

amounts with several ^{51}Cr-labeled tumor cells for 30 min at 37°C in the presence of 5 mM $CaCl_2$ (Fig. 8). CTLL-2 granules lyse El4 very efficiently, followed by S194 and BW 5147. YAC-1 and two types of P815 are also lysed, albeit at lower efficiency. Nonspecific release of ^{51}Cr in these experiments was <5%. No change in the sensitivity pattern was observed by varying the incubation time from 15 min to 2 h. 2 μg of granules added to 4 × 10^4 tumor cells in the cytolytic assay corresponds to a killer/target ratio of 3–6:1, assuming a recovery of granules of ~50%. The granules thus are comparable or even more active than the intact cell; virtually complete lysis of EL4 with 2 μg granule protein ensues in less than 30 min at 37°C.

Isolation of Poly Perforin 1 Complexes. In previous experiments it was found that CTLL-2 upon stimulation with Con A assembled poly P1. An increased generation of poly P was observed upon incubation of CTLL-2 with 10 μg/ml PMA in addition to Con A. After stimulation in this way of 10^9 cells for 16 h, the cell pellet was harvested, lysed with 5% sodium deoxycholate (DOC), and digested with DNAse. After removal of insoluble material by low-speed centrifugation, the DOC extract was purified by passage over a Sepharose CL4B column in the presence of 1% DOC. Fig. 9 shows the elution profile and the SDS-polyacrylamide gel analysis of the high molecular weight fractions pooled in pool I–III. Without reduction (*NR*, inset Fig. 9) the protein remains on top of the 2.5% stacking and separating gel, indicating a molecular weight of >3 × 10^6. Upon reduction, three major protein bands are detectable with molecular weights similar to those of K2–4. Fig. 10 depicts the ultrastructure of the high molecular weight pool I containing circular poly perforin complexes, seen in top view (arrows) and side view (arrowheads), in addition to linear polymers (open arrows). The poly P1 complexes often aggregate, even in the presence of detergent, in head to head or head to tail fashion to long tubular arrays (filled short arrow in
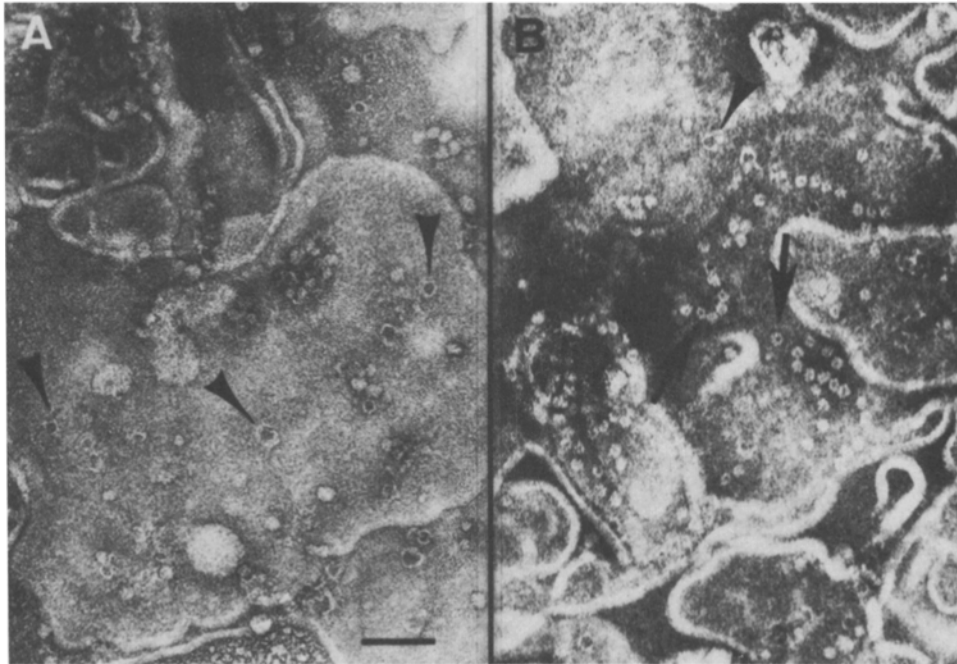
FIGURE 7.   Membrane lesions assembled by incubation of sheep erythrocytes with isolated granules. Arrowheads in *A* and *B* depict top views of poly P1. Poly P2 is seen in *B* (arrows). Granules were mixed with 200 μl 12.5% sheep erythrocytes to correspond to 50 *Z* in the presence of 5 mM $CaCl_2$. After incubation for 1 h at 37°C, erythrocyte ghosts were collected by centrifugation and, after 3 washes in 5 mM EDTA, pH 7.4, resuspended in isotonic Tris-buffered saline, pH 7.4, and digested with 100 μg/ml trypsin for 16 h at room temperature. After an additional wash, the membranes were mounted on grids and negatively stained with 2% uranyl formate. Bar, 133 nm.

panels *1* and panel *2*). This type of aggregation suggests the presence of hydrophobic domains on either end of the poly P1 tubule.

Discussion

This study is the first demonstration of strong, Ca-dependent cytolytic activity of isolated T cell granules and of their capacity to assemble poly P complexes forming membrane lesions. Based on the quantitative dose response (Fig. 8) of granule-mediated tumor cell lysis, it appears likely that the granules represent a major part of or the entire cytolytic principle of cytolytic T cells. Cytolytic activity is not expressed by granules isolated from T cell lymphomas and antisera raised against cytolytic granules are specific for cytolytic cells. These findings suggest that cytolytic T cell granules contain a highly specific set of proteins not present in other types of T cells.

Cytolytic granules lyse a variety of tumor target cells, however, with different efficiency. The finding that B6 granules lyse YAC1 and S194 cells in addition to sheep erythrocytes, whereas B6 cells lyse only S194 would suggest that granule lysis is unspecific and that target specificity is conferred by the T cell's plasma membrane. The different susceptibility of tumor targets to granule-mediated
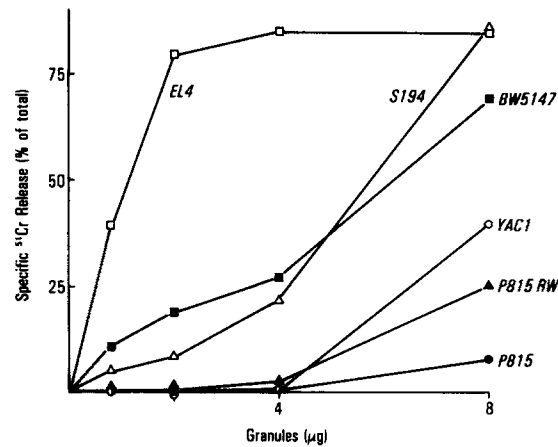
FIGURE 8. Tumoricidal activity of isolated granules. CTLL-2 granules isolated from Percoll
gradients were incubated with 4 × 10^4 ^{51}Cr-labeled target cells in a total volume of 50 μl for
30 min at 37°C. Controls received the Percoll solution from a gradient to which no granules
were applied. Nonspecific (spontaneous) release was <5%. Total release was determined by
lysis of target cells with SDS. P815 is a target susceptible to macrophage lysis, whereas P815
RW is a selected subline susceptible to NK-cell lysis.

lysis may simply be due to the requirement of larger numbers of membrane
lesions for the lysis of more resistant targets. However, a number of other
explanations are possible for this observation and further experiments are
required to clarify this point.

The isolation procedure for granules described yields essentially pure granules
with little contamination by other cellular organelles. Purity of granules was
evaluated by SDS polyacrylamide slab gel analysis (Fig. 3), by morphological
examination of the granule fraction by electron microscopy (Fig. 1) and by
functional analysis showing co-migration of cytolytic activity with dense granules
(Fig. 2) and an ~140-fold purification upon Percoll gradient centrifugation
(Table I). The peptide composition of CTLL-2 granules is complex and the
precise function of the individual proteins, designated K1 to K6, is not known
at present, except that they are related to the cytolytic activity of the granules.
It is probable that some of the proteins reside in the limiting granule membrane
and may be involved in the Ca-dependent activation and that others are located
in the granule interior and are responsible for polymerization and cytolysis.

Granule-mediated cytolysis is accompanied by the formation of tubular com-
plexes (poly perforins) that may cause target membrane disruption by membrane
insertion and transmembrane channel formation. The proteins comprising the
high molecular weight complexes in the poly P1 preparation are disulfide linked,
whereas the granule proteins are present as monomer in non-disulfide-linked
form. It appears, therefore, that polymerization and complex formation of
granule proteins is accompanied by the formation of disulfide bonds resulting in
covalent cross-linking of the poly P1 subunits. This property is analogous to
properties of poly C9 of complement (10).

Although it seems clear from these studies that the granules contain the
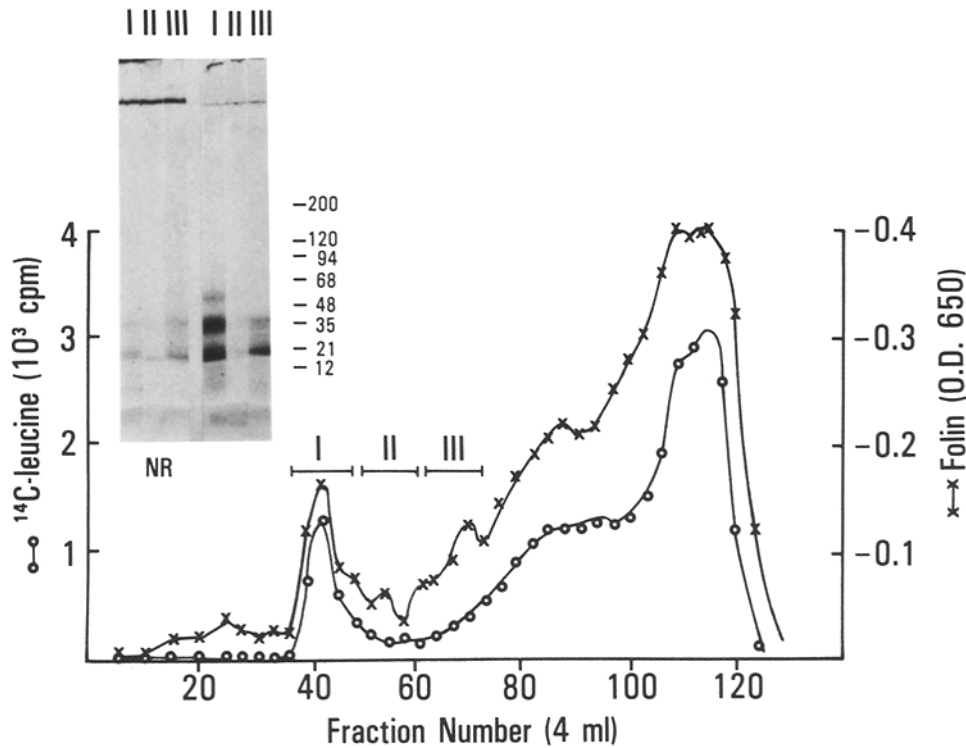precursor proteins and the activator system for poly perforin assembly, these

FIGURE 9. Purification of poly P1 by gel filtration. 10^9 CTLL-2 were grown in [^{14}C]leucine containing medium, stimulated with Con A and phorbol myristate acetate and extracted with DOC as described in Materials and Methods. The elution profile of protein and radiolabel from a Sepharose CL4B column is shown. The high molecular weight pools (I, II, III) were analyzed on 2.5 to 10% SDS-polyacrylamide gradient slabs (inset) under nonreducing (left, NR) and reducing (right) conditions.

studies do not exclude the possibility that other granule components mediate cytotoxicity independent of lesion formation or cooperate in cell damage with the poly perforins. It is conceivable, for instance, that the formation of membrane lesions provides access for other activities (e.g. nucleases, proteases, toxins, surface active proteins, etc. (13–15)), to the target cell interior, or that these activities are injected through these lesions into the cell.

It is of interest to note that the granules of CTLL-2 are endowed with highly active cytolytic activity, whereas the intact cell, although derived from a cytolytic T cell line, is not or is only marginally cytolytically active. CTLL-2 thus provides an example of a cell that contains the cytolytic machinery, but that seems to have lost the mechanism by which it can be activated. B6, in contrast, has cytolytic activity as intact cells, albeit with a specificity different from that of its isolated granules suggesting that T cell specificity is conferred by plasma membrane receptors and that the killing step per se is unspecific. Studies with three additional cloned cytolytic T cell lines (references 16, 17, and Podack, Konigsberg, Pircher, Acha-Orbea, Hengartner, manuscript in preparation) confirmed the cytolytic activity of isolated T cell granules described here. Cytolytic activity was also observed in granules isolated from a rat tumor cell line expressing NK-
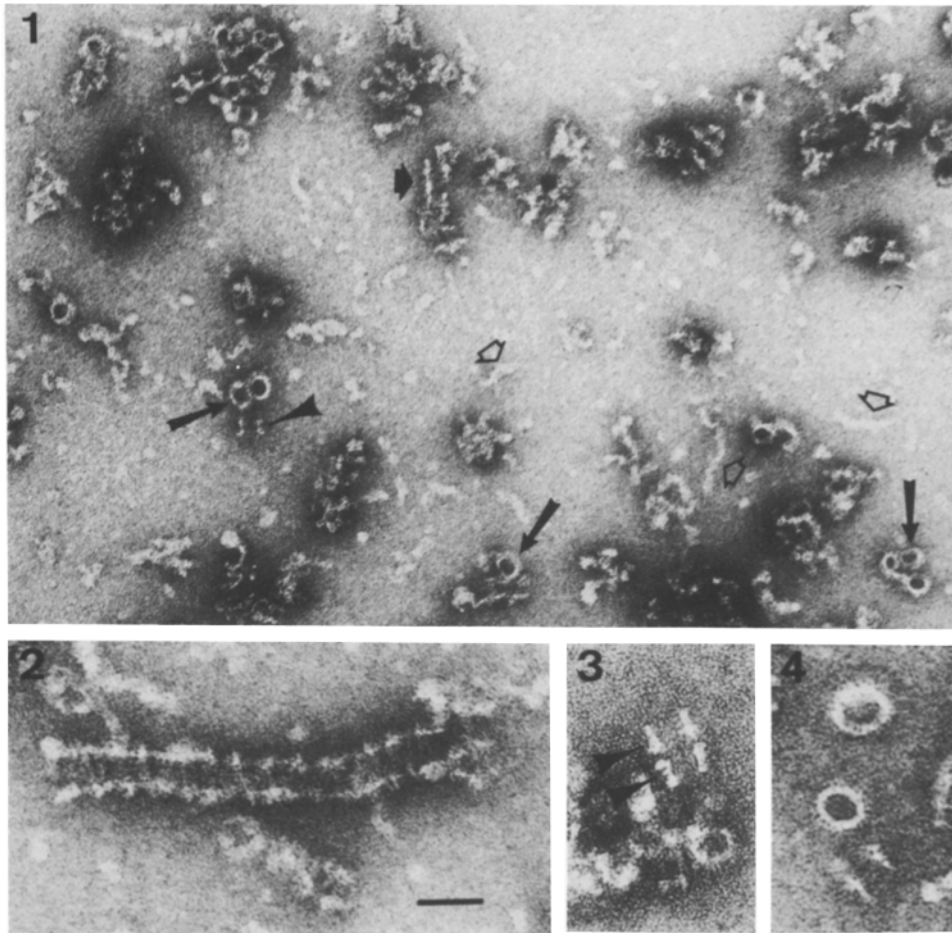
FIGURE 10.   Ultrastructure of purified poly P1 (peak I of Fig. 7). Panel *1*: field view. Arrows depict top views; arrowheads, side views of circular poly P1. Open arrows point to linear polymers. Panel *2*: Poly P1 aggregating in head to head and head to tail fashion forming structures of ladder like appearance. Panels *3*, and *4*: selected images as poly P1. Scale bar in panel 1 corresponds to 1,330 Å; in panels *2–4* to 665 Å.

like cytolytic activity (1). It thus appears that the isolation and biochemical characterization of killer lymphocyte-granules will provide the key to the understanding of the molecular mechanism of lymphocyte-mediated cytotoxic activity.

Summary

The cytoplasmic, dense granules of cloned T cell lines were isolated and analyzed for their functional and biochemical properties. Isolated granules of ~90% homogeneity, in the presence of Ca, effect strong tumoricidal and hemolytic activity. Tumor cell lysis is complete in less than 30 min, with <2 μg granule protein corresponding to a killer/target ratio of 3–6:1 by assuming 50% yield for granule isolation. The granules contain a set of unique proteins, responsible for cytolytic activity and designated K1 to K6, in the molecular weight range of

14,000 to 75,000, as defined by sodium dodecyl sulfate (SDS) polyacrylamide slab gel analysis under reducing and nonreducing conditions. Cytolysis mediated by isolated granules is accompanied by the assembly of tubular complexes of 160 Å (poly P1) and of ~70 Å width (poly P2) that are inserted into membranes and form ultrastructural membrane lesions.

As shown by immunofluorescence and by Percoll gradient fractionation, cytolytic granules are detected in cells of cytolytic T cell lineage and not in the T cell lymphomas El4 and S194.

Poly perforin 1 assembled by CTLL-2 upon stimulation with concanavalin A (Con A) and phorbol myristate acetate (PMA) was isolated by detergent extraction and gel filtration. Poly P1 is composed of disulfide-linked subunits that, after reduction, co-migrate with certain granule proteins. The results are compatible with the hypothesis that the dense granules of cytolytic T cells contain cytolytic proteins that polymerize to disulfide-linked tubular poly perforins in a Ca-dependent reaction and may cause cytolysis by membrane insertion and transmembrane channel formation.

References

1. Henkart, P., M. Henkart, P. Millard, and C. Reynolds. 1984. Isolation and cytolytic activity of granules from naturally occurring LGL tumors. *In* Natural Killer Activity and its Regulation. T. Hoshino, H. S. Koren, and A. Uchida, editors. Excerpta Medica, Tokyo, p. 150.
2. Podack, E. R., and G. Dennert. 1983. Cell mediated cytolysis: assembly of two types of tubules with putative cytolytic function by cloned natural killer cells. *Nature (Lond.).* 302:442.
3. Dennert, G., and Podack, E. R. 1982. Cytolysis by H2 specific T-killer cells: assembly of tubular complexes during the lytic reaction. *J. Exp. Med.* 157:1483.
4. Carpen, O., I. Virtanen, and E. Saksela. 1982. Ultrastructure of the human natural killer cells: nature of the cytolytic contact in relation to cellular secretion. *J. Immunol.* 128:2691.
5. Carpen, O., I. Virtanen, and E. Saksela. 1981. The cytolytic activity of human natural killer cells requires an intact secretory apparatus. *Cell. Immunol.* 58:97.
6. Podack, E. R. 1984. Assembly of transmembrane tubules (poly perforins) on target membranes by cloned NK-cells: comparison to poly C9 of complement. *In* Natural Killer Activity and Its Regulation. *Excerpta Med. Int. Congr. Ser.* 101:641.
7. Iscove, N., and F. Melchers. 1978. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide reactive B lymphocytes. *J. Exp. Med.* 147:926.
8. Iscove, N., L. Guilbert, and C. Weyman. 1980. Complete replacement of serum in primary cultures of erythropoietin-dependent red cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acid, lecithin and cholesterol. *Exp. Cell Res.* 12:121.
9. Borregard, N., J. M. Heiple, E. R. Simons, and R. A. Clark. 1983. Subcellular

localization of b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J. Cell. Biol.* 97:52.

10. Podack, E. R., and J. Tschopp. 1982. Circular polymerization of the ninth component of complement (poly C9): ring closure of the tubular complex confers resistance to detergent dissociation and to proteolytic degradation. *J. Biol. Chem.* 257:15204.

11. Baker, P. E., S. Gillis, and K. A. Smith. 1979. Monoclonal cytolytic T cell lines. *J. Exp. Med.* 149:273.

12. Gillis, S., and K. A. Smith. 1977. In vitro generation of tumor specific cytotoxic lymphocytes. *J. Exp. Med.* 147:468.

13. Russel, J. H., T. Masakowski, T. Rucinsky, and G. Phillips. 1982. Mechanism of immune lysis. III. Characterization of the nature and kinetics of the cytotoxic T lymphocyte-induced nuclear lesions in the target. *J. Immunol.* 128:2087.

14. Granger, G. A., R. S. Yamamoto, D. S. Fair, and J. C. Hiserodt. 1978. The human LT system. I. Physical-chemical heterogeneity of LT molecules released by mitogen activated human lymphocytes in vitro. *Cell. Immunol.* 38:388.

15. Aggarwalt, B. B., B. Moffat, R. N. Narkins. 1984. Human lymphotoxin. *J. Biol. Chem.* 259:686.

16. Acha-Orbea, M., P. Groscurth, R. Lang, L. Stitz, and H. Hengartner. 1983. Characterization of cloned cytotoxic lymphocytes with NK-like activity. *J. Immunol.* 130:2952.

17. Pircher, H., G. Hammerling, and M. Hengartner. 1984. Restriction fine specificity of long term, hapten-specific cytotoxic T-cell clones: analysis with H-2K^b-mutant mice and H-2K^b specific monoclonal antibodies. *Eur. J. Immunol.* In press.