

CYTOLYSIS BY H-2-SPECIFIC T KILLER CELLS Assembly of Tubular Complexes on Target Membranes*

By GUNTHER DENNERT AND ECKHARD R. PODACK

From the Autoimmune and Neoplastic Diseases Laboratory, The Salk Institute for Biological Studies, San Diego, California 92138; and the Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037

Cell lysis plays an important role in immune functions enabling the organism to eliminate unwanted indigenous or foreign cells. There are two pathways to accomplish this, one that makes use of humoral factors including complement components and the other utilizing cytolytic killer cells. The molecular mechanisms leading to complement lysis are fairly well understood. A series of reactions triggered by antibody-dependent or independent pathways results in the formation of a membrane-bound C5 convertase. Then enzymatic activation of C5 initiates self-assembly and membrane insertion of the tetramolecular C5b-8 complex, which in turn binds and polymerizes C9. Finally, polymerization of 12–18 C9 subunits leads to a tubular complex that, upon insertion into the target membrane, gives rise to membrane lesions and may create a transmembrane channel leading to cell death (1–3). Little is known about the molecular mechanisms of cell-mediated cytotoxicity (for reviews see references 4–8). Recently, however, Dourmashkin et al. (9) reported that incubation of human peripheral blood lymphocytes with target cells may lead to membrane lesions similar to the ones caused by complement. Using clones of natural killer (NK)¹ cells, we reported (10) that NK cells may form two types of membrane lesions on target cells which differ in size from mouse, bovine, or human complement lesions. The larger lesion is caused by a tubular complex (Fig. 1) referred to as tubule type 1 (T1). T1 has a length of ~160 Å, an inner diam of ~160 Å, and an outer diam of ~260 Å. The smaller lesion is caused by tubule type 2 (T2) which appears to have a length of 120 Å, an inner diam of ~50 Å, and an outer diam of ~120 Å (Fig. 1). Both T1 and T2 are structurally similar to poly C9. They possess a torus and a hydrophobic domain on both ends of the tubule.

Ultrastructural studies (10) have led us to a working hypothesis for cell-mediated lysis by NK cells, illustrated in Fig. 1. The dense granules and/or Golgi apparatus of NK cells migrate to the site of killer/target membrane contact. Subsequently the effector cell releases multibilayer membranes and single bilayer vesicles associated with the putative precursors of T1 and T2 into the interstitial space of killer/target conjugates. The vesicles associate or fuse with target membranes, triggering the

* Supported by grant IM 284 from the American Cancer Society, grants CA 15581, CA 19334, and AI 18525 from the U.S. Public Health Service, and by Established Investigatorship No. 79149 from the American Heart Association.

¹ Abbreviations used in this paper: Con A, concanavalin A; NK, natural killer; Pf 1 and Pf 2, perforin 1 and 2; poly P1 and poly P2, polyperforin 1 and 2; T1 and T2, tubule type 1 and 2, circular complexes with putative cytolytic function synonymous to poly P1 and poly P2.

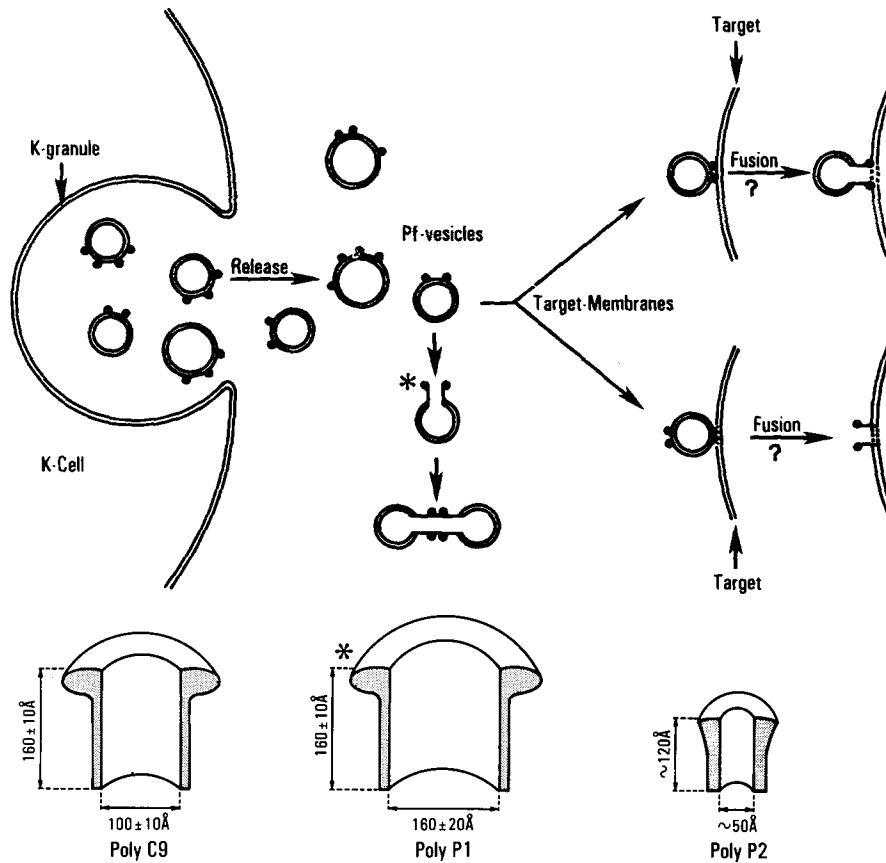


FIG. 1. A hypothetical model for cell-mediated killing. For better clarity, killer (K) and target membranes have been separated, although the reactions outlined probably occur in the narrow interstitial space of the killer/target conjugate. Dense granules or the Golgi of effector cells assembled bilayer vesicles bearing the perforins (Pf-vesicles) and released them at the conjugation site. Vesicles associated (fused?) with target membranes through either the perforins (upper right) or with the help of additional fusion or recognition proteins (lower right). In the lower part, the size of human poly C9, and mouse poly P1 and poly P2 are compared. The tubules are drawn sectioned parallel to their tubular axis. The shaded area corresponds to side view images in electron micrographs. Top views would appear as ring structures due to the torus that annulates the upper part of the tubules. Asterisks depict poly P1 in the upper and lower scheme.

assembly of T1 and T2 to tubules which in turn may be responsible for target cytolysis. Based on the putative function and assembly of the tubules, we propose the following nomenclature which will be used throughout the manuscript: The monomeric precursors of T1 are designated perforin 1 (Pf 1) and their tubular polymerization product, polyperforin 1 (poly P1). Similarly, the subunits of T2 and their assembly product will be referred to as Pf 2 and poly P2.

In this paper we investigate the mechanism of cell-mediated lysis by cloned H-2-specific T killer cells. Results show that a similar mechanism to the one suggested for NK cells is operative in this system.

Materials and Methods

Immunological Procedures. All mouse strains were bred at the Salk Institute and Scripps Clinic. Tumor cell lines were all grown in vitro: the BALB/c myeloma S194 in Dulbecco's minimum

essential medium-10% horse serum, as were the C57BL/6 thymic lymphomas EL4 and T1M1 4, and the AKR thymic lymphoma BW5147. The AKR thymic lymphoma TB-2 was grown in the presence of fetal bovine serum. The DBA/2 mastocytoma, P815, and A strain thymic lymphoma YAC-1 were grown in RPMI 1640-10% fetal bovine serum. Spleen blast cells to be used as targets were induced with 10 $\mu\text{g}/\text{ml}$ bacterial lipopolysaccharide in 2-d cultures. Cytotoxicity assays were done in microtiter plates at various ratios for 4 h using Cr^{51} -labeled targets (11). Percent cytotoxicity was calculated as releasable counts after subtraction of spontaneous release, which was $<3\%/h$ for tumor cells and up to $8\%/h$ on spleen blast cells. Three T killer clones were used. The CBA anti-H-2^d T killer clone MTL 2.8 was kindly provided by Dr. B. Caplan (Salk Institute) (12). Two C57BL/6 anti-H-2^d clones, B6.C.G3 and B6.C.G11, were induced in a mixed lymphocyte culture propagated and cloned by stimulation with H-2^d stimulator cells (13) and 30% concanavalin A (Con A) supernatant in RPMI 1640, 5×10^{-5} M β -mercaptoethanol, 5% fetal bovine serum. MTL 2.8 was propagated in T cell growth factor containing media without antigenic stimulation. Cell surface markers of T killer clones were determined by radioactive trace binding assay using a ^{125}I -labeled second antibody. The following antibodies were used: T24.31.7, a monoclonal rat antibody specific for Thy-1, kindly provided by Dr. I. Trowbridge (Salk Institute); monoclonal rat antibodies 53.7.313 and 53.6.72, specific for Lyt-1 and Lyt-2 (11), respectively, obtained from the Cell Distribution Center of the Salk Institute; a mouse antiserum specific for Ly-6.2, kindly provided by Dr. U. Hämmerling (Sloan-Kettering Institute, New York) (14); and mouse monoclonal 34.5.8, specific for H-2D^d (Cell Distribution Center, Salk Institute) which was grown as ascites. In some experiments, cytolysis was inhibited by monensin (1 $\mu\text{g}/\text{ml}$) kindly provided by Eli Lilly & Co., Indianapolis, IN.

Electron Microscopy Procedures. To prepare membranes for electron microscopy, killer and target cells were washed three times in serum-free tissue culture medium containing 0.5% purified human serum albumin, streptomycin, gentamycin, and fungizone before incubation. Cell mixtures containing killer/target ratios between 1:1 and 3:1 were lightly centrifuged, then incubated for 4 or 16 h at 37°C. Cell suspensions were vortexed for 1 min, incubated for 1 h at 37°C with 100 $\mu\text{g}/\text{ml}$ trypsin and again vortexed. Intact cells, nuclei and other debris were pelleted by 5 min centrifugation at 1,000 rpm and the supernatant was centrifuged for 15 min at 35,000 g . Membranes were suspended in 200 μl Tris/HCl-buffered saline, pH 7.2, containing 0.02% NaN_3 . In most cases membranes were then incubated for 16 h at 20°C with 100 $\mu\text{g}/\text{ml}$ trypsin and chymotrypsin to digest membrane proteins. This reduces background staining while leaving poly P1 and poly P2 intact. 2 ml H_2O were added to the membranes before sedimentation and resuspension in 50 μl Tris-buffered saline NaN_3 . Human complement lesions were assembled on rabbit erythrocytes as previously described (2). All membrane preparations were negatively stained with uranyl formate and then imaged in a Hitachi 12A microscope (Hitachi Ltd., Tokyo, Japan) at 75 kV accelerating voltage and $\times 35,000$ direct magnification. The monoclonal antibody 34.5.8 was adsorbed onto 30–60 Å diam colloidal gold particles (15) and aggregates separated by 15 min centrifugation at 35,000 g . 5 μg of gold-tagged IgG was added to the membrane preparation not proteolysed with trypsin and chymotrypsin. After incubation for 1 h at 37°C, membranes were washed twice to removed unbound gold IgG and imaged in the electron microscope.

Results

Properties of T Killer Clones Used. One-way mixed lymphocyte cultures were initiated with C57BL/6 (H-2^b) responder cells and BALB/c spleen cells in the presence of 30% Con A supernatant (11, 13). After 4 wk, cells were cloned by limiting dilution in microtiter wells (11). Cloned lines were tested for cell surface markers and cytolytic specificity. Two cloned lines, named B6.C.G11 and B6.C.G3, were chosen for further examination because of their excellent growth and good cytolytic activity. Table I shows the cell surface markers on these lines determined by radioimmune assays. They appear to be typical T killer cells because they are Thy-1⁺ and Lyt-1⁻²⁺.

Both lines were tested for their cytolytic specificity on a panel of normal and

TABLE I
Cell Surface Markers of T Killer Cells Used

Cell line	Thy-1 (T24.31.7)	T200 (I3.2.3)	Lyt-1 (53.7.313)	Lyt-2 (53.6.72)	Ly-6.2 (Serum)	H-2 ^b (288.6.5)	H-2 ^d (34.5.8)
B6.C.G11	+	+	-	+	+	+	-
B6.C.G3	+	+	-	+	+	+	-

Either rabbit, anti-mouse, or anti-rat Ig labeled with ¹²⁵I was used as a second antibody. Negatives (Lyt-1 and H-2^d) showed background binding with both antibodies. Background was determined by addition of the second antibody (¹²⁵I-labeled) only. Positives showed in excess of threefold background binding with both antibodies. The reagents used as first antibodies are given in parentheses.

TABLE II
Cytolytic Specificity of T Killer Clones

Target	H-2 haplotype of target								B6.C.G11			B6.C.G3		
	K	IA	IB	IJ	IE	IC	S	D	10:1	3:1	1:1	10:1	3:1	1:1
S194	<u>d</u>	(<u>d</u>)	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	87	70	56	75	72	59
YAC-1	<u>k</u>	(<u>k</u>)	<u>k</u>	<u>k</u>	<u>k</u>	<u>d</u>	<u>d</u>	<u>d</u>	87	78	51	50	42	28
P815	<u>d</u>	(<u>d</u>)	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	85	65	29	—	—	—
BALB/c BL	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	35	26	13	29	20	18
B10.A(18R)BL	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>d</u>	40	16	8	30	24	17
D ₂ GD BL	<u>d</u>	<u>d</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<1	<1	<1	<1	<1	<1
CBA BL	<u>k</u>	<u>k</u>	<u>k</u>	<u>k</u>	<u>k</u>	<u>k</u>	<u>k</u>	<u>k</u>	<1	<1	<1	4	<1	<1
C57BL/6 BL	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<u>b</u>	<1	<1	<1	<1	<1	<1

Cytolytic activity at 4 h was assayed on the targets shown at a/t ratios of 10:1, 3:1, and 1:1. SEM values for cytotoxicity were <2. H-2 determinants recognizable by the effectors are underlined. Spleen blast cells induced by bacterial lipopolysaccharide were used as normal targets. Parenthesis indicates that these antigenic determinants have not been shown to be on the respective targets. Also, the IB and IJ regions may not exist (16).

transformed target cells (Table II). They lyse the H-2^d tumor targets S194 and P815 and the H-2^a target YAC-1. This suggests that the determinants recognized are encoded by the D end of the H-2 gene complex. Further proof for this is that these lines lyse B10.A (18R) but not D2GD, C57Bl/6 (H-2^b), or CBA (H-2^k) splenocytes. These results show that the two cell lines do not express any NK-cell-like properties, but have both the antigenic and functional characteristics of T killer cells.

Polyperforins Are Deposited by T Killer Cells on Target Membranes. To examine the mechanism by which T killer cells lyse their targets, the two T killer clones were mixed with S194 targets at ratios between 4:1 and 1:1 and incubated for 4–16 h. Unlysed cells were pelleted by centrifugation and membranes examined by negative staining electron microscopy. At both time points, membranes showed multiple ring structures. They were either the large structures called T1 or poly P1 (Fig. 2A) or the smaller T2 and poly P2 (Fig. 2B) structures. Both structures were essentially identical in size to those previously seen with cloned NK cells (10). The inner and outer diameters were ~160 and ~260 Å for poly P1 and ~50–70 and 120 Å for poly P2.

Then we tested whether both poly P1 and poly P2 originate from T killer cells. T killer cells and S194 targets were incubated separately for 16 h with or without the T cell mitogen Con A. This lectin was used because it mediates nonspecific cell-mediated cytotoxicity. Membranes were isolated and examined by negative staining electron

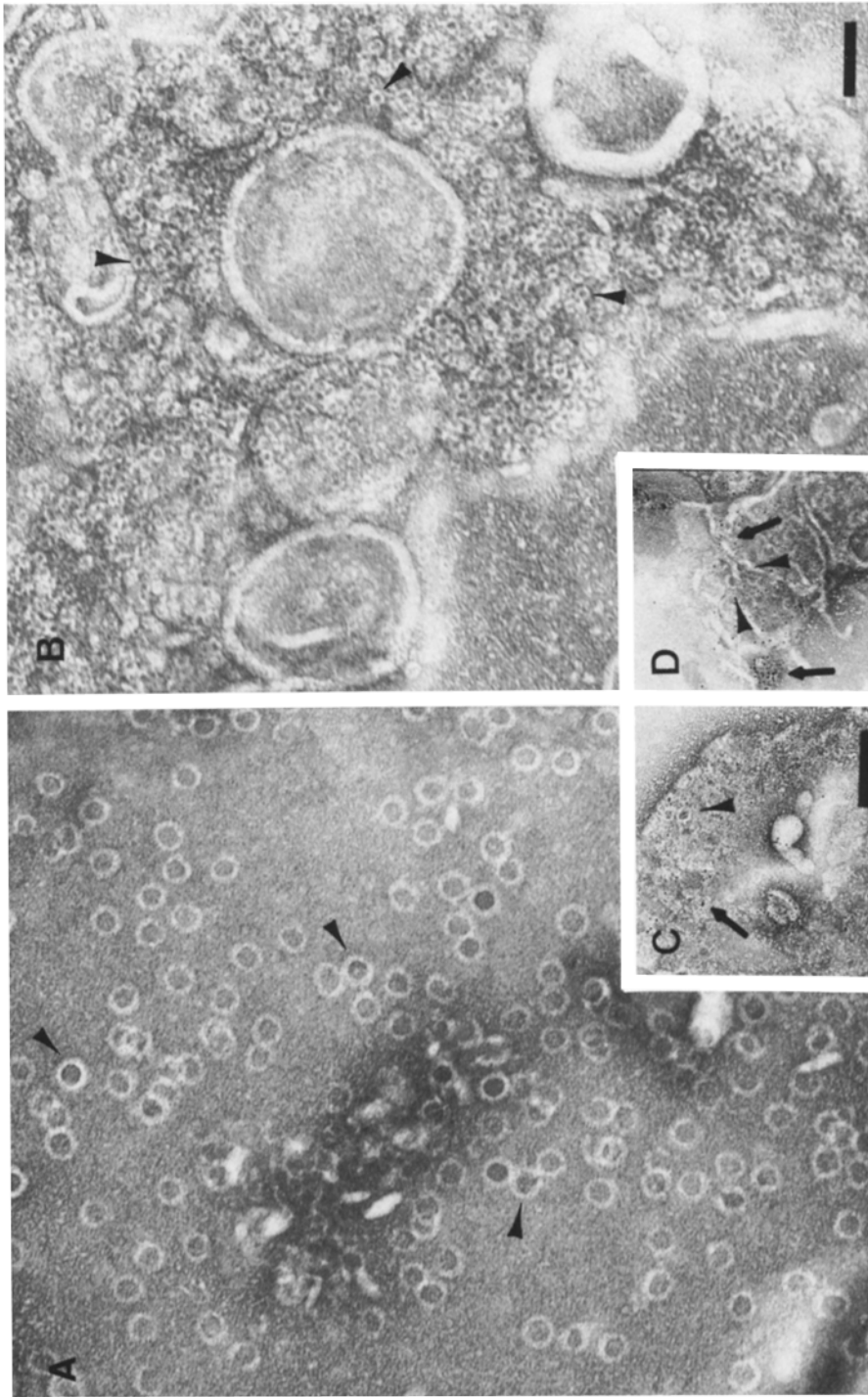


FIG. 2. Comparison of T killer-released poly P1 and poly P2. (A) Membrane-bound poly P1 (arrowheads) released by B6.C.G11. (B) Membrane-bound poly P2 (arrowheads) released by B6.C.G11. (C) and (D) Poly P1 (arrowheads) on S194 target membranes labeled with colloidal gold (arrows) coupled to anti-H-2^d antibody. The gold-labeled antibody is seen as a distinct black dot of ~50 Å diameter on the membrane fragments. Bar represents 57 nm in (A) and (B); 114 nm in (C) and (D).

microscopy. Results showed that T killer cells incubated with 5 $\mu\text{g}/\text{ml}$ Con A gave rise to membrane fragments carrying poly P1 and poly P2 complexes, but that S194 targets never showed these structures under any conditions (see also Table IV). This indicated that poly P are produced by T killer cells and that their appearance on cell membranes can be stimulated by Con A in the absence of target cells.

Next we tested whether both poly P1 and poly P2 are transferred from the killer to the target. We made use of a monoclonal antibody specific for H-2^d (34.5.8) that binds to S194 target cells but not to the T killer cell clones used (Table I). The antibody was labeled with colloidal gold (11) and reacted with membranes prepared from killer/target cell mixtures. In Fig. 2, C and D, membrane pieces identified as target membranes by gold-labeled 34.5.8 antibody show multiple poly P1 structures. Similarly, gold-labeled target membranes with poly P2 structures could be identified (not shown). This is compelling evidence that both poly P1 and poly P2 originate from effector cells and are deposited on target membranes.

Appearance of Polyperforins Is Dependent on the Expression of Cell-mediated Lysis. Having demonstrated that polyperforins originated in the T killer cells and were then transferred to the membrane surface of target cells, we next sought to determine whether these structures actually caused lysis of the target cells. Monensin, a carboxylic ionophor, inhibits cytotoxicity by NK cells at 1 $\mu\text{g}/\text{ml}$ without exerting detectable cytotoxicity. Monensin at this concentration also inhibited cytotoxicity by T killer cells by $\sim 70\%$ (Table III): three times higher effector to target ratios had to be employed to cause comparable cytotoxicity in its presence. Membranes isolated from killer/target mixtures after 4 h showed a reduction of the number of membranes carrying poly P1 complexes from 40 to 20% in the presence of monensin. Therefore, the degree of cytotoxicity appears to be roughly proportional to the number of poly P complexes deposited on the target.

The correlation between deposition of poly P and cytotoxicity was further tested using a number of different effector cell populations. Primary mixed lymphocyte cultures were set up and tested for both their cytolytic activity and the appearance of ring structures. It was observed that cultures with high cytolytic activity gave rise to membrane-associated polyperforins while cultures with no or very low activity did not (Table IV). T killer cells were also induced *in vivo* by injecting C57BL/6 mice with allogeneic P815 cells intraperitoneally. Peritoneal exudate lymphocytes obtained on day 10 had a very high cytolytic activity and were further enriched for T killer cells by passage over nylon wool columns. When these effector cells were passaged

TABLE III
Effect of Monensin on T Cell-Mediated Lysis and Poly P Complexes

Killer cells	Monensin	a/t	Percent cytotoxicity at 4 h	Membranes with poly P1 complexes at 4 h
B6.C.G11	—	1:1	73 \pm 1	40/100
B6.C.G11	—	0.3:1	46 \pm 4	—
B6.C.G11	—	0.1:1	19 \pm 2	—
B6.C.G11	1 $\mu\text{g}/\text{ml}$	1:1	44 \pm 1	20/100
B6.C.G11	1 $\mu\text{g}/\text{ml}$	0.3:1	19 \pm 2	—
B6.C.G11	1 $\mu\text{g}/\text{ml}$	0.1:1	5 \pm 1	—

TABLE IV
Correlation Between Cytolytic Activity of Effector Cells and Presence of Poly P1 Structures

Effector cells	Targets	Percent Cytotoxicity			Time* of incu- bation	Membranes with‡ Poly P1 complexes at 16 h
		10:1	3:1	1:1		
B6.C.G11	P815	85	65	29	4	15-30/50
B6 anti-BALB/c MLR§	S194	—	—	51	16	10-20/50
BALB/c anti-C3H MLR	YAC-1	—	—	<1	16	0/50
B6 anti-P815 PEL¶	P815	94	75	35	4	15-20/50
B6 Spleen	P815	<1	<1	<1	4	0/50
MTL 2.8.2	S194	93	76	68	16	15-25/50
CTLL-2	S194	<1	<1	<1	4	0/50
CTLL-2 + Con A	S194	<1	<1	<1	4	5-10/50
CD1/1.10G5 + Con A**	S194	<1	<1	<1	4	0/50
CD1/3.1C6 + Con A**	S194	<1	<1	<1	4	0/50
TB-2 + Con A‡‡	S194	<1	<1	<1	4	0/50
T1M1 4 + Con A‡‡	S194	<1	<1	<1	4	0/50
BW5147 + Con A‡‡	S194	<1	<1	<1	4	0/50
S194 + Con A§§	—	<1	<1	<1	4	0/50
P815 + Con A§§	—	<1	<1	<1	4	0/50
YAC-1 + Con A§§	—	<1	<1	<1	4	0/50

* Time at which cytotoxicity assays were terminated.

‡ Membrane lesions were determined after 16 h since more lesions were visible than at 4 h. About 50 membrane fragments were examined and membranes with more than two lesions were called positive. The numbers given have to be considered semiquantitative because recovery of membrane is not quantitative and may vary.

§ Spleen cells were induced in a 5-d, one-way mixed lymphocyte reaction (MLR), harvested, and incubated with either Cr⁵¹-labeled P815 or unlabeled P815 (a/t = 1:1) for assay of cytolytic activity and lesions, respectively.

|| BALB/c anti-C3H mixed lymphocyte culture had no cytolytic activity.

¶ C57BL/6 mice were injected intraperitoneally with 10⁷ P815 cells. Peritoneal exudate lymphocytes (PEL) were harvested on day 10, and purified on a nylon wool column. Assay for cytotoxicity and complex formation was as described for B6 anti-BALB/c MLR.

** CD1/1.10G6 and CD1/3.1C6 are transformed derivatives from a BALB/c anti-DBA/1 allospecific T killer line. These two lines as well as others isolated from different allospecific T killer lines have lost their cytolytic activity in the process of transformation.

‡‡ TB-2, T1M1 4, and BW5147 are thymus-derived tumors. None of these tumors shows target lysis in the absence or presence of 5 µg/ml Con A.

§§ S194, P815, and YAC-1 were used as targets. They do not show poly P1 complexes when incubated with or without Con A for 16 hr.

over nylon columns and then incubated with P815 targets, numerous poly P1 and poly P2 structures were seen on membranes. In contrast, spleen from unimmunized animals neither had cytolytic activity nor gave rise to poly P1 complexes. An interesting situation exists with the "T killer" line, CTLL-2 (17). This line is of H-2^b haplotype and was specific for H-2^d targets. Since its isolation, it has mutated into a noncytolytic line. When incubated with targets, it did not lyse and no circular complexes appeared on membrane fragments (Table IV). When stimulated with Con A however, this line produced circular complexes in low numbers (Table IV). Therefore CTLL-2 may have a defect in the lytic mechanism in an as yet unknown way or may produce the circular complexes in insufficient quantities for detectable cytolysis. Recently we derived transformed derivatives from allospecific T cell lines

that did not express cytolytic activity either in the presence or absence of lectins (unpublished results). None of these lines, including the two examples given in Table IV, gave rise to poly P structures (Table IV). A similar negative result was obtained with a panel of thymic lymphomas, among them BW5147, TB-2, and T1M1 4, none of which showed cytolytic activity nor gave rise to tubular structures as a result of incubation with targets in the presence or absence of Con A (Table IV). The targets P815, S194, and YAC-1 were also negative (Table IV). Thus there was a good correlation between the cytotoxicity of effector cells and the appearance of polyperforin tubules on membranes. It should be emphasized, however, that the isolation of membranes from effector/target mixtures is not quantitative nor is the counting of polyperforins on electron micrographs very accurate. The data in Tables III and IV therefore have to be considered semiquantitative.

Molecular Mechanism of T Killer Cell Lysis. It is well known that NK cells isolated from lymphoid tissue or grown as cell lines in vitro possess dense granules that can be seen by thin sectioning electron microscopy (18). Study of NK/target mixtures (10) had shown that these granules appear to participate in the lytic reaction in that vesicles are formed in these granules that are secreted into the interstitial space between killer and target. It was also observed that poly P tubules can be seen on the vesicles once they are secreted and it was hypothesized that they may be the vehicles for the transport of poly P to the target membrane. A schematic model for these events is shown in Fig. 1.

Experiments presented below suggest that a similar, if not identical, mechanism may be operative in the T killer model. The cloned T killer cell lines, B6.C.G11, B6.C.G3, MTL.2.8, all showed dense granules in thin sections (Fig. 3A). In killer/target mixtures or in the presence of Con A, these granules characteristically generated multilayer membranes (Fig. 3, B and C). It appears that these multilayer membranes give rise to many vesicles and that they may be secreted into the intercellular space subsequent to target/effector binding. A persistent finding in mixtures of killers and targets is the close association of vesicles with target membranes. In Fig. 4, A and C, vesicles (V) with poly P1 (arrowhead) are bound to a target membrane tagged with gold-labeled antibody specific for H-2D^d (arrow). The consistent lack of gold binding to the vesicular membranes indicates that they originated from killer cells because the T killer cells did not react with this antibody (Table I). The two types of membranes differentially labeled with the gold-tagged antibody provided a good internal control for antibody specificity and unspecific binding. There were several other types of structures on membranes not labeled with colloidal gold and therefore probably derived from killer cells. In one, it appeared that poly P1 (Fig. 4E) polymerized from subunits (Pf 1). Some complexes appear as rings, while others appear as half rings or figure eights. Similarly, in Fig. 4F, it appeared that poly P2 complexes were being assembled from individual subunits (Pf 2) into ring-like structures (arrowheads). In Fig. 4, B and C (open arrow), vesicles are imaged that may contain polyperforins in the early stages of assembly. There also appears a regular arrangement of spherical particles in Figure 4D and in the center of 4C. Whether these latter structures are related to poly P is not known at present. These images are compatible with the interpretation that poly P tubules are assembled from monomers associated with granule or Golgi membranes that fuse with target cells.

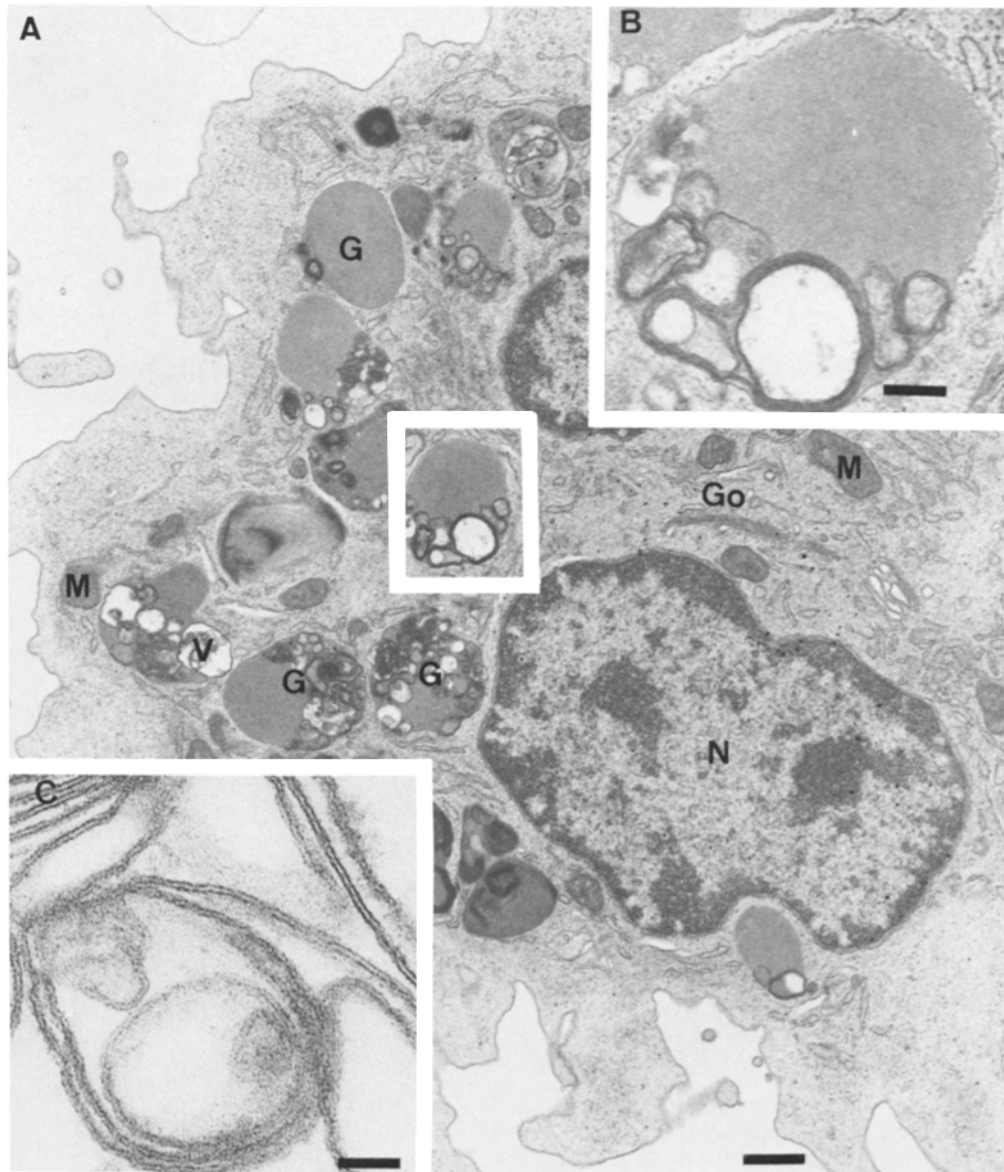


FIG. 3. (A) Thin section of T killer B6.C.G11 (N, nucleus; Go, Golgi; G, granules; M, mitochondria; V, vesicles [within granule]). There are many dense granules of various morphology. One granule containing membranous material is magnified (B). Intracellular multilayer membranes of another cell are shown in (C). Bars: (A) 666 nm; (B) 222 nm; (C) 57 nm.

Discussion

The results of this study show that cloned H-2 specific T killer cells grown in lymphokine-containing medium in the presence of stimulator cells possess dense granules similar to those of cloned NK cells (10, 18). Incubation of these effector cells with either Con A or target cells resulted in assembly of two types of tubular structures, poly P1 and poly P2, that could subsequently be demonstrated on target

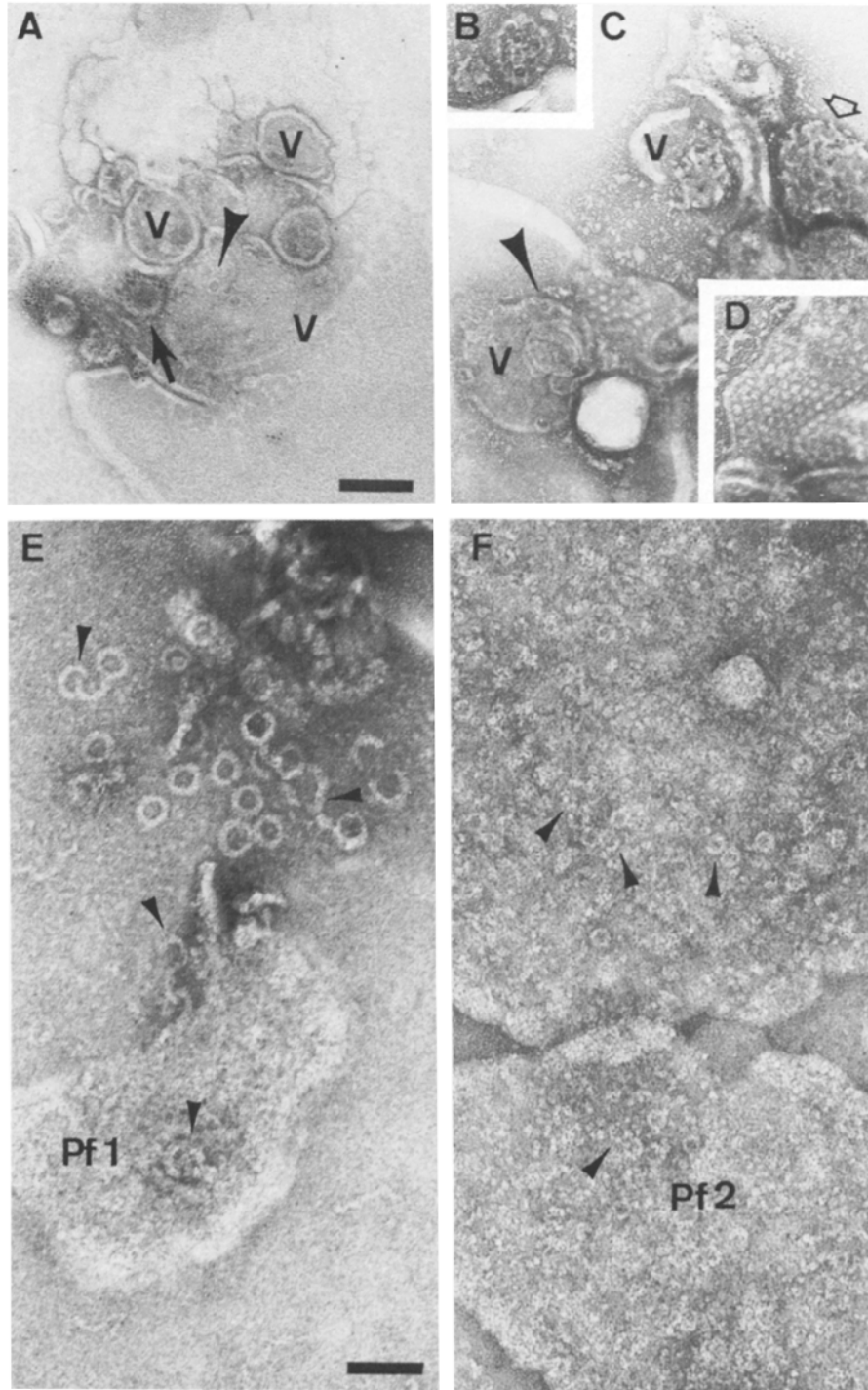


FIG. 4. Association of target membranes with vesicles derived from killer cells and containing polymerizing poly P complexes. (A) and (C) Numerous unlabeled killer cell vesicles (V) are associated with gold-tagged target membranes (arrows). Poly P1 is indicated with black arrowheads. (B) Vesicles that may contain poly P1 in the process of formation (also see [C] open arrow). (D) Regular arrays often found in killer/target membrane mixtures. (also see center of [C]) (E) Membrane fragment that appears to contain perforins in the process of polymerization. Arrowheads depict irregular and incomplete rings. The granular material labeled Pf 1 may represent the putative precursors of poly P1 associated with membranes. (F) Membrane fragments bearing poly P2 and structures that may represent monomeric precursors (Pf 2) and polymerizing tubules (arrowheads). Bar: (A-D) 100 nm; (E) and (F) 57 nm.

membranes. The dimensions of both poly P1 and poly P2 (Fig. 1) were different from those of mouse complement lesions, which have inner and outer diam of 50 and 200 Å, respectively (10). Poly P1 and poly P2 were also different in size from bovine and human complement lesions (10). To avoid any potential artifacts due to lesions assembled by either complement or bacteria, all experiments were performed in the presence of purified human serum albumin to replace serum and in the presence of antibiotics.

From our observation that poly P1 and poly P2 appear to be structures unique to cytolytic effector cells and that they were transferred from the effector cell to the target membranes during the lytic reaction, we have hypothesized that the two structures play a part in the lytic reaction. By analogy to the function of the membrane attack complex of complement, we have hypothesized that the function of the poly P structures is to produce ion channels in target membranes that lead to cell death. Two additional pieces of information support this hypothesis: (a) T killer cells that have lost their cytolytic activity assemble few if any poly P1 and poly P2 complexes; and (b) monensin, a drug that inhibits cell lysis, also inhibits poly P formation (19). One must emphasize, however, that the evidence accumulated thus far for the hypothesis that polyperforins play a central role in cell-mediated lysis is circumstantial. Substantial biochemical experimentation is required to obtain more conclusive results.

The structural studies on poly P1 and poly P2 support the hypothesis that both structures are polymerization products. The sequence of events leading to the polymerization and deposition of these complexes on the target membrane are quite speculative at this point. The electron micrographs suggest that the granules and Golgi of T killers play an important role in the lytic event and that they form membranes and vesicles. Poly P1 and poly P2 tubules could be seen on extracellular vesicles that are in close contact with target membranes. The vesicles may fuse with target membranes, as suggested for the gold labeling experiments in Figs. 2 and 4. From these observations a sequence of events can be constructed (shown schematically in Fig. 1). The granules (or Golgi) of killer cells contain both the precursors for membrane and the precursor perforins. The vesicles may function as transport vehicles for perforins and may contain entrapped substances that enable the killer cells to inject effector molecules into the target cell. The insertion mechanism of polyperforin may involve vesicle fusion or association with target membranes.

Whatever the precise mechanism of polyperforin insertion may be, our data may point to striking analogies in the final steps of complement- and cell-mediated cytolysis. Circular (tubular) polymerization of monomeric precursors and insertion of transmembrane tubules may constitute a general principle for cytolysis not only by complement and cytolytic effector cells but also by bacterial toxins (20, 21).

Summary

Cloned T killer cells derived from one-way mixed lymphocyte reactions were characterized with regard to their Lyt phenotype and specificity. Two clones of Lyt-1⁻2⁺ phenotype that recognized H-2D^d were selected and examined for their cytolytic function by negative staining and thin section electron microscopy. When incubated with the H-2^d target S194, they assemble two types of tubular complexes, polyperforin 1 and 2. Both structures appear to arise by polymerization of precursors that may originate in dense granules and/or Golgi of T killer cells. Polyperforins appear to be

associated with vesicles that are released during the lytic reaction and transferred to target membranes as shown by immunoelectron microscopy. Since there is a close correlation between target lysis and the appearance of polyperforins on target membranes, it is suggested that polyperforins take part in the cell-mediated killing reaction. Although polyperforins are different in size and several molecular properties from complement, there are striking similarities between these circular complexes and polyperforin (C9). It is therefore possible that they belong to a closely related family of cytolytic effector molecules.

The excellent technical assistance of Carol Gay Anderson is acknowledged.

Received for publication 20 December 1983.

References

1. Podack, E. R., and J. Tschopp. 1982. Polymerization of the ninth component of complement (C9): formation of poly C9 with a tubular ultrastructure resembling the membrane attack complex of complement. *Proc. Natl. Acad. Sci. USA.* **79**:574.
2. Podack, E. R., J. Tschopp, and H. J. Muller-Eberhard. 1982. Molecular organization of C9 within the membrane attack complex of complement. *J. Exp. Med.* **156**:268.
3. Tschopp, J., H. J. Muller-Eberhard, and E. R. Podack. 1982. Formation of transmembrane tubules by spontaneous polymerization of the hydrophobic complement protein C9. *Nature (Lond.)* **298**:534.
4. Berke, G. 1980. Interaction of cytotoxic T lymphocytes and target cells. *Prog. Allergy.* **27**:69.
5. Golstein, P., and E. Smith. 1977. Mechanism of T cell-mediated cytotoxicity: the lethal hit stage. *Contemp. Top. Immunobiol.* **7**:273.
6. Martz, E. R. 1977. Mechanism of specific tumor cell lysis by alloimmune T-lymphocytes: resolution and characterization of discrete steps in cellular interaction. *Contemp. Top. Immunobiol.* **7**:301.
7. Henney, C. S. 1977. T cell-mediated cytotoxicity: an overview of some current issues. *Contemp. Top. Immunobiol.* **7**:245.
8. Perlmann, P., and J.-C. Cerottini. 1979. Cytotoxic lymphocytes. In *The Antigens*. M. Sela, editor. Academic Press, Inc., New York. **V**:173.
9. Dourmashkin, R. R., P. Deteix, C. B. Simone, and P. A. Henkart. 1980. Electron microscopic demonstration of lesions in target cell membranes associated with antibody-dependent cellular cytotoxicity. *Clin. Exp. Immunol.* **42**:554.
10. Podack, E. R., and G. Dennert. 1983. Cell-mediated cytotoxicity: assembly of two types of tubules with putative cytolytic function by cloned natural killer cells. *Nature (Lond.)*. In press.
11. Dennert, G., G. Yogeewaran, and S. Yamagata. 1981. Cloned cell lines with natural killer activity. Specificity, function and cell surface markers. *J. Exp. Med.* **153**:545.
12. Bleackley, R. C., C. Havele, and V. Paetkau. 1982. Cellular and molecular properties of an antigen specific cytotoxic T lymphocyte line. *J. Immunol.* **128**:758.
13. Dennert, G., and M. DeRose. 1976. Continuously proliferating T killer cells specific for H-2^b targets: selection and characterization. *J. Immunol.* **116**:1601.
14. Koo, G. C., J. B. Jacobson, G. J. Hammerling, and U. Hammerling. 1981. Antigen profile of murine natural killer cells. *J. Immunol.* **125**:1003.
15. Tschopp, J., E. R. Podack, and H. J. Muller-Eberhard. 1982. Ultrastructure of the membrane attack complex of complement: detection of the tetramolecular C9 polymerizing complex C5b-8. *Proc. Natl. Acad. Sci. USA.* **79**:7474.
16. Steinmetz, M., K. Minard, S. Horvath, J. McNicholas, J. Frelinger, C. Wake, E. Long, B.

- Mach, and L. Hood. 1982. A molecular map of the immune response region from the major histocompatibility complex of the mouse. *Nature (Lond.)*. **300**:35.
17. Baker, P. E., S. Gillis, and K. A. Smith. 1979. Monoclonal cytolytic T-cell lines. *J. Exp. Med.* **140**:273.
 18. Grossi, C. E., and M. Ferranini. 1982. Morphology and cytochemistry of human large granular lymphocytes. *In NK Cells and Other Natural Effector Cells*. R. Herberman, editor. Academic Press, Inc., New York. 1-8.
 19. Carpen, O., I. Virtanen, and E. Saksela. 1982. Ultrastructure of human natural killer cells: nature of the cytolytic contacts in relation to cellular secretion. *J. Immunol.* **128**:2691.
 20. Bhakdi, S., R. Füssle, and J. Tranum-Jensen. 1981. Staphylococcal α -toxin: oligomerization of hydrophilic monomers to form amphiphilic hexamers induced through contact with deoxycholate detergent micelles. *Proc. Natl. Acad. Sci. USA.* **78**:5475.
 21. Mitsui, K., T. Sekiya, Y. Nozawa, and J. Hase. 1979. Alteration of human erythrocyte plasma membranes by perfringolysin 0 as revealed by freeze-fracture electron microscopy. *Biochim. Biophys. Acta.* **554**:68.