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Role of Granule Proteins in Lymphocyte-Mediated Killing

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The mechanism(s) by which effector killer cells lyse their targets remains as one of the more important and intriguing problems in biology today. Killer cells that lyse targets by a contact-dependent process are of particular interest to immunologists. This surface contact can be mediated via the Fc-dependent mechanism in the case of antibody-dependent cell-mediated cytotoxicity (ADCC), through binding of antibody-coated targets to cells that bear surface Fc receptors, such as macrophages, neutrophils, eosinophils, and natural killer (NK) cells, or alternatively, via specific and nonspecific receptors that recognize target cell surface constituents, as in the case of activated macrophages and cytotoxic T lymphocytes. In either instance, it has been assumed that the binding of the target elicits release of cytotoxic mediators by the effector cell that may become concentrated in the intercellular space of contact. The search for mediators of cytolysis has been actively implemented in numerous laboratories over the past few years.

Several criteria have been used to implicate a mediator in the cytolytic reaction, the details of which are given elsewhere [1,2]. Briefly, (1) the purified or highly enriched mediator should reproduce the cytolytic reaction mediated by intact cells. The kinetics of killing should be comparable to that observed with whole cells. (2) The expression and release of the mediator should correlate with the expression of activation and cytolytic capability of the effector cells. (3) The contact between the effector and target cells should prompt the release of the mediator. Alternatively, it is possible that nonspecific surface-active reagents, such as calcium ionophores, lipopolysaccharides, phorbol esters, and concanavalin A, may also trigger the release of the mediator into the extracellular medium. It should be pointed out that the identification of only low levels of secreted mediator does not preclude its role in cell killing since the same mediators may become highly concentrated in the diffusion-limited intercellular space of contact between the cells. (4) Inhibitors of the mediator may block whole cell killing. However, negative results in such experiments are also difficult to interpret, especially since the inhibitor may not have had access to the

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contact space between the cells. (5) Cell lines deficient in the synthesis or release of the mediator may have correspondingly lower cytolytic capability.

NATURE OF CYTOLYSIS MEDIATED BY CTL AND NK CELLS

The mechanism(s) by which cytotoxic T lymphocytes (CTL) and natural killer (NK) cells lyse tumor cells have been extensively studied in several laboratories over the past few years [see 3–10 for recent reviews on this topic]. A permeability increase in the target membrane occurring in association with effector cells was first demonstrated by Henkart and Blumenthal [11]. The involvement of pore-formation in target membrane damage, particularly in ADCC, was substantiated by ultrastructural demonstration of tubular lesions with an internal diameter of 15 nm that were formed on target membranes [12]. The ultrastructural demonstration of ring-like lesions on the surface of target cells was later confirmed and extended to cloned CTL [13] and NK cells [14].

Other functional studies that support a role for pore-formation in lymphocyte-mediated killing included: (1) the dose–response curve of cell killing suggesting a one-hit mechanism [15]; (2) the release of small molecular weight markers preceding the release of cellular contents of large molecular weight [16]; (3) studies on the inhibition of T-cell-mediated lysis using extracellular macromolecules of different sizes indicating the presence of either an osmotic or diffusion-limiting mechanism of protection [17]; (4) following initial contact, effector cells being no longer required for the completion of target lysis [18,19]; (5) functional sizing of the lymphocyte-mediated lesions giving estimates comparable to the size of the pores generated by the complement cascade [20,21].

The role of other mediators in T-cell-mediated cytolysis, such as oxygen metabolism intermediates has also been investigated [22]. Although O_2 is required for T-cell-mediated killing, reactive oxygen intermediates do not seem to play a direct role in the killing process [22]. Similar results have recently been reported for NK cells [23,24] that also cannot confirm a role for reactive oxygen intermediates in the killing mediated by these cells.

Proteolytic enzymes have also been implicated in killing mediated by peripheral blood lymphocytes [25,26], T cells [27–29], and NK cells [30–34]. These studies have investigated mainly the effect of protease inhibitors on cell-mediated killing. Based on these experiments, the involvement of serine esterase(s) has been suggested. Studies with inhibitors have also implicated the delivery of protease-sensitive structures from NK cells to the targets during cell killing [35–38]. More recently, it has been shown that CTL express specifically a serine esterase of 28 kDa that is present at much lower levels in helper T cells or resident thymocytes [39]. Parallel studies with NK cells showed the presence of serine esterase activity that is induced following conjugation of effector and target cells [40]. The specific expression of serine esterase activity by CTL has recently been confirmed and extended by two laboratories that have succeeded in isolating CTL-specific cDNA copies [41–43]. The deduced amino acid sequences of these cDNA copies are partially homologous to those of other well-known serine esterases. The exact role for serine esterase in mediating killing however is not yet known.

Recently, the role of a soluble cytotoxic factor from NK cells (NKCF) in killing has been extensively investigated [44–48]. This factor may be responsible at least in

part for the cytolysis mediated by NK cells [44–48]. The subcellular localization of this factor in NK cells is presently being investigated by Bonavida and his colleagues. Studies on a putative role of NKCF in cell killing have been hampered in the past by the scarce availability of this factor for biochemical analysis. It is possible that this NKCF may be related in some way to tumor necrosis factor (TNF) or lymphotoxin (LT) that have recently been cloned and expressed in *E coli* [49–52].

Multiple cytokines are also known to be produced by human large granular lymphocytes (LGL), such as interleukins 1 and 2, interferons α and γ and B-cell growth factor [53]. The human LGL appear to correspond morphologically and functionally to NK cells [54,55].

Recently, cloned cell lines of CTL and NK cells have become available [56–65]. It has now become possible to grow large numbers of homogeneous populations of cells for biochemical analysis. This feature has recently allowed work on lymphocyte killing to be extended to subcellular levels and to purified proteins. Here, the cytolytic properties of the granules and granule proteins isolated from these cells are reviewed.

FUNCTIONAL ASSAYS USED TO STUDY PORE FORMATION MEDIATED BY LYMPHOCYTES

The aim of this and other laboratories have consisted of isolating an active principle from CTL and NK-like cells that would explain the mechanism of cell lysis. Because of several indirect lines of evidence implicating a putative role for pore-forming proteins (PFP) in killing, several functional assays have been designed to screen for the membrane lytic activity produced by pore-formers. The details of the experimental strategies used to study PFP have been outlined elsewhere [66]. Briefly, a putative PFP produced by killer cells would be expected to change the balance of transmembrane electrolytes in a significant and measurable way allowing this membrane perturbation to be studied at several levels of cellular organization. First, at the cell level, PFP should lyse anucleated erythrocytes and depolarize the resting membrane potential of nucleated cells. Since most nucleated cells have an efficient membrane repair mechanism, PFP alone may not be sufficient to lyse nucleated cells unless it is present at sufficiently high concentrations. A simple automated hemolysis microassay, useful in the rapid and quantitative screening of large numbers of subcellular and chromatography fractions, has been designed [67]. Membrane potential changes may be assessed by using voltage-sensitive probes [68–70], direct impalement of cells with microelectrodes [70–72] and whole cell recordings using a patch-clamp system [73]. The lysis of nucleated cells can be assessed using standard ^{51}Cr release cytotoxicity assays [74,75].

Putative PFP may also be assayed and examined in greater details by using lipid vesicles as model target membranes [68–70,76–78]. The lipid vesicles may be labeled with fluorescent markers [77], voltage-sensitive probes [68–70,76] and labeled macromolecules [70,72,78] and studied to score the extent of membrane leakiness produced by PFP. Resealed erythrocyte ghosts may also be used in assaying for the extent of release of trapped macromolecules [20,21,78].

The most sensitive functional assay for PFP, however, is direct single channel recording using planar lipid bilayers or patch-clamped membranes [68–70,72,73,79]. Because of the high-impedance of the bilayers examined by these techniques, even

minute current fluctuations associated with individual PFP molecules can be measured with high resolution.

The putative PFP may also form tubular structures that are large enough for direct ultrastructural examination, which would provide important morphological information not available by other techniques. Since the effector cells studied here have been shown to produce tubular lesions on target membranes [12–14], the putative PFP should also mediate the assembly of similar lesions on membranes.

CYTOLYTIC GRANULES OF CTL AND NK CELLS

Presence of Cytoplasmic Granules

Some of the more prominent features of cloned CTL and NK cells or cell lines are the large cytoplasmic granules that show a well-defined internum and externum [13,14,40,63,65,80–84]. The internum, made up of a fine amorphous matrix, is usually surrounded by electron-dense membrane-bound vesicular material (Fig. 1).

Isolation of Cytoplasmic Granules

Work from several laboratories has implicated a role for the granule population of CTL, NK-like cells, and LGL as the primary lytic apparatus involved in killing [69,70,72,73,85–88]. The expression of cytolytic granules may be related to the action of the interleukins (IL). A T-cell hybrid (PC60) that can grow independently of IL-2, becomes cytolytic and acquires cytoplasmic granules following induction with a combination of IL-1 and IL-2 [88–90]. With the possibility of growing homogeneous populations of cytolytic cells now to at least 10^9 cells, several investigators have succeeded in isolating granule populations of those cells [67,69,70,72,77,78,85–88]. The isolation procedure usually involves rupturing cells, followed by centrifugation of the nucleus-free cell lysate through continuous or discontinuous Percoll gradients. The different fractions from the same Percoll gra-

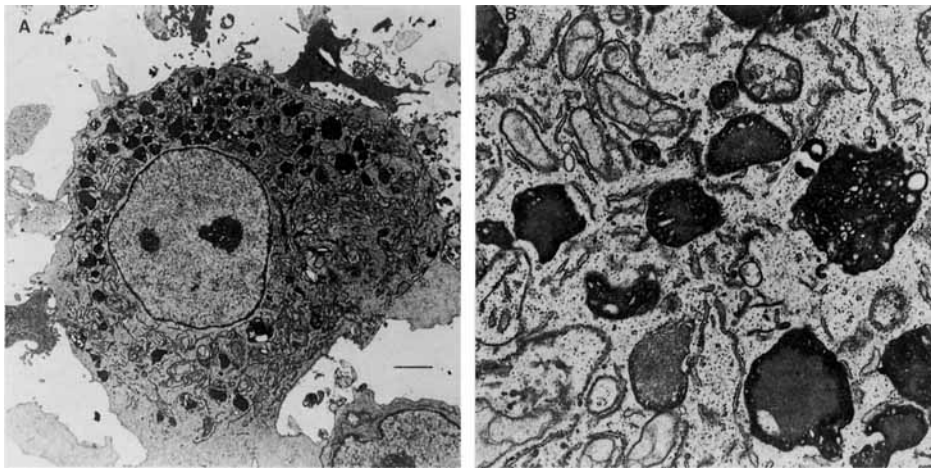

Fig. 1. (A) Morphology of the cytolytic Hy 3-Ag3 cell, with high NK-like activity, showing numerous electron-dense granules in the cytoplasm. (B) An enlarged view of granule region [70]. Scale: 35 μm (A) and 657 nm (B).

dient are then tested for hemolytic activities. Since the granules are lysosomal in nature, they are also enriched for a number of lysosomal markers [85]. Recent work from this laboratory has also shown that a potent neutral serine esterase activity may be used as a convenient and specific granule marker for CTL and NK cells [91,92]. The isolated granules show a morphology which is indistinct from that of intact cells (Fig. 2A).

Structural and Functional Lesions Produced by Granules

The isolated granules are capable of mediating the assembly of tubular lesions on target membranes at 37°C and only when millimolar amounts of Ca^{2+} are present (Fig. 2b–d). Tubular lesions with an internal diameter ranging 150–170 Å are observed. These lesions resemble closely those formed by whole effector cells on the surface of target cells, indicating the granules contain the cytolytic PFP.

As pointed out, granule extracts contain potent hemolytic activity, which is only observed in the presence of Ca^{2+} (Fig. 3). The granule lytic activity can be solubilized by extracting granules with buffers containing high amounts of salts [69,70,77,78,85–87]. Kinetics studies performed on the hemolytic reaction mediated by the granules, measured as a continuous decrease of turbidity of the erythrocyte suspension at 700 nm, reveal that hemolysis is usually complete within 10 min at 37°C (Fig. 3). The hemolysis is completely abolished by chelating Ca^{2+} in the erythrocyte medium with EGTA, but can be restored readily with subsequent addition of Ca^{2+} (Fig. 3). The

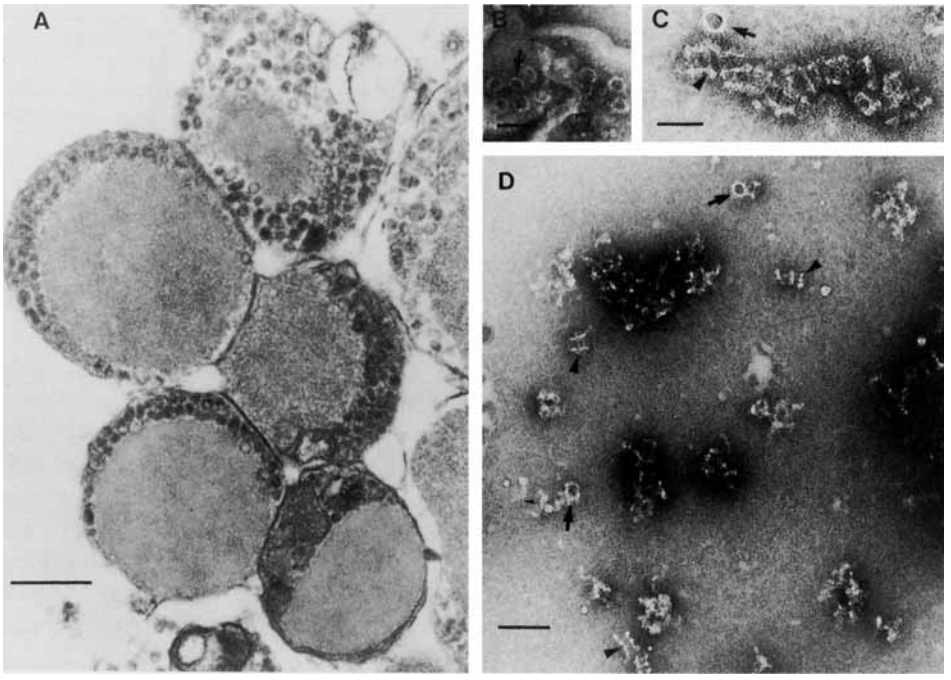

Fig. 2. (A) Granules isolated from Hy 3-Ag3 cells by centrifugation through Percoll gradients. (B–D) Membrane lesions produced by granules on sheep erythrocyte membranes. Top (arrows) and longitudinal (arrowheads) views of tubular lesions are seen [70]. Scale: 270 nm (A); 57 nm (B); 38 nm (C); 87 nm (D).

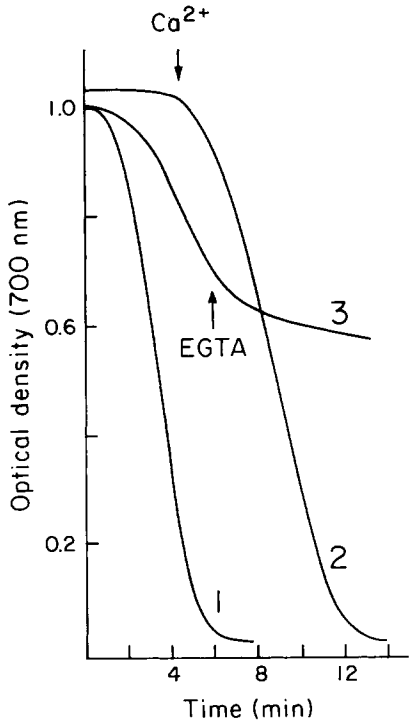

Fig. 3. Hemolytic activity associated with granules of Hy 3-Ag3 killer cells. Sheep erythrocytes were resuspended in buffer containing 1 mM Ca^{2+} (curve 1), 0.1 mM Ca^{2+} (curve 3), and no Ca^{2+} (curve 2). Granules were added to the granule suspension at time 0 to a final protein concentration of 6 μg/ml. In curve 2, 1 mM Ca^{2+} was added where indicated. In curve 3, EGTA was added to a final concentration of 5 mM (arrow). The temperature of all experiments was 37°C [70].

extent of hemolysis is markedly decreased when the hemolytic reaction is carried out at room temperature.

Granules depolarize rapidly the membrane potential of nucleated cells [69,70,72] and induce marker release from lipid vesicles and resealed erythrocyte ghosts [69,70,77,78]. Granules are also capable of lysing tumor cells in the presence of Ca^{2+}, without showing any cell specificity [85–87]. When tested against high-impedance planar bilayers, granule proteins induce a rapid change in membrane resistance [69,70]. The change in membrane resistance occurs as a progressive incorporation of discrete ion channels into the bilayer, which usually continues until the membrane breaks down (Fig. 4). The sizes of the channel steps are heterogeneous, ranging 0.4–6 nS per channel in 0.1 M NaCl (1 S = ampere/volt). This range of unit conductance reflects a flow rate of at least 10^9 ions/s/channel. The scatter obtained for the unit conductances may be due to the multiple sizes of the polymerized tubular complexes (which would include partially polymerized complexes, half-rings, double rings, etc).

The channels formed by the granule proteins remain permanently open and are highly resistant to closing by increasing the transmembrane potential (Fig. 5). A significant amount of channel closing is only observed at voltages exceeding 70 mV. The current–voltage relationships for these channels show a linear curve (Fig. 5B),

Fig. 4. Effect of granule-derived material on bilayer conductance. Planar bilayer was made in 0.1 M NaCl and clamped at 30 mV. Granule proteins from CTLL A2 were introduced into the cis compartment (the current being considered positive when flowing from cis to trans). Note the increase of membrane current in discrete steps; current trace (top, right) is continued at the bottom (left) [69].

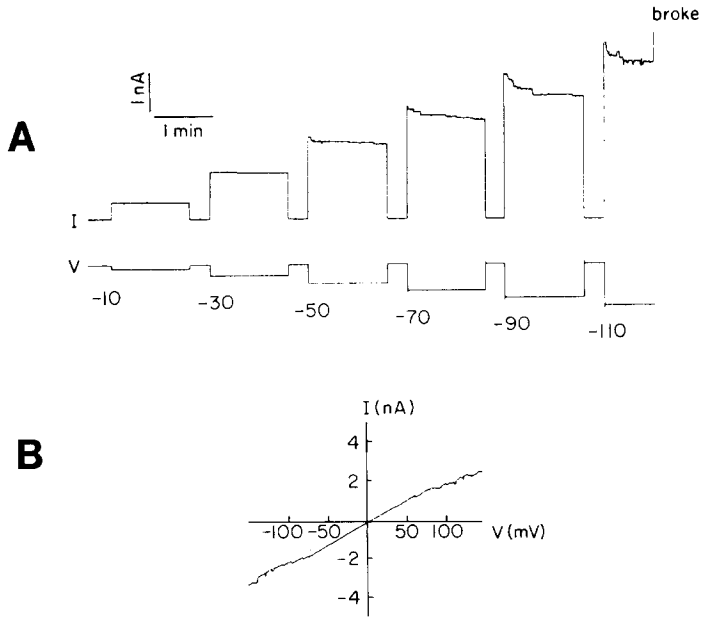

Fig. 5. A) Steady-state membrane current (I) as a function of membrane voltage (V). Bilayer in 0.1 M NaCl exposed to granule proteins was challenged with increments of voltage. B) Current-voltage plot generated by passing a continuous voltage ramp from −150 to +150 mV in 1 s, with bilayer containing granule-derived pores [69].

with noise and only slight deviation from linearity (ie, reflecting channel closing) being observed at voltages that exceed 70 mV. Since channels close only at high voltages, we have been able to obtain single channel recordings with bilayers clamped at 120–150 mV [69,70]. This behavior indicates that large, stable, and voltage-resistant channels are formed by the granule-derived proteins, which are attributes that would favor an active role of these channels in mediating cytolysis.

The calcium dependence of killing mediated by the intact granules may be two-fold [70]: Calcium appears to be required for both fusion of granules with target membranes and assembly of the tubular complexes.

A PORE-FORMING PROTEIN ISOLATED FROM GRANULES AND ITS ROLE IN CELL KILLING

Isolation of the Pore-Forming Protein (PFP)

The putative PFP from lymphocyte granules has been given different names, following the morphological description of the tubular lesions mediated by intact effector cells and by isolated granules. Henkart and colleagues have named the putative pore-former of rat LGL tumor as cytolysin [77,85,86]. Podack and Dennert have named the mouse CTL and NK cell pore-formers as perforin 1 and 2, and their polymerized species as polyperforin 1 and polyperforin 2, respectively [13,14]. Since we have identified and purified pore-forming proteins from a variety of effector cell-types (including the protozoan parasite *Entamoeba histolytica* [66,68], human eosinophils [93], mouse lymphocytes with NK-like activity [70], and human NK cells [94]) we will refer generically to these proteins as pore-forming proteins (PFP) in this review.

The PFP from mouse cytotoxic T cells and NK-like lymphocytes have recently been isolated [67,70,72,73,95]. In our laboratories, we have used a combination of molecular sieving and ion exchange chromatography steps [70,72,73]. The eluted fractions from the columns are assayed for hemolytic activity using the microassay described earlier [67] and also for pore-forming activity in planar bilayers [70,72].

The monomeric protein migrates with a M_r of 66–68 kDa [95] and 70–75 kDa [67,70,72,73] under reducing conditions when analysed by SDS-polyacrylamide gel electrophoresis (Fig. 6B, lanes 1 and 2). The nonreduced form of this protein has an apparent M_r of 60–66 kDa as observed by gel electrophoresis (Fig. 6B, lane 4) and by molecular sieving chromatography (Fig. 6A). Sizing PFP by HPLC (Fig. 6A) provides an independent estimate of its apparent M_r, excluding the possibility that the single band observed here may have been contaminated with the closely comigrating bovine serum albumin or that the activity may correspond to some other minor species not identified on the gel.

Similar polypeptides (in M_r, and functional and structural properties) have been isolated from CTL and NK-like lymphocytes [70,72,73]. We have also recently identified a 70–75 kDa lytic polypeptide in human peripheral blood NK cells maintained in IL-2-containing medium and in a human NK cell line [94].

Polymerization of the Lymphocyte PFP

The purified protein polymerizes in the presence of Ca^{2+} and at 37°C, resulting in the formation of a one million Dalton species that resists at least partially dissociation by boiling and by treatments with SDS and reducing agents (Fig. 6B, lane 3). On Sephacryl S-200 column, the polymerized material elutes in the void volume. The polymerized species resembles the tubular lesions observed before with granule extracts (Fig. 7). Ring-like structures with an internal diameter averaging 160 Å are typically seen. In the absence of Ca^{2+}, polymerization does not occur (Fig. 6).

Functional Lesions Produced by Lymphocyte PFP

The purified protein shows potent hemolytic activity: 1 ng of protein lyses completely 10^8 sheep erythrocytes [67]. Ca^{2+} is apparently required for both attachment of the protein to the target membrane and for its subsequent polymerization to form functional pores [70]. It is thought that Ca^{2+} may result in the unfolding of PFP

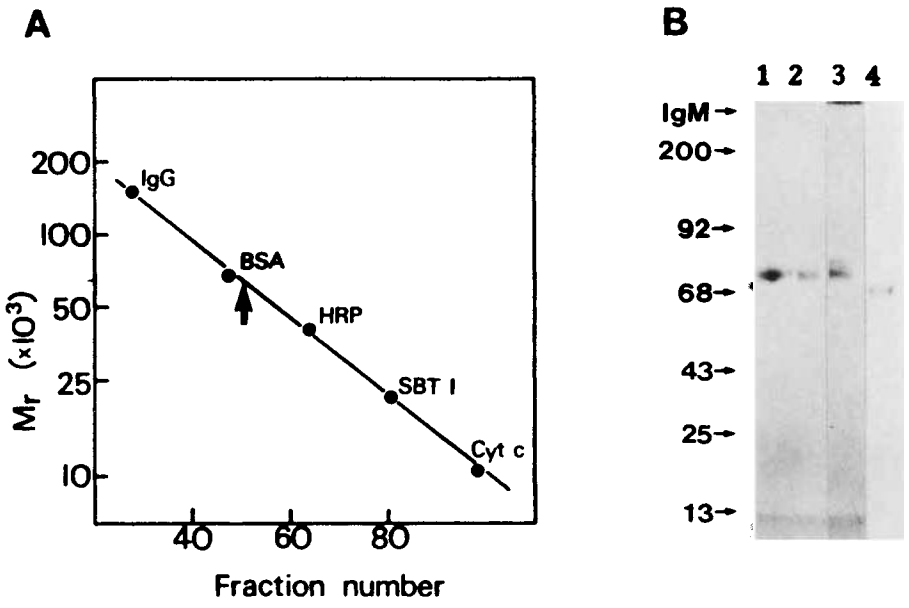

Fig. 6. A) Position of PFP eluted through a TSK-G3000 column, as determined by the protein profile and the hemolytic activity. The injected sample was previously purified by Sephacryl S-200 and Mono Q columns. B) SDS-PAGE profile of lymphocyte PFP. PFP from Mono Q column (5 μg protein applied, lane 1) and G3000 column (2 μg, lanes 2 and 4). We incubated 10 μg of PFP from G3000 column were incubated at 37°C, 48 hr, with 1 mM $CaCl_2$, 0.1% deoxycholate, 0.05 mM PMSF, 0.1 IU/ml of aprotinin, and applied this to lane 3. Lanes 1–3 were run under reduced conditions; lane 4, unreduced. Note the high M_r band in lane 3, corresponding to most of the material applied [70].

in solution with exposure of lipid-binding domains. The purified protein also lyses a variety of tumor cells [70,72]. The amount of protein required to lyse nucleated cells, however, is several times higher than that required to lyse a similar number of erythrocytes, consistent with the well-known observation that nucleated cells but not anucleated erythrocytes are capable of repairing their damaged membranes.

The first effect of PFP on cells appears to be a rapid depolarization of the resting membrane potential [70,72,73]. This effect can be demonstrated by using macrophages and chicken embryo myoblasts as targets cells [70,72,73]. Ca^{2+} is required for maximal depolarization activity (Fig. 8). PFP that has been incubated at 37°C (ie, after polymerization) loses its surface-active effect (Fig. 8).

PFP also inserts spontaneously into cell membranes, as measured by patch clamp, in the whole cell configuration (Fig. 9). PFP mediates increase of membrane current in discrete steps, indicative of incorporation of ion channels into the cell membrane.

Lipid vesicles that have been exposed to PFP also become leaky to electrolytes and certain macromolecules (lucifer yellow, molecular weight 457; sucrose, molecular weight 342) [70,72,73]. The leakiness induced by PFP is nonspecific to a variety of monovalent and divalent ions tested.

Like the granule extracts, the isolated PFP also induces the formation of similar functional ion channels in planar lipid bilayers (Fig. 10). Similar voltage–current relationships and ion selectivity values are observed with the purified protein. The channels produced by PFP also have large unit conductances (0.4 nS in 0.1 M NaCl)

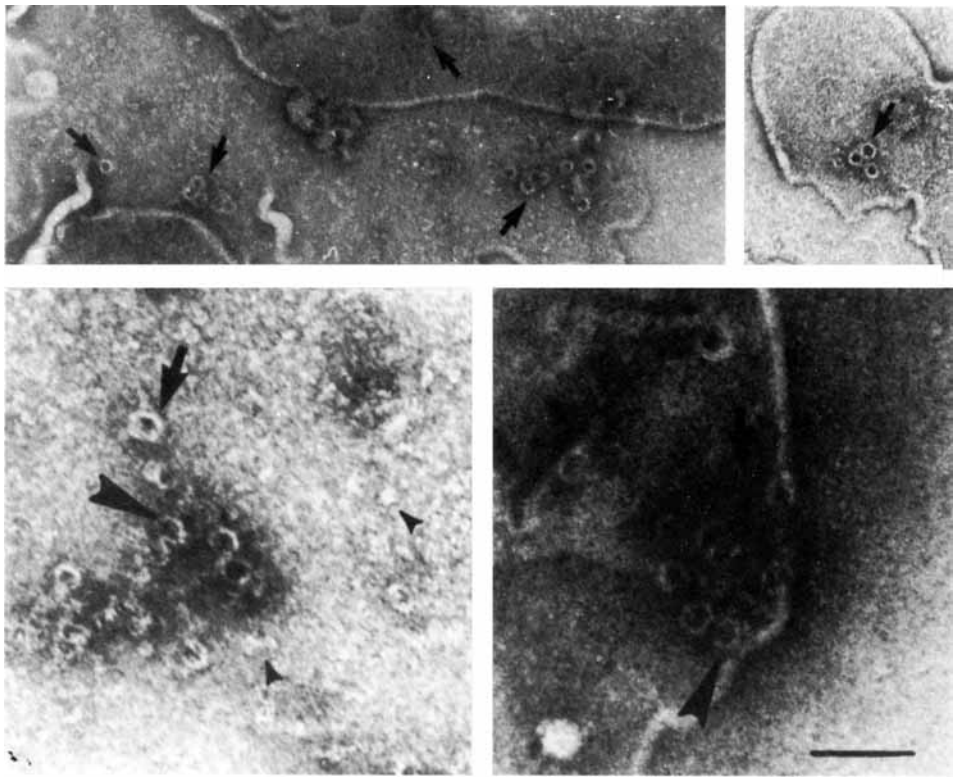

Fig. 7. Selected images of PFP polymerized on sheep erythrocyte membranes. Typical tubules of 160 Å internal diameter are observed. Complete rings (arrows) and incompletely polymerized (arrowheads) tubules are observed [72]. Scale bar: upper panels, 250 nm; lower panels, 85 nm.

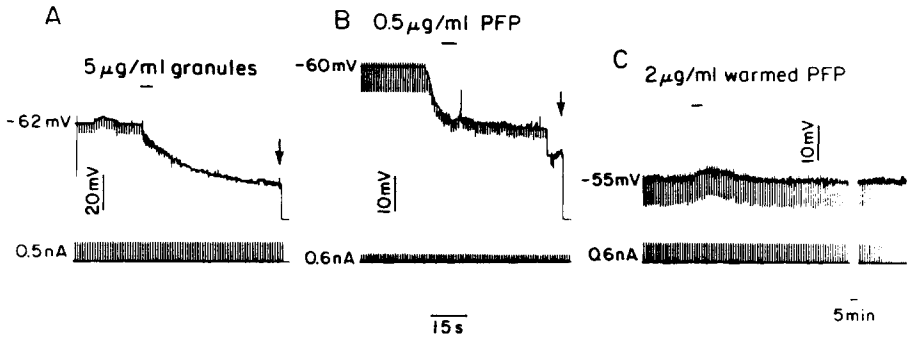

Fig. 8. Depolarization of cultured chicken muscle cells by PFP. Cells were exposed to Hy 3-Ag 3 granules (A) and PFP (B) (indicated by bars). Arrows indicated withdrawal of microelectrode. (C) PFP was incubated at 37°C for 4 hr in the presence of 1 mM Ca^{2+} prior to addition to cells. The lower traces show the applied current pulses [70].

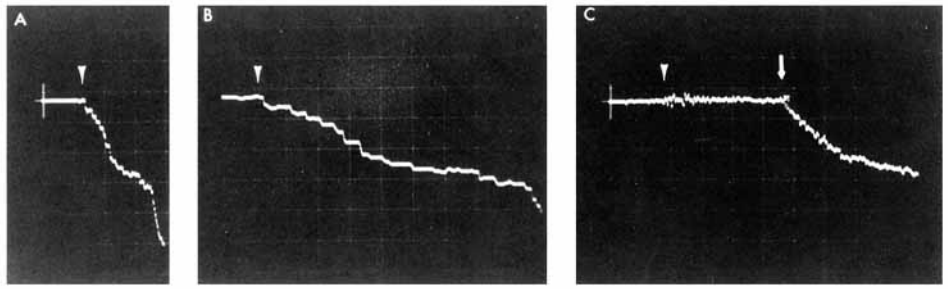

Fig. 9. Patch-clamp of S49.1 cells, in the whole-cell configuration. Cells were clamped at −60 mV during PFP application. PFP (to 0.1 μm/ml, from CTLL All) was added from a second micropipette at a distance of about 100 μm from the cell (arrowheads point to beginning of perfusion). Scales per box: vertical, 200 pA; horizontal, 5 s. B) Horizontal expansion of (A), ×4. C) Cell batched in Ca^{2+}-free medium. Ca^{2+} was subsequently added from a third pipette to a final concentration of 0.5 mM (arrow). Same scale as in A. Downward deflections represent inward currents [73].

and are relatively voltage-resistant to closing (Fig. 10). Bilayers treated with PFP become leaky to glucosamaine (which has a Stokes diameter of 8 Å), $Tris^-$ and $EGTA^{2-}$, implying in a large functional diameter for the assembled pores.

It is also possible to demonstrate that PFP produces functional pores in bilayers without undergoing complete polymerization to form the ultrastructurally visible tubular lesions [70,72,96]. The isolated protein forms functional pores at room temperature (22–24°C), under conditions in which ring-like lesions are not observed. This observation is further substantiated by the finding of a large scatter in channel sizes, indicating that channels of different sizes are associated with multiple polymerization forms of this protein. As noted, the visualization of tubular lesions has been taken as a definitive proof for pore-formation during effector cell killing [12–14]. Our observations imply that this protein may form functional channels and damage effectively target membranes without the need to form these tubular lesions which are visible by electron microscopy.

PFP Is a Secretory Protein

The cytoskeleton [97] and the granules [98] of CTL reorient to become polarized towards the target cell following contact of the two cell types. These observations provide additional support for the contention that killing by lymphocytes may involve a secretory phenomenon, with a vectorial and localized release of PFP into the intercellular zone of contact occuring as a result of selective recognition and binding to the target cell. CTL stimulated with the calcium ionophore A23187 release PFP into the extracellular medium [96], suggesting that this protein is a secretory protein. Release of PFP is accompanied simultaneously by cell degranulation and functional pore-formation. The protein released by mouse CTL assembles into tubular lesions, binds to lipids and has been identified as the lymphocyte PFP using specific polyclonal antibodies as immunoadsorbents [96]. It is possible that during cell killing of target cells, the cytosolic levels of Ca^{2+} may also increase to promote degranulation and secretion. We are currently investigating the release of PFP by NK cells following treatment of cells with immunocomplexes and anti-Fc receptor antibodies. The binding of the NK cell surface Fc receptors may be actively involved in triggering secretion of granule contents by these cells. In this respect, it is interesting that

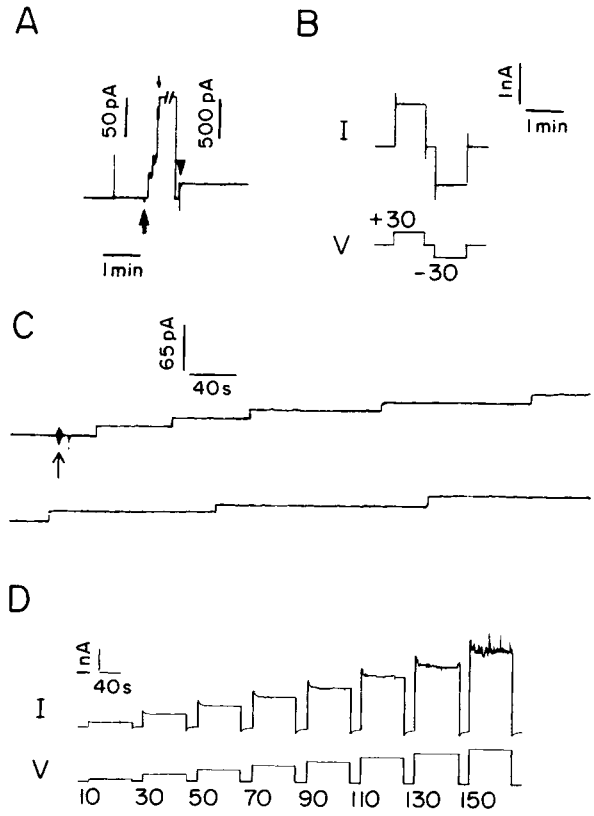

Fig. 10. A) Conductance increase in planar bilayer mediated by CTLL R8 PFP (75 ng/ml). The bilayer (in 0.1 M NaCl, 1 mM $CaCl_2$, 10 mM Hepes, pH 7) was clamped at constant +30 mV. Current went off-scale (arrow) for 10 min (parallel bars), until the amplifier again was turned down ×10 (arrowhead). B) Symmetry of response of PFP-mediated current to voltage steps of opposite polarities. Lower part shows voltage trace (V) and upper panel the current record (I). C) Step increments of current associated with incorporation of 12 ng/ml of PFP. The bilayer was prepared as in (A) and clamped at + 35 mV. D) Steady-state current values obtained with different voltage pulses. Bilayer as in (A). Note the increase of noise in the current record only at voltages higher than 110 mV [72].

binding to the mouse macrophage Fc receptor raises rapidly the cytosolic levels of free calcium to micromolar levels [99].

Immunological Cross-Reactivity Between C9 and Lymphocyte PPF

The lymphocyte PFP shows a number of structural and functional characteristics similar to those displayed by polymerized C9 [100–103]. Polymerized C9 also forms large, voltage-resistant channels with poor ion-selectivity [103]. Moreover, the monomeric C9 also migrates with a M_r of 70–75 kDa (reduced) and 62–66 kDa (nonreduced). The possibility that these two molecules may also share homologous amino acid domains has recently been assessed by using polyclonal antibodies raised against C9 and lymphocyte PFP in immunoblots [96,103]. Our data indicate that these two molecules share immunological cross-reactivity. The homology is limited to a cysteine-rich domain that is only antigenically exposed when the two proteins are chemically reduced.

Involvement of Other Granule Constituents in Cell Killing

Involvement of neutral serine esterases has recently been implicated in cell killing mediated by CTL and NK cells. A protease with a M_r of 28 kDa has been identified in CTL that is expressed at least 300 times higher in CTL than in other lymphocytes, including B cells, noncytotoxic T cells, and clones of helper T cells [39]. Several CTL-specific cDNA copies encoding for putative serine esterases have already been identified and cloned [41–43]. The serine esterase activity is contained in the granule population of these cells [91,92]. We have identified two proteins that are labeled with the specific serine esterase label [^{3}H]DFP, migrating at 34–36 kDa and 28–30 kDa on SDS-PAGE, under reducing conditions. Only the 34–36 kDa protein is found to have functional esterase activity, as measured using chromogenic substrates in an automated microassay system [91,92]. Under nonreducing conditions the 34–36 kDa protein migrates with a M_r of 60–64 kDa. This protein may therefore consist of a homodimer of two 34–36 kDa subunits. The serine esterase activity is released by CTL that have been stimulated with A23187 or target cells, again indicating that like the lymphocyte PFP, the serine esterase is also a secretory protein.

Proteoglycans have also recently been identified in the granules of NK cells [104,105] and CTL (Young and Leong, unpublished observations) and appear to be released by cells during cell killing. These acidic molecules may function as a substratum to which the other granule cationic proteins may bind. The serine esterases may perform some function related to cytolysis, perhaps in the processing of other lytic granule proteins that may be required for their activation. However, the exact role of the serine esterase activity and proteoglycans remain to be elucidated.

During the killing mediated by CTL, there is a reported early onset of fragmentation and release of target cell DNA that parallel the release of cytoplasmic ^{51}Cr label [106–113]. This pattern of response is strikingly different from the pattern observed when the same target cells are lysed by antibody and complement. The early digestion of DNA proceeds with the generation of fragments that are multiples of approximately 200 base pairs, suggesting the involvement of an endonuclease activity that is triggered during cell killing [111,112]. Such kinetic analysis of this process reveals that damage of the target cell nuclear membrane may occur as an early event in CTL-mediated killing. Recent experiments indicate that this type of nuclear damage may involve the participation of lymphotoxin (LT) [113]. LT introduced into nucleated cells produced similar fragmentation of DNA, leading to the suggestion that this protein may be injected through the pore formed by lymphocyte PFP (perforin, cytolysin) [113]. Several observations from this laboratory support an involvement of LT in killing by this proposed mechanism. Polyclonal antibodies directed against recombinant LT and TNF react with CTL in immunofluorescence studies (Young, Liu, and Palladino, unpublished observations). Analogously, anti-TNF antibodies react with the granules of NK cells, suggesting that these mediators may be localized in granules that are then released during the killing process.

CONCLUSION

The killing of tumor cells mediated by CTL and NK cells appears to involve the formation of pores in the target membranes. The cytolytic protein responsible for this pore-formation has been purified and several of its biochemical and functional properties have been studied. This pore-forming protein (PFP/perforin/cytolysin) is thought

to be released into the intercellular space at the zone of contact between the effector and target cells. The released protein is thought to assemble into tubular lesions on the target membrane in the presence of millimolar amounts of calcium found in the extracellular medium. The strategical localization of this pore-forming protein in the effector cell granules provides the cell with an apparatus to produce a directed and localized secretion and membrane injury on the target. It appears that calcium may be required for both secretion of PFP from cells and for its assembly into tubular complexes.

Pore-formation is not restricted to lymphocyte killing and has been associated with the mechanism of action of a number of bacterial toxins and antibiotics [114,115]. Recently, PFP have also been identified in other cell types that mediate cytolysis by a contact-dependent mechanism. The human eosinophil cationic protein, a major protein of eosinophil granules, also shows pore-forming properties that resemble closely those of lymphocyte PFP and poly C9 [94]. Similar PFP have been identified in the distantly related and pathogenic protozoan *Entamoeba histolytica*, which also kill cells by a contact mechanism [66,68,116,117]. Thus pore-formation may represent a common mechanism of killing shared by a variety of different effector cell types. The killing process may differ from cell to cell mainly in the mechanism of target cell recognition. Once triggered, the killing event may develop in a similar fashion. How the effector cell protects itself from self-inflicting injury remains to be elucidated. The intriguing link between PFP of immune cells and those of distantly related killer protozoan parasites further substantiate our view that these effector molecules may play an important role during cell killing.

The immunological cross-reactivity between the mouse lymphocyte PFP and the human C9 suggests the possibility that these proteins may have all evolved from the same ancestral protein, diverging thereafter to become specialized for their effector roles in either humoral or cellular immunity.

Other proteins localized in the granules may also play a vital role in cell killing and may make use of the transmembrane tubule formed by PFP to gain access to the target cell interior. With our ability to purify all these granule constituents to homogeneity, we are now in a position to study their role in cell killing in more detail.

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