

# DISSOCIATION OF MEMBRANE BINDING AND LYTIC ACTIVITIES OF THE LYMPHOCYTE PORE-FORMING PROTEIN (PERFORIN)

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Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells damage target membranes through the membrane assembly of structural and functional pores (reviewed in references 1-4). Granules isolated from these cells contain proteins that are cytolytic and membrane-active, indicating that the lytic apparatus of these effector cells resides in their cytoplasmic granules (5-13). Recently, a pore-forming protein (PFP,<sup>1</sup> also named perforin or cytolyisin) that mediates this type of membrane damage has been isolated from lymphocyte granules and partially characterized (13-16).

Since intact granules isolated from effector cells are cytolytic, it is not clear how the lytic polypeptides are transferred from granules to target membranes. Moreover, no information is available on the intermediate steps involved in the assembly of the pore by soluble PFP/perforin that has been secreted by effector cells or released from the granules. To understand better the assembly mechanism, we describe here some of the intermediate steps involved in this cytolytic event. We also report on the role of certain modulatory factors such as pH, Ca<sup>2+</sup>, serum (and serum proteins), and proteoglycans on the lytic activity of granules and of soluble PFP/perforin. During cell killing, these factors may play a regulatory function on the membranolytic activity produced by CTL and NK cells.

## Materials and Methods

*CTLL, Granule Isolation and Purification of PFP.* The cytotoxic T lymphocyte cell line (CTLL) R8 was maintained in IL-2-containing medium (16). Granules were isolated by centrifugation of nucleus-free cell lysate through a Percoll gradient, as described (12), with the exception that a discontinuous Percoll gradient of 14 ml of 1.08 g/ml, 7 ml of 1.05 g/ml, and 7 ml of 1.03 g/ml was used. 10 ml of cell lysate (2-3 × 10<sup>7</sup> cell equivalents/ml) were layered on top and the tube was centrifuged at 39,000 *g* for 18.5 min in a Sorvall SS34 rotor. Fractions of 1 ml were collected and numbered from the

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<sup>1</sup> *Abbreviations used in this paper:* BLT, *N*, $\alpha$ -benzyloxycarbonyl-L-lysine-thiobenzyl ester; CTLL, cytotoxic T lymphocyte cell line; DMB, dimethylene blue; HDL, high-density lipoprotein; H<sub>50</sub>, 50% hemolysis; HU, hemolytic units; LDL, low-density lipoprotein; PFP, pore-forming protein.

top. Membrane-free granule extracts were obtained by incubation of granule fractions with 0.5 M NaKHPO<sub>4</sub>, pH 7.4, 10 mM benzamidine on ice (15), and purified PFP/perforin was obtained as described previously (13, 15, 16). Intact granules were obtained by high-speed centrifugation of Percoll fractions (180,000 g for 2 h), which resulted in the sedimentation of granules as a fluffy layer on top of the firm Percoll pellet. This material was gently aspirated into relaxation buffer (16) and immediately used afterwards as a source of intact granules.

*Microassay System.* The hemolytic activity of the fractions was assessed by a turbidimetric microassay as described (17). Briefly, 5–20  $\mu$ l of hemolytically active samples were incubated with 200  $\mu$ l of PBS, pH 7.4, containing 1 mM CaCl<sub>2</sub> and SRBC ( $5 \times 10^7$ /ml). After a 30 min incubation at 37°C, the microtiter plates were read at A<sub>700</sub>. One hemolytic unit (HU) was defined as the amount of material required to achieve 50% hemolysis (H<sub>50</sub>) of  $5 \times 10^7$  SRBC/ml in the presence of 1 mM CaCl<sub>2</sub> and pH 7.4. When buffers of other compositions were used as specified in the text, the SRBC suspension was always washed three times in the final buffer immediately before use. In all experiments, the granules and granule extracts were diluted at least 100-fold with the various buffers described in the text.

To dissociate the membrane binding and the lytic activities of granule extracts and purified PFP, SRBC were incubated with active samples on ice for 20 min in Eppendorf tubes. The cell suspension was then washed three times with ice-cold Ca<sup>2+</sup>-free PBS by centrifugation at 4°C to remove unbound proteins. The final cell pellet was resuspended to original volume with PBS containing 1 mM CaCl<sub>2</sub> and aliquots of 200  $\mu$ l of cell suspension were added to microtiter wells on ice. The A<sub>700</sub>, determined at this point as a control, showed virtually no hemolysis. The remaining wells were incubated for additional 30 min at 37°C before final A<sub>700</sub> reading of the wells. Inhibitors (5–20  $\mu$ l) were added to the hemolytically active samples and allowed to incubate for 20 min on ice before addition of SRBC. To study the effect of inhibitors directly on the pore formation step, the inhibitors were added to SRBC suspensions that have been exposed to membrane-active proteins and have been washed free of unbound proteins; incubation with inhibitors at this step was done on ice for 20 min before cells were warmed up to 37°C and A<sub>700</sub> was determined. The serum used in the text refers to heat-inactivated pooled human serum (Bio-Bee, Boston, MA), which had a protein concentration of 9.9 mg/ml. High-density lipoprotein (HDL) subpopulation 3 from normal human plasma was purified essentially as described (18). Human plasma low-density lipoproteins (LDL) were generously provided by Dr. R. Montgomery (19). Both HDL<sub>s</sub> and LDL were shown to be electrophoretically pure before use. Chondroitin sulfate used consisted of mixed isomers (grade III) and types A, B, and C (Sigma Chemical Co., St. Louis, MO). Heparin (grade II) was also obtained from Sigma Chemical Co.

The pH dependence of the hemolytic activities (Fig. 2) was performed using 25 mM Hepes buffer containing 0.14 M NaCl and 5 mM CaCl<sub>2</sub>, adjusted to the final pH given in the text with Tris-base. To determine the effect of pH on membrane binding and lytic activities (Fig. 4), SRBC were allowed to bind to granule extracts on ice for 20 min in four different combinations of Hepes-Tris buffer, containing either 10 mM CaCl<sub>2</sub> or 0 CaCl<sub>2</sub> (supplemented with 1 mM EGTA), with the final pH adjusted to 6.0 or 7.4, as indicated in the text. After incubation, cells were washed three times in ice-cold buffer containing the following recipes: (a) for cells initially incubated on ice with Hepes-Tris buffer, without CaCl<sub>2</sub> (and 1 mM EGTA), pH 6, the washing buffer consisted of Hepes-Tris buffer, 10 mM CaCl<sub>2</sub>, adjusted to pH 6 or 7.4 (as noted in the text); (b) for initial incubation with Hepes-Tris buffer, 10 mM CaCl<sub>2</sub>, pH 6, cells were washed with Hepes-Tris buffer, no CaCl<sub>2</sub>, pH 6 or 7.4; (c) for initial incubation with Hepes-Tris buffer, no CaCl<sub>2</sub>, pH 7.4, cells were washed with Hepes-Tris buffer, 10 mM CaCl<sub>2</sub>, pH 6 or 7.4; and (d) for initial incubation with Hepes-Tris buffer, 10 mM CaCl<sub>2</sub>, pH 7.4, cells were washed with Hepes-Tris buffer, 10 mM CaCl<sub>2</sub>, pH 6 or 7.4. After the last wash, cells were resuspended to original volume with the same washing buffers and incubated at 37°C for 30 min, and the wells were then read at A<sub>700</sub>.

The Ca<sup>2+</sup> dependence of the hemolytic activities (Fig. 3) was performed using the

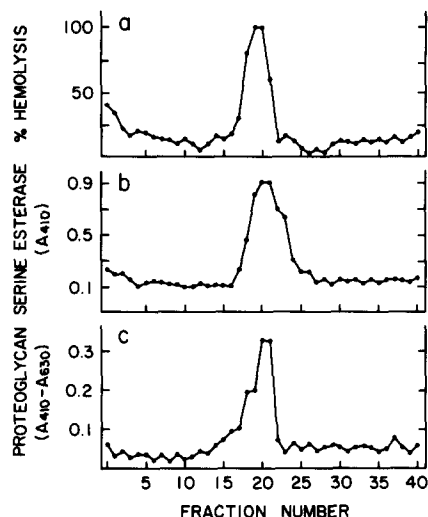


FIGURE 1. Profile of subcellular markers of CTLL-R8 cell lysate separated by a discontinuous Percoll gradient. Hemolysis (a), serine esterase (b) and proteoglycan (c) microassays were performed as described in Materials and Methods, using 20  $\mu$ l of cell sample per well. Data points represent average of duplicates. (c) An absorbance of 0.2 ( $A_{410}$  to  $A_{650}$ ) corresponds to 7.5  $\mu$ g of chondroitin sulfate and 6  $\mu$ g of heparin. (b) The background  $A_{410}$  observed was due to Percoll and was not subtracted.

following buffer: 10 mM Hepes, 0.15 M NaCl, 0.8 mM  $MgCl_2$ ; 0.5 mM EGTA, with pH adjusted to pH 7.4 or 6.8 with Tris-base as indicated in the text. The various concentrations of free  $Ca^{2+}$  were adjusted with  $CaCl_2$  following calculations obtained with a computer program kindly provided by Dr. A. Aderem of The Rockefeller University. The program made use of constants and relationships published elsewhere (20).

Microassays for protein content and enzymatic activities (serine esterase,  $\beta$ -glucuronidase, acid phosphatase, and succinate dehydrogenase) were performed essentially as described (21). Granules of CTL and lymphocytes with high NK-like activity have recently been shown to be markedly enriched for a serine esterase activity (21, 22), and a microassay for this marker has since been used as a convenient granule marker (21). Briefly, the reaction mixture for serine esterase assay consisted of 10  $\mu$ l of enzyme sample and 200  $\mu$ l of substrate ( $2 \times 10^{-4}$  M *N*, $\alpha$ -benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT; Calbiochem-Behring Corp., La Jolla, CA),  $2.2 \times 10^{-4}$  M nitrobenzoic acid, 0.1 M Tris-HCl, pH 8). After incubation for 30 min at 25°C, the  $A_{410}$  of the wells was determined.

An automated microassay for proteoglycans was modified from a published procedure (23). The assay was based on quantitative binding of the dye dimethylene blue (DMB) (Polysciences, Inc., Warrington, PA) by proteoglycans. 20  $\mu$ l of sample were mixed with 200  $\mu$ l of the dye solution (0.25% by weight of DMB, 3% formic acid) and the product was immediately read as the difference between  $A_{410}$  and  $A_{650}$ . The differential absorbance reading allows the partial elimination of the background due to the presence of Percoll. Heparin and chondroitin sulfate (grade III, mixed isomers; Sigma Chemical Co.) were used as standards.

## Results

*Granule Isolation: Presence of Serine Esterase and Proteoglycans in Granules of CTLL.* Granules from CTLL-R8 cells were enriched by subcellular fractionation, as assessed by hemolytic and serine esterase activities (Fig. 1). Granules of CTLL-R8 cells also contained proteoglycans (Fig. 1), a finding consistent with recent reports (24–26) that have described the presence of proteoglycans in the granules of NK cells. In addition to the granule markers shown in Fig. 1, granule fractions were also enriched for  $\beta$ -glucuronidase and acid phosphatase activities (data not shown). Granules could be separated from mitochondrial, lysosomal, and plasma membrane fractions based on the differential distribution of succinate dehydrogenase,  $\beta$ -glucuronidase, acid phosphatase, 5'-nucleotidase, alkaline

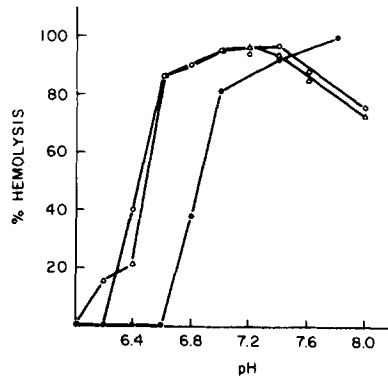


FIGURE 2. pH dependence of hemolysis by intact granules (●), granule extracts (○), and purified PFP (Δ) obtained from the Mono Q column. Active material was obtained as described in Materials and Methods. 2 HU of sample/20  $\mu$ l were used per well in triplicates.

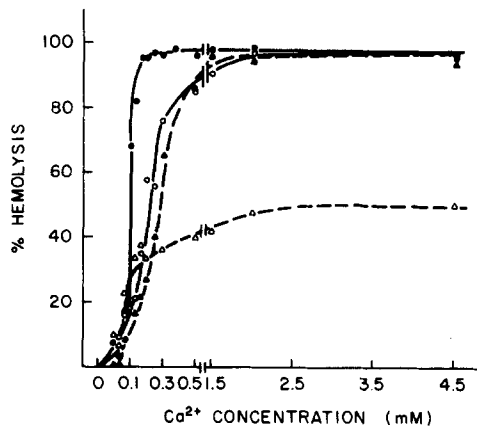


FIGURE 3. Ca<sup>2+</sup> dependence of hemolytic activities of granule extracts (○, ●) and intact granules (Δ, ▲). (●, ▲) pH 7.4; (○, Δ) pH 6.8. 2 HU of active sample were used per well in triplicates.

phosphodiesterase (reference 21; enzyme profiles not shown here). The enriched granule fractions were then used for functional assays as described next.

*Membranolytic Activities of Granules and PFP/Perforin Show Different Requirements for Ca<sup>2+</sup> and pH.* It has previously been shown (13) that intact granules, isolated from NK-like cells, lyse targets without the need to undergo granule rupture or release of granule contents into the supernatant before their contact with cells. To study this property in further detail, we performed experiments using granules in their intact form that have been isolated by subcellular fractionation and have been washed free of lysed granule material by high-speed centrifugation (referred to as intact granules). The hemolytic activity of intact granules was then compared with that of granule proteins that have been solubilized from the granule core by using high salts (referred to as solubilized granule proteins). As shown in Figs. 2 and 3, the hemolytic activities of intact granules and solubilized granule proteins showed different dependence curves for Ca<sup>2+</sup> and pH. Intact granules were only hemolytically active at pH >6.6, in contrast to solubilized granule proteins that were hemolytically active in a broader range of pH (activity observed in the pH range 6–8) (Fig. 2). The pH optimum for both these hemolytic activities, however, was close to neutral pH (7.0–7.4) (Fig. 2). As expected, the purified PFP obtained from the Mono Q column produced a pH dependence profile similar to that of solubilized granule proteins

(Fig. 2). Soluble granule proteins also required lower amounts of free  $\text{Ca}^{2+}$  to produce the same amount of hemolysis as compared with that required by intact granules present in equivalent hemolytic units (Fig. 3). For 2 HU of solubilized granule proteins,  $H_{50}$  was achieved at  $100 \mu\text{M}$  of free  $\text{Ca}^{2+}$ , whereas intact granules required  $300 \mu\text{M}$  to produce  $H_{50}$  under similar experimental conditions (Fig. 3). These observations suggest that intact granules may require an additional  $\text{Ca}^{2+}$  and pH-dependent step to effect cell lysis, an event that can be distinguished from the  $\text{Ca}^{2+}$  and pH-dependent lysis produced by granule proteins. The results presented here also demonstrate that lowering the pH blocks the lytic activity of the granules.

The inhibitory effect on the hemolytic activities associated with lowering the pH of the medium could be attributed in part by interference of low pH with the  $\text{Ca}^{2+}$  requirement. This inference was supported by the following observations. The hemolytic activity of soluble granule proteins was examined as a function of the  $\text{Ca}^{2+}$  concentration at the two fixed pH of 7.4 and 6.8 (Fig. 3). Lowering pH from 7.4 to 6.8 shifted the hemolytic curve to the right (Fig. 3), indicating that higher concentrations of free  $\text{Ca}^{2+}$  were required at pH 6.8 to attain the same levels of hemolysis attained at pH 7.4. A dichotomy of effects was again observed depending whether intact granules or solubilized granule proteins were used as the active samples. With intact granules, the inhibitory effect of lowering the pH was only partially reversed by an increase in the concentration of  $\text{Ca}^{2+}$ ; thus, even increasing  $\text{Ca}^{2+}$  to 4.5 mM, the total hemolytic activity observed at pH 6.8 was lower than that obtained at pH 7.4 (Fig. 3). At pH 6, the hemolytic activities associated with both intact granules and soluble granule proteins were completely abolished and could not be activated even with 10 mM  $\text{CaCl}_2$ . This inhibitory effect was reversible since raising the pH again from 6 to 7.4 promptly restored the hemolytic activity. Similar results were obtained with purified PFP.

*Dissociation of Membrane Binding and Lytic Activities of PFP/Perforin.* We next studied the possibility that PFP may need to bind to target membranes prior to its insertion into the bilayer to form a functional pore. To distinguish membrane binding from pore insertion, we investigated conditions in which PFP binds to target membranes without necessarily inflicting membrane damage. SRBC were incubated with solubilized granule proteins and purified PFP/perforin on ice (Fig. 4). Hemolysis was not observed under these conditions. However, the same treated SRBC, washed three times with ice-cold buffer to remove any residual unbound PFP in the medium, completely lysed upon subsequent incubation at  $37^\circ\text{C}$  (Fig. 4). These results indicate that PFP must have bound to erythrocyte membranes on ice but were incapable of either membrane insertion or pore formation. Hemolysis also occurred when SRBC bound with PFP on ice were subsequently incubated at room temperature ( $20\text{--}22^\circ\text{C}$ ), rather than  $37^\circ\text{C}$ . However, the kinetics of lysis observed at room temperature was 3–5 fold slower than that observed at  $37^\circ\text{C}$ . Thus, binding of PFP to lipid membranes is temperature-insensitive and appears to precede the temperature-dependent, membrane insertion/pore-formation stage that leads to cell lysis.

We next studied separately the  $\text{Ca}^{2+}$  and the pH dependence of the membrane-binding and pore-insertion steps (Fig. 4). Membrane binding by PFP occurred

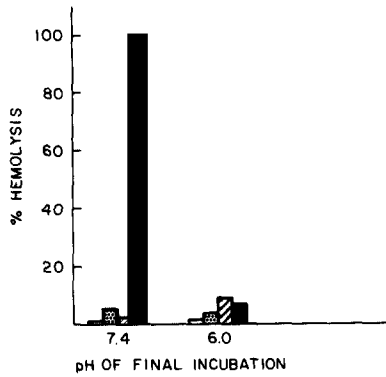


FIGURE 4. Dissociation of membrane binding from pore-insertion activities associated with PFP in granule extracts. The conditions for the experiment are outlined in Materials and Methods. Initial incubation refers to exposure of SRBC to samples on ice for 20 min under the conditions indicated in the graph, followed by washing and resuspension of cell pellet in the indicated buffer (*final incubation*), and subsequent incubation at 37°C for 30 min before determination of  $A_{700}$ . Experiments were performed in triplicates, with 10 HU of granule extracts applied to each well. (□) Initial incubation: on ice, 0 Ca<sup>2+</sup>, pH 6.0; final incubation: 37°C, 10 mM Ca<sup>2+</sup>, pH 6.0 or 7.4. (▤) Initial incubation: on ice, 10 mM Ca<sup>2+</sup>, pH 6.0; final incubation: 37°C, 0 Ca<sup>2+</sup>, pH 6.0 or 7.4. (▥) Initial incubation: on ice, 0 Ca<sup>2+</sup>, pH 7.4; final incubation: 37°C, 10 mM Ca<sup>2+</sup>, pH 6.0 or 7.4. (▧) Initial incubation: on ice, 10 mM Ca<sup>2+</sup>, pH 7.4; final incubation: 37°C, 10 mM Ca<sup>2+</sup>, pH 6.0 or 7.4.

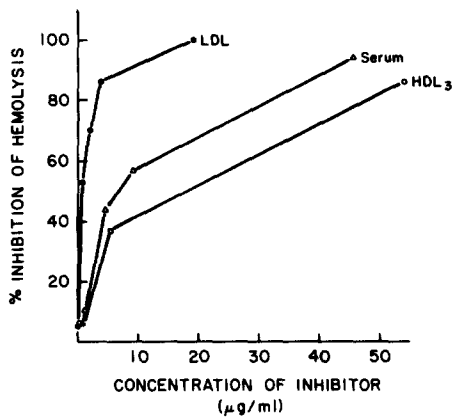


FIGURE 5. Inhibition of hemolytic activity of granule extracts by serum, LDL, and HDL<sub>3</sub>. The concentration of protein in the undiluted serum used was 9.9 mg/ml. 60 HU of granule extract were applied in triplicates to each well under the conditions outlined in Materials and Methods. The concentration of inhibitor corresponds to the final concentration of each inhibitor after addition of the SRBC volume. Control incubation with buffer did not produce any inhibitor (not shown).

only at neutral pH (pH >6.6) and in the presence of submillimolar amounts of Ca<sup>2+</sup> (free Ca<sup>2+</sup> >50 μM). PFP did not bind to membranes at pH 7.4 in the absence of Ca<sup>2+</sup> (with 1 mM EGTA) nor at pH 6 in the presence of 10 mM Ca<sup>2+</sup> (Fig. 4). Alternatively, SRBC that effectively bound PFP on ice (at pH 7.4 in the presence of 10 mM Ca<sup>2+</sup>) did not lyse upon subsequent incubation at 37°C if Ca<sup>2+</sup> was deleted or if the pH was lowered to pH 6 during the subsequent temperature-sensitive stage (Fig. 4). These results indicate that both a neutral pH environment and the presence of submillimolar amounts of Ca<sup>2+</sup> are required for both membrane binding and pore insertion by PFP.

*Selective Inhibition of Membrane Binding Activity of PFP.* Given our ability to distinguish between the stages of membrane binding and pore insertion associated with PFP, we next investigated the mechanisms of action of several inhibitors of PFP. Serum and serum lipoproteins have recently been shown to inhibit the lytic activity of PFP isolated from a mouse CTLL (27). In accord with these results, <1% human serum inhibited completely the hemolytic activity of 112 HU of CTLL-R8 PFP (Fig. 5). Human LDL and HDL<sub>3</sub> at microgram levels also produced a remarkable dose-dependent inhibition of the hemolytic activity (Fig.

TABLE I  
*Effects of Serum, HDL<sub>3</sub>, LDL, and Heparin on the Binding and the Pore Insertion Stages during Hemolysis by Granule PFP\**

Effect on:	Percent hemolysis				
	Control	Serum	HDL	LDL	Heparin
Membrane binding*	100	6	7	0	5
Pore insertion <sup>‡</sup>	100	95	91	93	98

\* 5 HU of purified PFP in 10  $\mu$ l were incubated with 10  $\mu$ l of serum, HDL or LDL (at final 50  $\mu$ g/ml each), or heparin (1 mg/ml) in triplicate wells for 20 min on ice. SRBC (200  $\mu$ l) were added to each well and an additional incubation of 40 min at 37°C was made before reading of the wells at A<sub>700</sub>.

<sup>‡</sup> 5 HU of purified PFP in 10  $\mu$ l were added to 200  $\mu$ l of SRBC suspension in PBS containing 1 mM CaCl<sub>2</sub>. The mixture was incubated on ice for 20 min. Cells were then washed in ice-cold buffer three times and resuspended to original volumes. 10  $\mu$ l of inhibitor, from stocks of 0.4 mg/ml, or 10  $\mu$ l of control PBS were added to each well. The microtiter plate was incubated with agitation first at 4°C for 20 min, followed by incubation at 37°C for 30 min. Data points represent average of triplicates. Similar results were obtained with total solubilized granule proteins.

5). In our hands, LDL was much more effective than HDL<sub>3</sub> in producing the same level of inhibition (Fig. 5). The inhibitory effect on PFP mediated by serum, LDL, and HDL<sub>3</sub> occurred only at the stage of membrane binding, as evidenced by the following observations: SRBC treated with PFP on ice in the presence of one of these inhibitors did not lyse upon subsequent incubation at 37°C even under optimal conditions of Ca<sup>2+</sup> and pH (Table I). However, SRBC that had bound PFP on ice in the absence of any inhibitor lysed effectively upon subsequent incubation at 37°C even when serum, HDL<sub>3</sub>, and LDL were present during this last incubation (Table I). These results indicate that the serum lipoproteins inhibit only the binding of PFP to membranes, probably by competing with lipid bilayers for lipid-binding domain(s) of PFP. Our results also suggest that the lipophilic domain(s) of PFP are only accessible when PFP is free in solution but that these domains become masked after the attachment of PFP to lipid bilayers. Further experiments are necessary to show whether serum lipoproteins may account entirely for the inhibitory effect of whole serum on PFP. In marked contrast to the action of these lipoproteins, albumin, for example, had no inhibitory effect on PFP (data not shown).

As noted, granules of CTLL-R8 cells contain proteoglycans. It is possible that these highly acidic molecules bind to PFP in the granules to form a stable and inactive complex. We tested the effects of heparin and chondroitin sulfate (mixed isomers and types A, B, and C) on the hemolytic activity mediated by PFP (Fig. 6). Heparin alone produced partial inhibitory effect only at 0.5 mg/ml, whereas chondroitin sulfate was totally ineffective on PFP (Fig. 6). Like the serum lipoproteins HDL and LDL, heparin at these concentrations only interfered with membrane binding by PFP (Table I). Addition of heparin to SRBC that have already bound PFP on ice did not inhibit lysis of these cells upon subsequent incubation at 37°C (Table I).

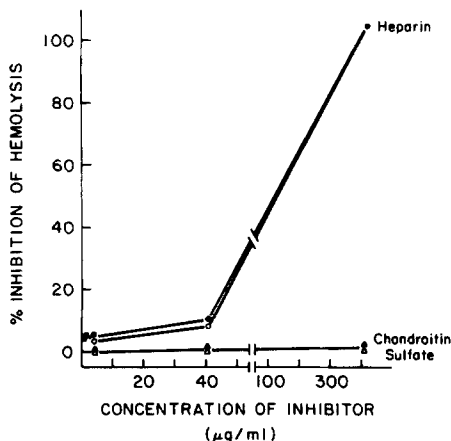


FIGURE 6. Inhibition of hemolytic activity of granule extracts and purified PFP by proteoglycans. 60 HU of solubilized granule proteins (O,  $\Delta$ ) or purified PFP ( $\bullet$ ,  $\blacktriangle$ ) were applied in triplicates per well and were incubated with heparin or chondroitin sulfate (mixed isomers) as described in Materials and Methods. The concentration of inhibitor refers to the final concentration, after addition of SRBC. Results obtained with chondroitin sulfate A, B, or C were similar to that obtained with the mixed isomers.

### Discussion

To understand better the mechanism by which the lymphocyte PFP/perforin monomers assemble into transmembrane lesions, we have attempted here to define the intermediate steps involved in this type of lytic assembly. At least two intermediate events involved in the lytic assembly of membrane lesions can be resolved by these experiments that have not been described previously. We have shown that PFP binds to erythrocyte membranes under conditions (on ice) that do not lead to membrane damage. The lytic step produced by membrane-bound PFP occurs only at higher temperatures.

Both membrane binding and pore formation mediated by PFP are dependent on the presence of submillimolar amounts of  $\text{Ca}^{2+}$  and neutral pH. A modulatory effect of pH on the lytic activity of CTL granules was also recently reported by Criado et al. (11). The strict requirements for neutral pH and millimolar amounts of  $\text{Ca}^{2+}$  in the expression of membranolytic activity would ensure that PFP is packaged in the granules of killer cells in an inactive form, since these compartments are thought to be acidic in nature and low in free  $\text{Ca}^{2+}$ . It is also noteworthy to point out that the effect of pH on PFP is totally reversible between pH 6 and 8, precluding the possibility that PFP that has been stored in an acidic microenvironment (granules) would be completely inactivated. The role of pH in the assembly of membrane lesions by PFP is not clear at this time.  $\text{H}^+$  appears to interfere first with  $\text{Ca}^{2+}$  binding by PFP and at high levels directly but reversibly inactivates PFP.

The membrane-binding and pore-insertion steps are differentially susceptible to various inhibitors. We have found that serum and the serum components HDL and LDL inhibit membrane binding by PFP in a dose-dependent manner without interfering with pore insertion by membrane-bound PFP. Presumably the serum lipoproteins compete for lipid-binding domain(s) of PFP, rendering it inactive to bind to lipid bilayers. This mechanism may play an important protective function during cell-mediated killing, whereby the extracellular serum could avoid the accidental injury of innocent bystander cells mediated by PFP that has been released from lymphocytes. During cell killing, the close apposition of effector/target cell membranes may favor the binding of the released PFP to the



target membrane. However, any unbound PFP would be rapidly inactivated by serum, preventing its further cytotoxic use. The lack of effect of serum and serum HDL and LDL on membrane-bound PFP suggests that once PFP has bound to bilayers, lysis would proceed to completion in the presence of a calcium and neutral pH environment even when the killer cells are no longer in close attachment with the targets. This possibility would be consistent with previous reports (28, 29) describing a self-programmed,  $\text{Ca}^{2+}$ -dependent, but killer cell-independent stage in lymphocyte-mediated killing which occurs after contact and dissolution of the lymphocyte from the target.

Recently, proteoglycans of the chondroitin sulfate A type have been identified in the granules of NK cells and shown to be released from cells during cytotoxicity (25, 26). As shown here, proteoglycans may also be used as a granule marker for CTL. Proteoglycans of CTL-R8 cell granules can also be visualized on polyacrylamide gels stained with Alcian Blue, which reveal several bands of different molecular masses (profile not shown here). Because proteoglycans have previously been speculated to exert a protective function during cell-mediated killing, tentatively through their binding to PFP/perforin released from cells (25, 26), we tested the effects of heparin and chondroitin sulfate on the hemolytic activity mediated by PFP. Only heparin inhibited hemolysis at high concentrations (0.5 mg/ml), whereas chondroitin sulfate (and the three subtypes A, B, and C) produced no effect in the wide concentration range tested in our experiments. Heparin at these concentrations also blocked only membrane binding by PFP. The lack of effect of chondroitin sulfate and the relatively high concentrations of heparin required to block the cytolytic function of PFP do not seem to favor the possibility of a protective function for proteoglycans during cell killing, but rather suggest that more likely these complex macromolecules play a different role.

Intact granules isolated from CTL and NK-like lymphocytes have previously been shown (13) to be hemolytically active. The rupture of granule membranes and the release of soluble granule contents into the supernatant are not required for the expression of the hemolytic activity. Moreover, the hemolytic activities of intact granules and solubilized PFP have different requirements for  $\text{Ca}^{2+}$  and pH, suggesting that intact granules may mediate an additional event to produce cytotoxicity that is also  $\text{Ca}^{2+}$ - and pH-dependent. Thus, isolated granules may have to fuse first with erythrocyte membranes before releasing their contents into the target cell interior. In close analogy, granules may also have to fuse with lymphocyte plasma membranes during cell-mediated killing to release granule contents into the extracellular medium.  $\text{Ca}^{2+}$  may be required for this form of membrane fusion. Previous studies (30) have already shown that stimulation of lymphocytes with A23187, a calcium ionophore, triggers cells to secrete PFP. It would be interesting to determine whether the interaction of lymphocytes with targets also leads to an abrupt rise of the cytoplasmic levels of free calcium that initiates granule fusion and degranulation.

After conjugation of CTL and NK cells with lysable targets, it is proposed that at least three distinct  $\text{Ca}^{2+}$ - and pH-dependent events may be required to produce cytotoxicity: (a) fusion of cytoplasmic granules with the plasma membrane of lymphocytes, resulting in secretion of PFP into the immediate area of cell-to-cell

contact; (b) binding of the released PFP monomers to the target cell membrane; and (c) assembly of the membrane-bound monomers into functional transmembrane pores. It should be noted that at this time we cannot distinguish between the stages of membrane insertion and pore formation mediated by membrane-bound PFP. If each monomer molecule forms a functional pore, then membrane insertion would also be synonymous with pore formation. On the other hand, if monomers need to self aggregate and polymerize in the plane of the bilayer before functional pore formation, then membrane insertion would represent another intermediate step required for the final assembly of the multimeric pore. Experiments designed to address this issue are currently in progress in our laboratory.

### Summary

Granules isolated from CTL and NK cells contain a cytolytic pore-forming protein (PFP/perforin). At low temperatures (on ice), PFP binds to erythrocyte membranes without producing hemolysis. Hemolysis occurs when the PFP-bound erythrocytes are warmed up to 37°C, which defines a temperature-dependent, lytic (pore-formation) step distinct from the membrane-binding event. Ca<sup>2+</sup> and neutral pH are required for both membrane binding and pore formation by PFP. Serum, LDL, HDL, and heparin inhibit the hemolytic activity of PFP by blocking its binding to lipid membranes. Lysis by PFP that has bound to erythrocyte membranes is no longer susceptible to the effect of these inhibitors. The hemolytic activities associated with intact granules and solubilized PFP show different requirements for Ca<sup>2+</sup> and pH, indicating that cytolysis produced by isolated granules may involve an additional step, possibly fusion of granules with membranes. It is suggested that three distinct Ca<sup>2+</sup>- and pH-dependent events may be involved during cell killing by CTL and NK cells: (a) fusion of cytoplasmic granules of effector cells with their plasma membrane, releasing PFP from cells; (b) binding of the released PFP to target membranes; and (c) insertion of monomers and the subsequent formation of lytic pores in the target membrane. The serum-mediated inhibition of membrane binding by PFP could prevent the accidental injury of bystander cells by cell-released PFP, but would allow cytolysis to proceed to completion once PFP has bound to the target membrane.

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