BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 44–49 (1996)
ARTICLE NO. 1755

Human Perforin: Rapid Enrichment by Immobilized Metal Affinity Chromatography (IMAC) for Whole Cell Cytotoxicity Assays

Christopher J. Froelich,[1] Jane Turbov, and William Hanna

Department of Medicine, Evanston Hospital, Northwestern University, Evanston, Illinois 60201

Received October 30, 1996

Perforin, a potent pore-forming protein, plays an important role in lymphocyte-mediated cytolysis causing necrosis or, when combined with the granzymes, apoptosis. The studies on perforin, although already extensive, have been hampered by the limited amount of material available from killer lymphocytes. Using a cell line that expresses high levels of human perforin, we describe a straightforward purification scheme that allows isolation of the lytic protein in approximately 8 hours. Perforin is enriched from the YT-INDY cell line by cavitation followed by differential centrifugation and ion metal affinity chromatography. In addition to demonstrating the lytic activity of human perforin toward various cell lines, we show that the conditions of the cytotoxicity assay influence both the kinetics and magnitude of perforin-mediated cytotoxicity.

Cytotoxic lymphocytes mediate cell death via two pathways: Fas and granule-mediated (perforin/granzymes). Fas controls normal cell renewal by inducing programmed cell death of actively proliferating cells. In contrast, the biological function of perforin and associated granule proteins is to maintain immune surveillance against both exogenous and endogenous ''non-self'' cells (e.g. viral infection, tumorigenesis). Although perforin was first isolated more than 10 years ago, the description of the molecular mechanism that underlies the synergy of this pore forming protein and the granzymes in apoptosis remains conjectural. Building on the work of Winkler and Hudig (1), we describe here a procedure in which perforin is highly enriched from a human cell line by nitrogen cavitation followed by differential centrifugation and ion metal affinity chromatography. Evidence is presented which indicates that the performance of perforin is influenced by the conditions under which the targets cells are exposed to the lytic protein.

MATERIAL AND METHODS

Reagents. RPMI-1640 and 2 mM L-glutamine were from Gibco Labs (Grand Island, NY) and bovine serum (FBS) was purchased from Hyclone (Ogden, UT). HEPES and fatty acid free BSA were purchased from Calbiochem, Inc. (San Diego, CA). Radioisotope $^{51}NaCrO_4$ was from Amersham, Inc. (Arlington Hts, IL). Anti-perforin was kindly provided by Dr. M. Nagata, Sumitomo Electric Industries, LTD, Osaka, Japan. Unless indicated all reagents were purchased from Sigma Chemical, St. Louis, Mo.

Cell lines. YT-INDY (provided by Z. Brahmi, Indiana University), K562, YAC-1 and U937 used in this study were maintained in continuous passage using standard tissue culture technique (5% CO_2, 37°C). Complete medium (CM) used for cell culture consisted of RPMI-1640/10% heat inactivated FCS supplemented with 0.1 mM MEM non-essential amino acids solution, 2 mM L-glutamine, 100 U/ml penicillin, and 50 ug/ml streptomycin. YT-INDY cells were seeded into a 2.0 L Fenwall flasks at 1×10^5/ml (total volume-1.5L). In approximately one week the cell density increases to 1×10^6/ml/flask (total-15 $\times 10^8$) which is suitable for a cavitation.

Perforin purification. Human perforin was extracted in 1M NaCl from YT granules and purified by ion metal

[1] To whom correspondence should be addressed at Evanston Hospital, Research Department, WH Building, Rm. B624, 2650 Ridge Ave., Evanston, IL 60201. Fax: 847.570.1253. E-mail: granzyme@merle.acns.nwu.edu.

0006-291X/96 $18.00

affinity chromatography by following protocol. Approximately 5×10^9 YT cells were collected, washed twice in Hank's buffered salt solution (HBSS) with 1% bovine serum albumin (BSA), and resuspended at 1×10^8 cells/ml in ice cold relaxation buffer (10 mM PIPES buffer, 0.1 M KCl, 3.5 mM $MgCl_2$, 1 mM ATP, 1.25 mM EGTA, pH 6.8, adjusted to isotonicity [285 mOsm/l] with 4 M NaCl and containing 0.05 % BSA). The cells were disrupted by gradual decompression following pressurization in a nitrogen cavitation bomb (Parr Instrument Co., Moline IL, Model 4639) at 250 p.s.i. at 0°C for 9 minutes.

High density granules were prepared for salt extraction from the disrupted cells by spinning at 400 × g for 7 minutes to remove nuclei and unbroken cells. The postnuclear supernatant was then spun at 14,500 × g for 15 minutes (Sorvall Superspeed centrifuge, SS-34 rotor) to yield the granule pellet and the cytosolic supernatant. The granule pellet was then extracted by mixing with 1M NaCl in 20 mM Na Acetate, pH 4.5 (10 ml) containing 2 mM EDTA for 60 min at 4°C (on ice). The extract was spun at 8,500 × g for 10 minutes. The supernatant was filtered through a 0.8 μm filter apparatus, applied to PD-10 columns containing P-6 matrix (BioRad, Inc.) and eluted with 1M NaCl, 20 mM HEPES, 10% betaine. The granule extract now exchanged into HEPES buffer was immediately injected onto a HR 5/5 column (Pharmacia, Inc.) loaded with Poros 20 MC metal chelate affinity media (PerSeptive Biosystems, Cambridge, MA) to perform ion metal affinity chromatography (IMAC). Prior to use, the column is charged with the following 20 mM sodium Acetate, 0.1M CoCl (pH 4.5). After eluting unbound protein with buffer A (1M NaCl, 20 mM HEPES, 10% betaine (pH 7.5), the column was subjected to a linear 10 min imidazole gradient (0-0.2 M) by introduction of buffer B (buffer A plus 0.2 M imidazole). Eluted fractions were continuously monitored at 280 nm using a Macintosh computer compatible integrator (MacIntegrator, Rainin, Inc., Emeryville, CA) connected to a Varian UV spectrophotometer. All gradient fractions were individually assayed for hemolytic activity (2). Protein concentrations were determined by the Protein Gold assay (ISS, Natick, MA) using BSA as the standard. The fractions with the highest hemolytic activity, usually two, were concentrated tenfold by Centricon ultrafiltration in the presence of EDTA and fatty acid free BSA to yield final concentrations of 2 mM and 100 μg/ml respectively.

SDS-polyacrylamide gel electrophoresis. Proteins were separated by SDS-PAGE in a 10% acrylamide slab gel with a 4% gel stacker as described by Laemmli (3) and then underwent silver stain with a kit from BioRad.

Immunoblotting. Western analysis was carried out as previously described (4). Briefly, samples were first electrophoresed on 10% SDS-PAGE. Electro-transfer to nitrocellulose membrane was performed with a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) that was set at a constant voltage of 150 for 90 minutes. The membrane was blocked for 2-3 h at 37°C with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0) containing 1% (vol/vol) BSA and subsequently treated with anti-perforin antibody for 1 h at room temperature using the Fast-BLOT Developer System (Pierce Chemical, Inc., Rockford, IL). After washing three times, the blot was reacted with the appropriate anti-mouse IgG horseradish peroxidase conjugate (1:1000 dilution) for 1 h at room temperature and then washed again 5 times with TBST. The membrane then was developed with ECL system (Amersham, Inc.) according to the manufacturer's directions.

Cytolytic chromium release assay. The assay was performed as described (5) with the following modifications. Target cells (2×10^6) were incubated with 100 μCi of ^{51}Cr (Amersham, Arlington Hts., IL) for 1h, washed 3 times with RPMI/0.05% BSA and resuspended at 1×10^5/ml. One hundred microliters of cells was added to round bottom 96 well plates containing 50 μl of buffer control. Then 50 μl of perforin or perforin buffer (HEPES) was added to the microwells. After incubation, supernatants were harvested after centrifugation of the microwell plates (300 ×g for 5 min) and radioactivity determined with a Beckman Gamma counter (Beckman Instruments, CA). Maximum release was defined as cpm in 1×10^4 labeled target cells and spontaneous release reflected cpm in supernatants of targets incubated with designated buffer controls. Percent lysis was calculated as follows: % lysis = (cpm Sample-cpm Spontaneous)/ (cpm Maximum-cpm Spontaneous) × 100.

RESULTS AND DISCUSSION

Building on a previous study (1) we report here a simple protocol that consistently yields quantities of human perforin that are sufficient to perform multiple cytotoxicity assays. Previous attempts to isolate perforin have been complicated by the variable quantity of pore forming protein present in the normal cell populations chosen for isolation. The original YT cell line, derived from a 15 year old boy with acute lymphoblastic lymphoma/thymoma, is an IL-2-independent, non-MHC restricted NK-like cell line that is highly granular (6). YT-INDY is similar to the original line except that it has lost the ability to kill K562 cells. We have observed by Northern and Western analysis that the YT-INDY line expresses abundant mRNA and protein for perforin (5). Unlike LAK cells which need exogenous growth factors for proliferation, CTL lines which require periodic stimulation with adherent cells and rat NK cell

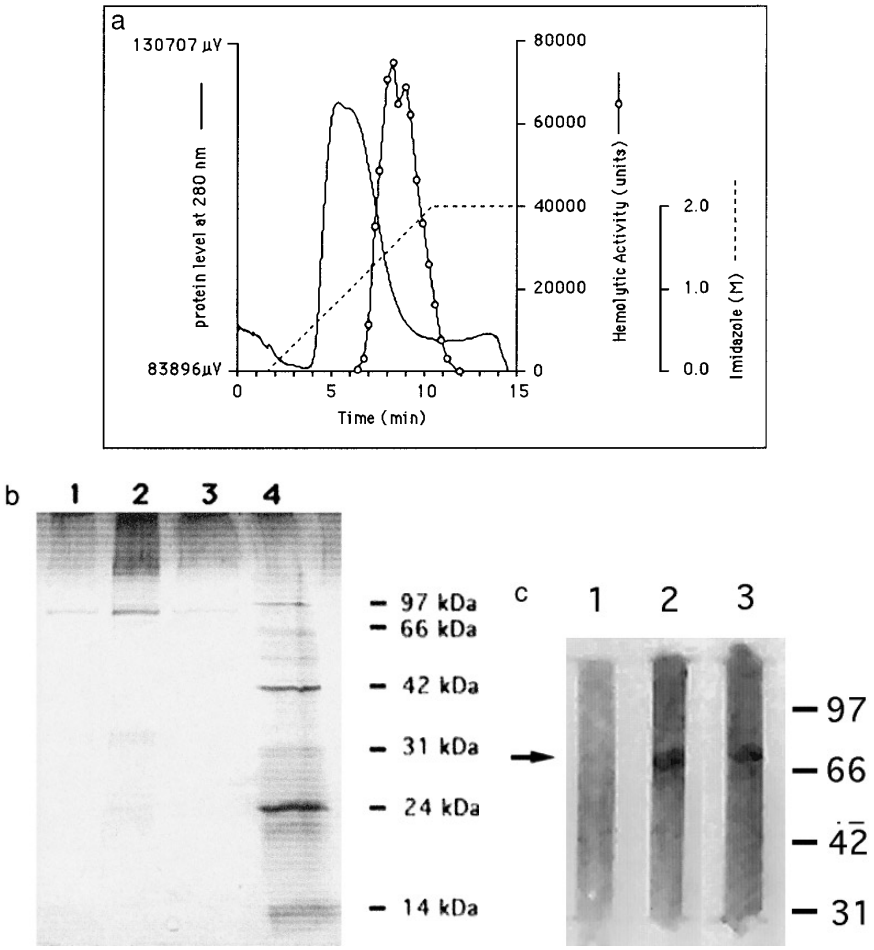

FIG. 1. Purification of perforin from granule extract of YT-INDY cells. (a) Cobalt ion metal affinity chromatography of granule extract demonstrating elution of a single peak of hemolytic activity. After exchanging the 1 M NaCl granule extract on a PD-10 column into buffer A (20 mM HEPES, 1M NaCl, 10% betaine, pH-7.5), 10 ml of the preparation was injected onto a cobalt-charged IMAC column (HR/5) and the column was subsequently washed (0.7 ml/min) with buffer A until the protein level returned to baseline. Then a 10-minute imidazole gradient (0-0.2 M) was applied at a flow rate of 1.5 ml/min. Sequential fractions were tested for hemolytic activity as described under Material and Methods. (b) Silver stain of 12% SDS-PAGE gel in which each lane was loaded with the two fractions that contained peak hemolytic activity Perforin isolated by IMAC in three separate preparations: Lane 1, ID # 95.03.28; Lane 2, ID # 95.07.04; Lane 3, ID # 95.04.20; Lane 4, pre-stained standards (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor). (c) Immunoblot of 12% SDS-PAGE gel reacted with mouse anti-perforin mAb and developed with ECL system: Lane 1 was loaded with 105 hemolytic units (74 ng) while Lanes 2 and 3 were loaded respectively with 25,350 hemolytic units (protein N.D.) and 11,830 hemolytic units (protein N.D.). Arrow indicates position of developed band corresponding to band observed by SDS-PAGE and sieve staining.

lines which may need to be propagated *in vivo*, YT-INDY readily proliferates in vitro offering a reliable source of human perforin.

The published protocols used to isolate perforin rely on density gradient centrifugation to enrich cytotoxic granules and two or more chromatographic to purify the protein to homogeneity (7,8). We show that the time consuming density gradient centrifugation step as well as multiple chromatographic procedures can be eliminated if IMAC is used to isolate perforin

TABLE 1
Perforin Purification from YT-INDY Cell Line[a]

Purification step	Total protein (mg)	Hemolytic activity (units)	Spec activity (U/mg)	Purification (fold)
Cavitate (10×10^8 cells)	771.000	41,970	0.02	—
1 M NaCl granule extract	0.145	2,467	1.82×10^4	9.1×10^5
Ion metal chromatography	0.037	117,875	3.20×10^6	1.6×10^8

[a] Measurement of protein level and hemolytic activity were performed as described under Material and Methods.

from the crude granule extract. As shown in Fig. 1a, the application of a linear imidazole gradient results in a single peak of hemolytic activity that elutes on the tail of the broad-based protein peak. The two fractions with highest hemolytic activity were concentrated and submitted to 12%-SDS PAGE. Silver staining showed the presence of a single protein band that migrates with an Mr typical for perforin. Immunoblotting of other preparations with an anti-perforin mAb (Fig. 1c) verified that this protein band was indeed the pore forming protein. Table 1 describes the yield of perforin during a typical isolation from the YT-INDY line when the two peak fractions were retained.

Stabilization of the isolated perforin was problematic. This is likely due to the dissociation of perforin from its chaperone, calreticulin, and the low concentration of the protein (9). Efforts to remove the imidazole and betaine by dialysis against 0.15 M NaCl resulted in complete loss of hemolytic activity. Similarly attempts to concentrate the perforin in active form failed unless a protein stabilizer was present. The eluted perforin was concentrated tenfold in the presence of fatty acid free BSA to yield a final concentration of stabilizer at 0.1 mg/ml. Under this condition, the perforin was completely stable for one week at 4°C and after thawing of samples frozen at −70°C (data not shown).

Inasmuch NH2-terminal sequencing was not performed, we cannot ensure the perforin was isolated entirely to homogeneity. Nevertheless, the peak fractions appear to be devoid of

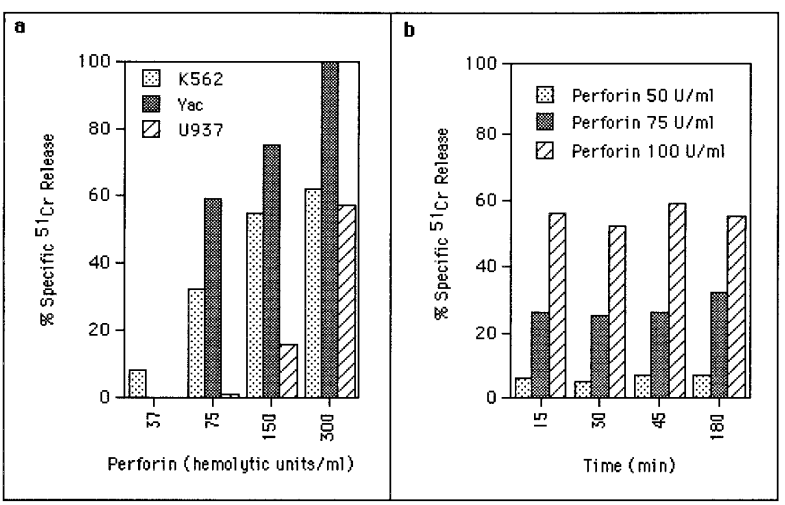

FIG. 2. Target cell lysis mediated by human perforin concentration (a) and time (b) dependence of perforin-induced cytotoxicity. Assay performed as described under Material and Methods.

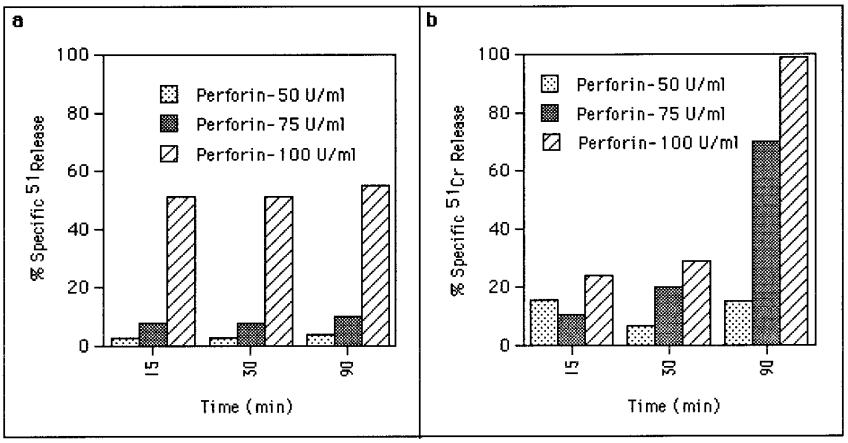

FIG. 3. Effect of assay conditions on kinetics of perforin-mediated lysis of U937 cells. In (a), stabilized perforin (50 μl) was added first to the microwells followed immediately by HEPES buffer and then radiolabeled U937 cells (1×10^4 cells per 100 μl in RPMI 1640 and 0.05% BSA). In (b), labeled U937 cells were added to round-bottom microwells followed by 50 μl of HEPES buffer and then stabilized perforin (50 μl).

major contaminating proteins by SDS-PAGE and silver staining. Our typical stock preparation contained an average of 1-3 $\times10^5$ hemolytic units in 30-50 μg. Approximately 100-300 hemolytic units/ml or 30-50 ng/ml of protein has been sufficient to lyse upwards to 10^6 nucleated cells (see below). A contaminating protein present at the limits of detection of the silver stain (e.g., 100 ng) would be diluted approximately 1000 fold in the working concentration of perforin yielding a potential value of 100 pg/ml. Since the perforin was stabilized with BSA at 0.1 mg/ml, the contaminants in this reagent probably exceed those that might exist in the perforin preparation. Thus the described purification scheme probably provides a source of perforin sufficient to perform standard cytotoxicity assays.

Figure 2a demonstrates typical dose response curves when perforin was added to targets, K562, YAC-1 and U937. Generally hemolytic activity in the range of 100 to 300 hemolytic U/ml was sufficient to produce 50% lysis of the targets. The dilute betaine and imidazole appeared to have no untoward effects. When kinetics of lysis was examined, we unexpectedly learned that maximal lysis occurred within 15 min (Fig. 2b). In an attempt to comprehend the failure to observe a time dependent increase in perforin mediated lysis, the order of addition of cells and perforin was evaluated. When the targets were added to microwells containing perforin under the conditions used for Fig. 2, maximal lysis again occurred within 15-30 min (Fig. 3a). A time dependent increase in Cr release, however, was noted when perforin was added to microwells which already contained the labeled targets (Fig. 3b). Similar results were observed when K562 and YAC cells were tested (data not shown).

In summary, we have delineated a reliable, straightforward method that highly enriches human perforin. The availability of this technique should facilitate the performance of studies to further characterize the biologic function of this enigmatic molecule in granule mediated apoptosis.

ACKNOWLEDGMENT

This work was supported by the Rice Foundation.

REFERENCES

1. Winkler, U., Pickett, T. M., and Hudig, D. (1996) *J. Immunol. Methods* **191,** 11–20.
2. Hudig, D., Allison, N. J., Pickett, T. M., Winkler, U., Kam, C.-M., and Powers, J. C. (1991) *J. Immunol.* **147,** 1360–1368.

3. Laemmli, U. K. (1970) *Nature* **227,** 680–682.
4. Hanna, W. L., Zhang, X., Turbov, J., Winkler, U., Hudig, D., and Froelich, C. J. (1993) *Protein Purif. Exp.* **4,** 398–402.
5. Su, B., Bochan, M. R., Hanna, W. L., Froelich, C. J., and Brahmi, Z. (1994) *Eur. J. Immunol.* **24,** 2073–2080.
6. Yodoi, J., Teshigawara, K., Nikaido, T., Fukui, J. K., Noma, T., Honjo, T., Takigawa, M., Minato, M., Tsudo, M., Uchiyama, T., and Maeda, M. (1985) *J. Immunol.* **164,** 1623–1629.
7. Shi, L., Kraut, R. P., Aebersold, R., and Greenberg, A. H. (1992) *J. Exp. Med.* **175,** 553–566.
8. Young, J. D.-E., Hengartner, H., Podack, E. R., and Cohn, Z. A. (1986) *Cell* **44,** 849–854.
9. Dupuis, M., Schaerer, E., Krause, K.-H., and Tschopp, J. (1993) *J. Exp. Med.* **177,** 1–7.