Brief Definitive Report

PURIFIED PERFORIN INDUCES TARGET CELL LYSIS BUT NOT DNA FRAGMENTATION

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A striking characteristic of CTL-mediated cytolysis, and one that distinguishes it from complement-mediated killing, involves extensive nuclear damage in the target cell (1, 2). Killer cell-derived lytic granules have been implicated as the mediators of events between CTL and target cells (3, 4). Lytic granules contain a variety of components including a pore-forming protein (PFP, perforin or cytolysin), serine esterases, and proteoglycans (3, 4), and a polypeptide, related to TNF, that can induce slow DNA damage in susceptible cells (5). While it seems likely that granule contents could mediate membrane damage, their role in the induction of nuclear damage is controversial (6–9). To avoid potential errors in the interpretation of results obtained with intact granules, we studied the role of highly purified perforin in the induction of DNA fragmentation. Our results show that perforin alone is unable to cause the type of DNA fragmentation observed during CTL-mediated killing, suggesting that mediators other than perforin are likely to be involved in the induction of nuclear damage.

Materials and Methods

Lytic Granule Assay Medium (LGAM). LGAM consists of 135 mM NaCl, 5 mM KCl, 25 mM Hepes, 2 mM $MgCl_2$, 4 mM glucose, 1% BSA, pH 7.2 (9). LGAM was used in cytotoxic assays instead of normal tissue culture medium because perforin-mediated lysis is strongly inhibited by serum or calcium.

MLR Blasts and CTL Clones. CBA/J anti-BALB/cJ MLR blasts propagated in IL-2-containing media were prepared as described previously (9). The H-2^d anti-H-2^b CTL clones AB.1 and AB.2 were generously provided by Dr. W. R. Clark (10). The CTL clone CTLL-R8, obtained from Dr. M. A. Palladino, was used as the source of perforin (11).

Perforin Extraction and Purification. Perforin was purified from CTLL-R8 as previously described (11, 12). Briefly, nucleus-free lysates of 10^{10} CTLL-R8 cells, obtained after nitrogen cavitation, were centrifuged at 39,000 *g* for 20 min. The pellet was then subjected to a high-salt, pH-shift extraction (12). After a second centrifugation at 39,000 *g* for 30 min, the perforin-

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enriched supernatant was diluted in starting buffer (20 mM Tris-HCl, 1 mM EGTA, pH 7.2) and applied sequentially on DEAE-Sepharose, Q Sepharose, Polyanion SI, and Superose 12 columns (all from Pharmacia Fine Chemicals, Uppsala, Sweden). Perforin activity in the various column fractions was quantified in hemolytic units (HU) using a microassay (12). The purity of the perforin was ascertained by SDS-PAGE (Phastgel; Pharmacia Fine Chemicals; Fig. 1).

DNA Fragmentation and Cytotoxicity Assays. Murine CTLL-2, EL-4, P815, R1.1, WEHI 7.1, and YAC-1 tumor cells, labeled in their DNA with [^{125}I]UdR and in their cytoplasm with ^{51}Cr as previously described (9), were used as target cells. Targets and CTL were washed three times in warm (37°C) LGAM before use. For the assay of perforin-mediated killing, 10^4 double-labeled target cells in 50 μl LGAM were placed in 1.5-ml microfuge tubes and gently mixed with 100 μl LGAM (spontaneous) or with various amounts of perforin-containing samples in a total of 100 μl LGAM (experimental). After 10 min of incubation at 37°C, 50 μl of LGAM containing 8 mM $CaCl_2$ (final concentration, 2 mM) was added and the samples were returned to the incubator. CTL-mediated cytolysis was assayed in a similar fashion with various numbers of CTL in 100 μl LGAM being added instead of perforin in the experimental condition. CTL-target cell mixtures were centrifuged (50 *g* for 5 min) to establish cell contact and incubated at 37°C for 10 min before addition of $CaCl_2$ and further incubation. In some experiments, the target cells were incubated with 5 μg/ml Con A for 30 min before addition of CTL. For the assay of complement-mediated killing, 10^4 double-labeled target cells in 50 μl LGAM were placed in 1.5-ml microfuge tubes containing 50 μl of cell-free culture supernatants of the HO-13-4 B cell hybridoma, which secretes anti-Thy-1.2 IgM mAb. After a 30-min incubation at 4°C, 50 μl of guinea pig serum, as a source of complement, and 50 μl of 8 mM $CaCl_2$ were added and the samples were placed at 37°C. At the indicated times, percent specific fragmented DNA and ^{51}Cr release were calculated as described previously (9).

DNA Electrophoresis. [^{125}I]UdR-labeled chromatin from 13,000 *g* supernatants of hypotonically lysed target cells (13) was extracted with 50% isopropanol and 0.5 M NaCl overnight at −20°C. The precipitated DNA was air-dried, redissolved in 10 mM Tris, 1 mM EDTA, pH 7.4, and subjected to electrophoresis in 0.75% agarose for 2 h at 100 V with Tris-boric acid-EDTA running buffer (13). DNA was visualized by autoradiography.

Results

Lack of DNA Fragmentation During Killing Mediated by Purified Perforin. Perforin was extracted from granules isolated from the murine cytotoxic lymphocyte CTLL-R8 with high salt-containing buffers and applied sequentially to a series of chromatographic columns. The material obtained from the final column, Superose 12, migrated as a single band of 70 kD in SDS-PAGE (Fig. 1), which corresponds to the molecular mass of perforin (11). Fractions eluted from each column that contained hemolytic activity were tested for their ability to induce lysis and DNA fragmentation in nucleated target cells. The perforin-containing fractions eluted from the first ion exchange column, DEAE-Sepharose, induced lysis of all cells and some DNA fragmentation in EL-4, R1.1, and YAC-1 but not P815 (data not shown). However, when perforin-containing fractions were pooled and applied to other columns (Q-Sepharose, Polyanion SI, and Superose 12) and eluted fractions were similarly tested for lytic and DNA fragmentation activities, the correlation between these activities began to break down. Thus, very little DNA fragmentation was induced by perforin-containing fractions eluted from Q Sepharose (data not shown) and no activity was detected in those of Polyanion SI (Fig. 2, *bottom*). In contrast, MLR blasts and the CTL clone AB.2 induced rapid target cell DNA fragmentation and lysis when tested in the same experiments under the same conditions (Fig. 2, *top*). The lack of target cell DNA damage induced by perforin-containing fractions eluted from Polyanion SI column

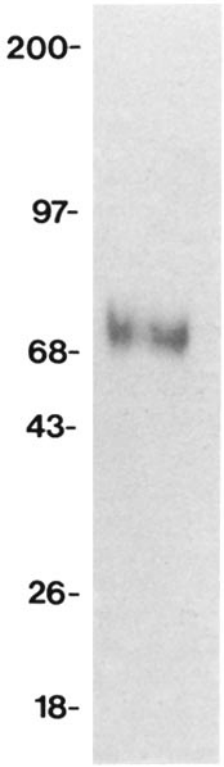

FIGURE 1. Silver-stained SDS-polyacrylamide gel profile of perforin eluted from Superose 12 column. 0.1 μg of purified perforin, reduced with 0.1% β-mercaptoethanol, was subjected to electrophoresis in 10% SDS-polyacrylamide. $M_r \times 10^{-3}$ shown at left.

as compared with the extensive fragmentation induced by CTL was confirmed by agarose gel electrophoresis (Fig. 3); DNA damage was not detectable in YAC-1 killed with PFP (lanes *3* and *4*), whereas DNA fragments consisting in oligonucleosomes (1, 2, 13) could be readily isolated from YAC-1 cells incubated with CTL (lane *2*).

For the experiment shown in Fig. 4, CTLL-2, EL-4, P815, R1.1, WEHI 7.1, and YAC-1, labeled with ^{51}Cr and [^{125}I]UdR, were incubated with either CTL clone

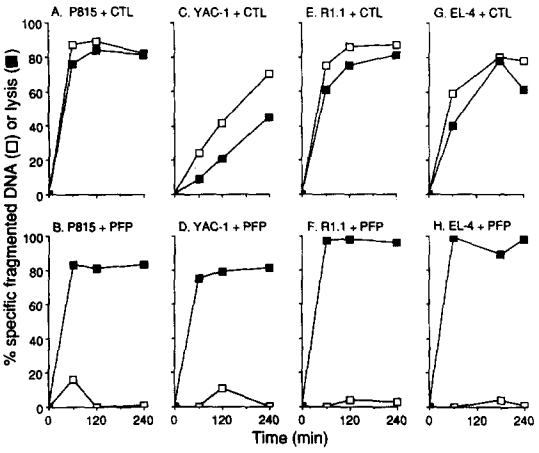

FIGURE 2. Comparison of target cell DNA fragmentation and lysis mediated by CTL or purified perforin (PFP). 10^4 P815, YAC-1, R1.1, or EL-4 target cells labeled in their DNA with [^{125}I]UdR and cytoplasmically with ^{51}Cr were incubated with either 5×10^5 CBA anti-BALB/c MLR blasts (*A*, *C*), 5×10^5 CTL clone AB.2 plus 5 μg/ml Con A (*E*, *G*), or with 64 HU partially purified perforin (*B*, *D*, *F*, *H*). Percent specific fragmented DNA (□) and lysis (■) were determined at the times indicated.

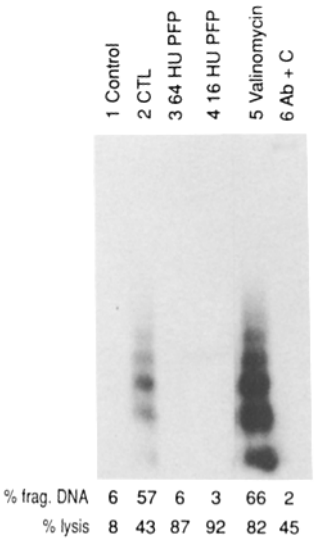

FIGURE 3. Agarose gel electrophoresis of nonsedimenting [^{125}I]UdR-labeled DNA obtained from 10^4 YAC-1 cells incubated for 4 h at 37°C alone (lane *1*), or with 5 × 10^5 CBA anti-BALB/c CTL (lane *2*), 64 HU partially purified perforin (lane *3*), 16 HU partially purified perforin (lane *4*), 100 μM valinomycin (lane *5*), or anti-Thy-1.2 + complement (lane *6*). Electrophoresis in 0.75% agarose was for 2 h at 100 V (22°C). DNA was visualized by autoradiography. (*% frag. DNA*) the proportion of the total ^{125}I recovered from [^{125}I]UdR-labeled YAC-1 cells as nonsedimenting DNA and that was applied to the agarose gel. (*Percent lysis*) ^{51}Cr release obtained in parallel studies using double-labeled YAC-1 cells.

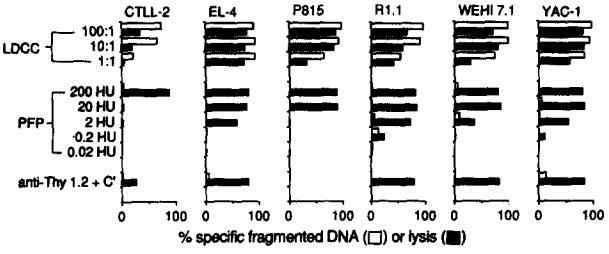

FIGURE 4. Lack of target cell DNA fragmentation during lysis mediated by purified perforin. CTLL-2, EL-4, P815, R1.1, WEHI 7.1, or YAC-1 target cells labeled in their DNA with [^{125}I]UdR and cytoplasmically with ^{51}Cr were incubated with various numbers of the CTL clone AB.1 plus 5 μg/ml Con A (LDCC); or with various amounts of purified perforin (PFP); or with anti-Thy-1.2 mAb plus complement. Percent specific lysis (■) and DNA fragmentation (□) were determined after 4 h of incubation at 37°C.

AB.1 plus 5 μg/ml Con A (LDCC), Superose 12-purified perforin (PFP), or anti-Thy-1.2 antibodies plus complement for 4 h before determination of DNA fragmentation or lysis. Our results show that while all three treatments resulted in cellular cytotoxicity as measured by ^{51}Cr-release, only incubation with CTL induced DNA fragmentation. Another positive control included in these experiments consisted of treatment of the six different targets with valinomycin (6), which produced both lysis and DNA fragmentation (data shown for YAC-1 cells only, Fig. 3, lane *5*). When the time of assay was increased to 8 h, neither purified perforin nor complement induced DNA fragmentation, although in 12–15-h assays, a small but significant amount of DNA fragmentation was observed in perforin- and complement-treated cells (data not shown).

Discussion

This study was performed to address the question of whether lytic granules or perforin isolated from CTL can induce DNA fragmentation as well as lysis of target

cells. Our results clearly show that highly purified perforin, like complement (1, 2), lyses target cells efficiently but does not induce DNA fragmentation. The preparations of purified perforin used here are most likely homogeneous since, in addition to migrating as single bands of 70 kD in SDS-PAGE that correspond precisely to the molecular mass of perforin (Fig. 1), they have already been used successfully for primary amino acid sequence determination (11). It should be noted that our results conflict with recently published findings (7) that have described DNA fragmentation activity associated with purified perforin. It is possible that the perforin preparations used in that study contained contaminating granule constituents other than perforin. In accordance with this possibility, our own experiments have revealed some DNA fragmentation-inducing activity associated with perforin-enriched fractions eluted from the DEAE- and Q-Sepharose ion-exchange columns (data not shown), while more purified preparations obtained from Polyanion SI and Superose 12 columns did not induce detectable DNA fragmentation (Figs. 2–4). Thus, it is possible that granule mediators other than perforin may be responsible for inducing DNA fragmentation. Preliminary results show that some, but not all, granule preparations obtained from various CTLL lines contain a DNA fragmentation–inducing activity that shows a time course of action comparable to that produced by intact CTL (data not shown). Since several investigators have reported that isolated granules can induce DNA fragmentation (6, 7), whereas other groups have failed to confirm these results with preparations that were nonetheless lytic (8, 9), our results suggest that a possible explanation for these disparities is that the lytic granules used by the individual groups varied significantly in their constituents due to differences in isolation techniques as well as the types of killer cells from which the granules were isolated. Furthermore, the target cell types, experimental conditions, and assay durations were markedly different in those studies, making comparisons difficult.

Hameed et al. (7) have suggested that pore-forming agents in general are capable of producing DNA fragmentation. In addition to the results shown here, several other pieces of information contradict this hypothesis; in our hands the well-known channel formers complement, *Staphylococcus aureus* α-toxin (up to 50 μg/ml), and mellitin (up to 100 μg/ml) induce cytolysis in most target cells tested *but not* DNA fragmentation (see also reference 14). Valinomycin, a potassium ionophore, on the other hand, causes both cytolysis and DNA fragmentation (Fig. 3, lane *5*; reference 6, 14). Thus, DNA fragmentation appears to represent a complex phenomenon that does not seem to bear a simple causal relationship with membrane channel formation. From the results presented here it is clear, however, that factors or mediators other than perforin must be considered as the basis of DNA fragmentation induced by intact CTL.

Summary

Rapid and extensive target cell DNA fragmentation is a unique characteristic of CTL-mediated killing. We studied the role of the granule pore-forming protein (PFP/perforin/cytolysin) of CTL in mediating lysis and DNA fragmentation of target cells. Perforin was isolated from murine CTL by sequential application of perforin-enriched granule fractions to four chromatographic columns: DEAE-Sepharose, Q-Sepharose, Polyanion SI, and Superose 12. Purified perforin was eluted as a single band of 70 kD in SDS-PAGE. While purified perforin produced potent lysis of a variety of target cells tested, it did not induce any measurable amount of DNA frag-

mentation. In parallel experiments, intact CTL produced marked DNA fragmentation of the same target cell populations. Our results suggest that perforin alone is not responsible for the DNA fragmentation observed during CTL-mediated killing and that other, as yet unknown, mediators or mechanisms are likely to be involved in the induction of target cell nuclear damage.

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