CYTOLYSIS BY Ca-PERMEABLE TRANSMEMBRANE CHANNELS

Pore Formation Causes Extensive DNA Degradation and Cell Lysis

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Cell death accompanied by the degradation and release of nuclear DNA has been observed in various biological systems (for review see references 1, 2). Two well-studied examples are the cell death induced by steroid hormones in immature lymphocytes and the cell death induced by cytolytic T cells and NK cells (3–7). Steroid hormone induced cell death requires active protein synthesis in the target cell before the onset of DNA degradation. In contrast, CTL-induced DNA breakdown is independent of target cell protein synthesis. However, CTL induced DNA degradation also requires active target participation and it has been shown that the final step(s) of DNA cleavage are target specific (8, 9). Further evidence indicates that hormone- and CTL-induced cell death trigger the same target cell suicidal pathway: steroid-resistant cells were also resistant to CTL killing (10); more recently it was shown, however, that a steroid resistant cell may only fail to trigger CTL rather than being intrinsically resistant to CTL (11).

The extent of DNA degradation of targets under CTL attack can be quite variable. Certain target cells, for instance P815, show substantial DNA release, whereas L929 exhibit, by comparison, much lower DNA release upon CTL attack (9). In some cases (3T3 cells), DNA release is barely detectable at all, despite target cell death as measured by chromium (Cr) release, emphasizing further the importance of the target contribution (9).

The molecular mechanism of DNA degradation is not understood. Several studies reported that complement-lysed cells do not undergo DNA degradation (3, 5, 10) even though an earlier report demonstrated single-strand breaks in the nuclear DNA of complement lysed cells (12). More recently (13) it was reported that cytolytic granules isolated from CTL failed to induce DNA degradation in target cells despite vigorous Cr release. These studies suggest that perforin, the pore-forming protein of CTL and NK with homology to C9 of complement (14–26), may not be involved

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in DNA degradation. On the other hand, Allbritton et al. (27) found that cytolytic granules cause DNA degradation in target cells that is preceded by the influx of Ca. These authors also observed target DNA breakdown mediated by the potassium ionophore valinomycin.

Both complement and granules are multicomponent systems. To understand the role of isolated pore formers per se in DNA degradation and in cytolysis, we used two purified pore-forming proteins and investigated their effect on DNA and Cr release in various tumor target cells. Staphylococcal α-toxin binding to membranes forms transmembrane channels of ~3 nm internal diameter in a metal independent polymerization reaction (28, 29). Perforin, obtained from CTL or NK cells, forms up to 16-nm large transmembrane channels in a Ca-dependent polymerization reaction (15, 20). We report here that both channel formers induce DNA degradation in target cells. The induction of DNA degradation by pore formers is enhanced in the presence of TNF and requires intracellular Ca and an acidic intracellular compartment.

Materials and Methods

Cells and Media. P815, L929, Yac-1, and EL-4 were maintained in Iscove's modified DMEM (Gibco Laboratories, Grand Island, NY) containing 10% FCS.

Preparation and Culture of Human LAK Cells. LAK cells were generated in 1,000 U/ml rIL2 (Hoffman-La Roche, Inc., Nutley, NJ) for 12–20 d from mononuclear cells from healthy volunteers as described previously (30, 31). HY3Ag3 cells, a cloned murine cytotoxic cell with NK-like specificity (32), were maintained in Iscove's media supplemented with 10% FCS and 500 U/ml of rIL-2.

Purification of Murine Perforin. Cytotoxic granules from human LAK cells and from murine cytotoxic HY3Ag3 cells and perforin were prepared as described previously (15, 20). Briefly, the granules were extracted with an equal volume of 1 M $NaKPO_4$ pH 6.5, containing 1 mM EGTA, 0.02% azide, and 1 mM $MgCl_2$. After 30 min of centrifugation at 20,000 rpm in a Sorvall centrifuge, the soluble extract was applied to Sephacryl S-300 equilibrated with 20 mM $NaKPO_4$, pH 6.5, 0.145 M NaCl, 1 mM EDTA, 3 mM NaN_3, and 1 mM benzamidine HCl. The fractions eluted were tested for both hemolytic and esterase activity as described (20, 33). The fractions showing hemolytic activity were pooled and used for further purification by Mono Q ion-exchange chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) as described (20). Murine perforin prepared in this way was free of *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl-ester (BLT)-esterase activity. In addition perforin was also free of DNase activity as measured by the lack of cleavage of plasmid DNA even after prolonged incubation.

Staphylococcus α-Toxin. Purified staphylococcus α-toxin was purchased from Calbiochem-Behring Corp. (San Diego, CA). 10 mg of α-toxin was dissolved in 10 ml of HBSS and aliquoted in 1-ml Eppendorf tubes and stored at −20°C until use.

Labeling of Target Cells. The target cells to be used in ^{51}Cr-release assays were suspended at 5×10^5/ml in Iscove's medium containing 10% FCS to which 100 μCi of ^{51}Cr (sodium chromate, New England Nuclear, Boston, MA) were added. The cells were incubated for 90 min at 37°C in the incubator, washed twice in Iscove's medium containing 0.5% BSA, and resuspended at 5×10^5/ml in the same medium. For $^{125}IUdR$ labeling of target cells, P815, L929, Yac-1, and El-4 were subcultured 24 h before use in 25-cm^2 Falcon Labware (Oxnard, CA) flasks. At the time of assay, the cells were resuspended at 5×10^5/ml in Iscove's medium containing 10% FCS in 15-ml conical sterile tubes. 10–15 μCi of $^{125}IUdR$ (New England Nuclear) was added to each tube and incubated 2–3 h at 37°C. After incubation, the cells were washed twice with Iscove's medium containing 0.5% BSA and resuspended at 5×10^5/ml in the same medium. The labeled cells were kept on ice until use.

^{51}Cr-release Assay. ^{51}Cr-labeled target cells (10^4 cells in 20 μl) were placed in 1-ml Eppendorf tubes. The assays were done in triplicate in a final volume of 200 μl Iscove's medium containing 0.5% BSA. Various concentrations of α-toxin, mouse and human purified per-

forin were added last as indicated. When LAK cells were used as effectors, various ratios of the effector cells from 100:1 to 3:1 were added. The tubes were incubated at 37°C in the incubator for various periods of time as indicated. At the end of incubation, the tubes were centrifuged at 2,000 rpm for 2 min in an Eppendorf centrifuge. 100 μl of cell-free supernatant was collected from each Eppendorf tube and counted in a gamma counter. Percent specific release was calculated by the following formula: Percent specific ^{51}Cr release = $100 \times [(cpm_{exp} - cpm_{spon})/(cpm_{max} - cpm_{spon})]$. Maximal release was determined from the supernatant of the cells that were lysed by HCl (10%, final concentration). Spontaneous release was determined from the supernatant of target cells incubated alone.

^{125}IUdR-release Assays. ^{125}IUdR-labeled targets (10^4 cells in 20 μl) were added to 1 ml Eppendorf tubes. The assays were done in triplicate in a final volume of 200 μl Iscove's medium containing 0.5% BSA exactly as described above. Iscove's medium contains 4.4 mM KCl and 1.5 mM CaCl. After incubation, the tubes were centrifuged at 3,000 rpm for 2 min. The supernatant of each tube was carefully withdrawn. The pelleted cells were lysed for 2 min with 0.5 ml ice-cold HBSS (without calcium) containing 0.2% NP-40. The cell lysate was centrifuged at 10,000 rpm for 5 min at 4°C to separate fragmented chromatin from intact chromatin. The radioactivity in the initial supernatant (incubation medium), in the 10,000 rpm supernatant, and in the 10,000 rpm pellet were determined in the gamma counter. The DNA release was calculated as described for ^{51}Cr release. Released DNA, corresponding to the minimal value of fragmented DNA, is expressed as the sum of radioactivity in the initial supernatant and the 10,000 rpm supernatant.

Effect of TNF on ^{125}IUdR Release and ^{51}Cr Release. Human rTNF was a gift from Genentech, Inc. (San Francisco, CA) TNF was added to the assays at concentrations between 500 and 5,000 U/ml (final concentration). In controls, TNF was added without α-toxin. Incubation was carried out for 3 h at 37°C.

Effects of Lysosomal and Metabolic Inhibitors on ^{125}IUdR and ^{51}Cr Release. Inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO). Labeled targets (^{125}IUdR and ^{51}Cr) were preincubated with lysosomal inhibitors (30 mM NH_4Cl, 5 μM chloroquine, 1 μg/ml monensin) for 2–3 h at 37°C in BSA-containing medium. Shorter incubation periods are not sufficient for complete equilibration and inhibition (34). After preincubation, the targets were washed once with 0.5% BSA in Iscove's medium and resuspended in the same medium containing the same concentrations of inhibitors. ^{125}IUdR and ^{51}Cr release was determined as described before. Controls contained the same inhibitor concentration; no toxicity was observed with inhibitors alone. In experiments using metabolic inhibitors (10 mM sodium azide; 20 mM deoxyglucose), the assays were carried out without preincubation of target cells. Stock solutions of various inhibitors were made in HBSS, except monensin, which was dissolved in absolute ethanol at 10 mg/ml, and Quin 2/AM, which was dissolved in DMSO (300 mM).

Calcium-depletion Assays. Ca depletion with the use of EGTA was done as described previously (35). Briefly, target cells were resuspended in 10 mM Hepes-buffered HBSS without Ca, containing 0.5% BSA and supplemented with 5 mM EGTA and preincubated for 1 h in order to deplete intracellular Ca pools (35). Assays were carried out in buffers of the same composition. As this treatment did not seem to completely deplete intracellular Ca, target cells were, in addition, loaded with the intracellular Ca chelator Quin 2 (36). Briefly, 5×10^5 targets were incubated with Quin 2/AM (3 mM) in Hepes-buffered HBSS without calcium at 37°C for 30 min. The cells then were diluted 10-fold and incubation continued for 60 min to allow intracellular esterolysis. After incubation, the cells were centrifuged at 1,000 rpm for 3 min. They were resuspended at 5×10^5/ml in Hepes-buffered HBSS without calcium and kept on ice for subsequent use. The intracellular Quin 2 concentration was determined to be 1.7 mM by lysis of cells and fluorescence compared with known standards of Quin 2.

Determination of DNA Degradation without the Use of Radioisotopes. These experiments were carried out to ascertain that DNA degradation was not caused by the manipulation of cells or by irradiation (37). P815 and L929 were harvested and resuspended in 25-cm^2 tissue culture flasks in 5 ml Iscove's medium containing 0.5% BSA at a density of 2×10^6/ml. α-toxin was added to a final concentration of 20 μg/ml or 0.5 mg/ml and the cells were incubated

for 3 h at 37°C. The cells were then pelleted and lysed with 0.5 ml ice cold 0.5% NP40 in Ca-free HBSS containing 5 mM EDTA. Nuclei were sedimented after 30 s of incubation on ice at 10,000 rpm for 0.5 min at 4°C. The supernatant of the lysate and the pellet after resuspension in 0.5 ml Ca-free HBSS were extracted for 10 min at room temperature with an equal volume of phenol chloroform, the aqueous phase was precipitated with ethanol, redissolved in 20 μl, and separated by electrophoresis on 1% agarose gels. The gels were stained with ethidium bromide and photographed under UV light. Ethidium bromide staining is proportional to the amount of DNA. Staining was quantitated by densitometry of the negatives in a video densitometer (Bio-Rad Laboratories, Richmond, CA). The plots are corrected for spontaneous DNA release in controls incubated under identical conditions without α-toxin.

Results

The Role of the Target Cell in DNA Degradation Induced by Pore Formers. The staphylococcal α-toxin was chosen as pore former for the investigation of transmembrane channel-induced Cr release and DNA release from nucleated target cells. α-Toxin, in purified form, is cytolytically active for erythrocytes (28, 29) and for nucleated cells. Its action is known to proceed via metal independent membrane insertion and polymerization to an electronmicroscopically detectable transmembrane pore of ~3 nm internal diameter (28). Three types of target cells were chosen based on their known properties as being active (P815), relatively inactive (L929), or intermediate (YAC) in DNA degradation induced by CTL (10). The target cells were labeled with ^{51}Cr as cytoplasmic marker and with $^{125}IUdR$ as marker for DNA, and then subjected to increasing amounts of α-toxin (Fig. 1, *A, C, E*) for various periods of time (Fig. 1, *B, D, F*). Controls were incubated in the same 0.5% BSA-containing tissue culture medium without added α-toxin. DNA release was expressed as the sum of extracellular and cytoplasmic DNA, as measured by NP-40 lysis at 4°C and sedimentation of nuclei (10). Spontaneous release for Cr was <5% and between 5 and 15% for DNA in a 3-h assay period. All values are corrected for spontaneous release.

Fig. 1 shows the dose-response and the kinetics of DNA and Cr release from three different target cells caused by α-toxin. Panels *A* and *B* show that P815 cells release large amounts of DNA from their nucleus at a dose and time when virtually no Cr release is seen. A dose of 20 μg toxin per assay in 3 h causes 30% specific DNA release and <5% specific Cr release. The DNA release is detectable as early as 20 min after addition of toxin when no Cr release above the control is seen. Cr release, however, reaches high levels also after 16 h of incubation. It appears that in P815 cells DNA degradation is primarily responsible for cell death by the pore former

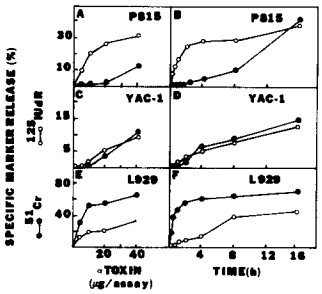

FIGURE 1. Dose-response and kinetics of Cr and DNA release in three target cells mediated by staphylococcal α-toxin. (*A, C, E*) Dose-response, incubation time 4 h. (*B, D, F*) Kinetic response from 0.5 to 16 h, dose 20 μg. (*Open symbols*) DNA release. (*Closed symbols*) Cr release. Spontaneous release in 3 h of Cr was 9% (P815), 8% (YAC1), and 12% (L929); and of DNA 15% (P815), 8% (YAC1), and 10% (L929). Duplicate samples in three separate experiments gave similar results.

α-toxin and Cr release may be a secondary event. The opposite appears to be the case when L929 cells are used as targets under identical conditions (Fig. 1, *E* and *F*). 10 μg toxin cause already 60% Cr release accompanied by <10% DNA release. Cr release is observed very early and only after 8 to 16 h accompanied by comparable DNA breakdown. YAC cells occupy a position between the two types of target cells described, in that Cr release and DNA release are parallel. YAC cells are more resistant to lysis by the same dose of toxin compared with P815, which in turn are more resistant than L929 cells.

To ascertain that the observed DNA release is not the result of artifactual DNA degradation caused by the manipulation of cells during the labeling procedure and to assure that the iodine γ-irradiation itself is not causing DNA degradation (37), the experiments were also carried out with unlabeled target cells. DNA degradation was determined directly by phenol extraction and agarose gel electrophoresis of soluble (cytoplasmic) and insoluble (nuclear) DNA. In these experiments, 10^7 cells were incubated in tissue culture flasks in a volume of 5 ml with 100 μg and 2.5 mg α-toxin (20 μg and 500 μg/ml) for 3 h. These values are in the range of the high and low end of the dose-response curve in Fig. 1. The cells after incubation were harvested, lysed with NP-40 as usual, and fractionated by centrifugation for 30 s at 10,000 rpm into nuclei (pellet) and cytoplasm (supernatant). Both fractions were extracted with phenol and the DNA separated by agarose gel electrophoresis. The results are shown in Fig. 2. Both L929 and P815 cells were used in this experiment. The control cells were treated the same way without adding α-toxin during the incubation period. The results are quantitated in the left panel of Fig. 2 by densitometric substraction of the spontaneous DNA degradation in control cells. It can be seen in Fig. 2 that DNA breakdown in L929 requires a high dose of toxin (500 μg/ml, lane *B*) when compared with P815 where maximal breakdown of DNA is observed with 20 μg/ml

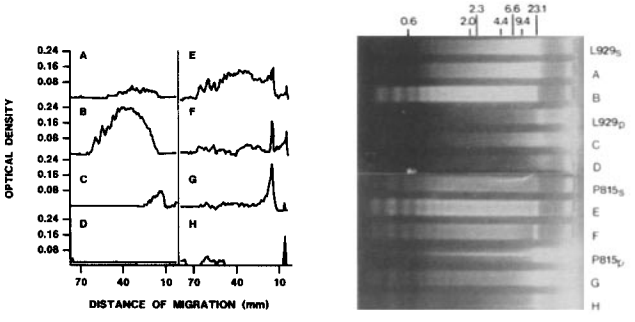

FIGURE 2. DNA degradation in L929 and P815 mediated by α-toxin as measured by nonisotopic assays. (*Right*) Analysis of DNA fragments by agarose gel electrophoresis and ethidium bromide staining. Lanes *L929*$_s$, *A*, and *B* show soluble (cytoplasmic) DNA in control cells, and after incubation with 20 μg/ml (*A*) and 500 μg/ml (*B*) α-toxin. *L929*$_p$, *C*, and *D* show the corresponding samples of insoluble (nuclear) DNA. *P815*$_s$, *E*, and *F* as well as *P815*$_p$, *G*, and *H* are soluble and insoluble DNA, respectively, of P815 incubated with 20 μg/ml and 500 μg/ml α-toxin. The numbers on top represent the position of DNA molecular weight markers in kilo base pairs. (*Left*) Densitometric scan of the samples in the right panel, subtracting simultaneously the background of cells incubated without α-toxin.

(lane *E*). The typical pattern of nucleosomal DNA fragments is observed. At a high dose of toxin in lane *F*, less DNA is detectable in the cytoplasmic fraction of P815 because most of the fragments have already been released into the tissue culture medium.

DNA degradation under these nonisotopic conditions is quite comparable to the data reported above in Fig. 1. In addition, it can be seen that the nuclear (insoluble) DNA of α-toxin-treated samples, although not released, is nevertheless substantially degraded in P815 (lanes *G, H*). As in the experiments with trace-labeled cells, P815 cells appear to be much more sensitive to DNA degradation than L929 cells at low α-toxin concentrations.

α-Toxin affords the opportunity to investigate several biochemical and cell physiological parameters of DNA degradation induced by pore formers. Fig. 3 summarizes an extensive series of such experiments with the two target cells P815 and L929 measuring both DNA and Cr release mediated by α-toxin. The dashed and dotted lines represent the control release of the two markers under standard conditions in the presence of Ca ions. If the target cells are pretreated for 1 h with 5 mM EGTA to deplete intracellular Ca, and the assay run in the presence of 1 mM EGTA, DNA release is eightfold decreased in P815 and completely abolished in L929. Cr release, in contrast, is increased twofold for P815, and from 60 to 80% for L929 cells. DNA release can be abolished also in P815 when the target cells are loaded with Quin 2, an intracellular Ca chelator (1.7 mM intracellular concentration). Again, Cr release is similarly enhanced as with EGTA alone. These experiments indicate that intracellular Ca in the target cell is essential for the triggering of DNA breakdown. Ca is not required for Cr release, indeed Cr release by α-toxin is enhanced in the absence of Ca (see also Fig. 4).

In the next group of experiments, lysosomotropic agents were tested for their effect on DNA degradation and Cr release. Pretreatment of target cells with chloroquine, NH_4Cl, and monensin reduces DNA degradation by two- to eightfold compared with the control level. Cr release is not affected by this treatment.

In the final group, metabolic blockers were tested for their effect on Cr and DNA release. Sodium azide, blocking the respiratory chain, and deoxyglucose, blocking glycolysis, alone and in combination, had no effect on the release of either marker by α-toxin.

Fig. 4 further defines the role of Ca in the Cr release caused by α-toxin. A com-

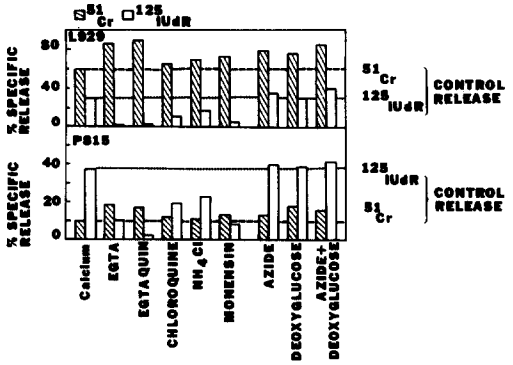

FIGURE 3. Effect of Ca chelators, lysosomotropic agents, and metabolic blockers on DNA degradation and Cr release by α-toxin. The concentration of the agents and incubation conditions are described in Materials and Methods. The release of Cr and of DNA under standard conditions (30 μg/assay α-toxin, 3 h, 1.3 mM Ca present) is indicated by the horizontal lines. (*Hatched bars*) Cr release; (*open bars*) DNA release.

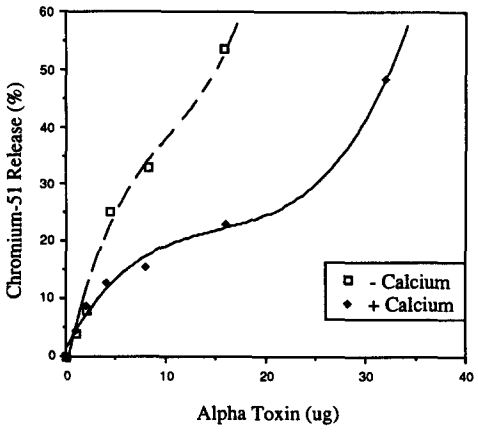

FIGURE 4. Effect of Ca depletion on the Cr release mediated by α-toxin. Cr-labeled Raji target cells were preincubated with EGTA (*open squares*, – *Calcium*) or in Ca-containing medium (*closed squares*, +Calcium) and then incubated with α-toxin in the absence or presence of Ca for 3 h at 37°C.

plete dose-response of α-toxin is shown using EGTA-pretreated and normal Raji cells as targets. In the presence of intra- and extracellular Ca, approximately twice the dose of α-toxin is required to achieve 50% Cr release compared with Ca-depleted cells. This indicates that in the absence of Ca, target cells are much more sensitive to lysis by pore formers in accordance with previous studies (38).

These studies together show that Ca ions on the target cell level have a dual effect: Lack of Ca in the target cell eliminates DNA degradation. At the same time, however, Ca depletion increases the susceptibility of the target cell to lysis by pore formers, as measured by Cr release.

In addition to intracellular Ca, an acidic, intracellular compartment is necessary for DNA degradation to ensue effectively, whereas metabolic blockers do not interfere.

Cooperation of TNF and Pore Formers in DNA Degradation. In previous studies, it was shown that lymphotoxin can induce DNA degradation in target cells (39, 40). The kinetics of DNA breakdown are accelerated when lymphotoxin is rapidly taken up by the target cell through pinocytosis (41). TNF is structurally and functionally related to lymphotoxin (42). We determined whether the combination of α-toxin and TNF had any influence on DNA degradation to test the question whether transmembrane channels can facilitate the uptake of other molecules. Using the two targets P815 and L929 increasing amounts of α-toxin were combined with a constant amount of TNF. As shown in Fig. 5, the addition of 2,000 U/ml rTNF to the assay almost doubled the amount of DNA release in both P815 and L929; Cr release was not affected. TNF alone without α-toxin (dashed line in Fig. 5) up to 5,000 U/ml did not cause DNA or Cr release within the 3-h period of the assay. The effect of TNF in combination with α-toxin was detectable down to 500 U/ml (not shown).

DNA Release Mediated by Isolated Perforin and LAK Cells. Isolated perforin was purified from granules as described (20) and used as pore former in the presence of Ca with the same target cells described before for α-toxin. DNA release and Cr release was determined. Fig. 6 shows that the ratio of DNA release and Cr release in the three target cells mediated by perforin is similar to that reported above for α-toxin.

To investigate the role of intracellular Ca in target cell DNA degradation mediated by intact LAK cells, target cells were loaded with Quin 2 (1.7 mM intracellular concentration) in order to chelate intracellular Ca. Either Ca entering the cell through

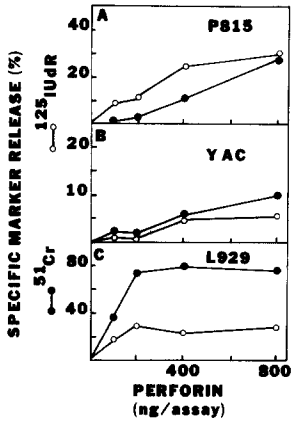

FIGURE 5. DNA and Cr release in P815, YAC1, and L929 mediated by purified murine perforin. The incubation time was 4 h; spontaneous Cr release was 8% (P815), 8% (YAC1), and 13% (L929); spontaneous DNA release was 13% (P815), 7% (YAC1), and 10% (L929).

transmembrane flux or liberated from intracellular Ca stores will be chelated by the intracellularly trapped Ca chelator. As target cells, EL4 were selected because they are efficiently lysed in the xenogeneic combination of human LAK and mouse targets even without addition of lectins. Murine targets are advantageous because DNA release is easy to determine owing to double strand breaks of DNA in these cells. Fig. 7 shows that intracellular chelation of Ca in the target cell with Quin 2 strongly inhibits DNA degradation induced by LAK cells (panels *C* and *D*). In contrast, Cr release is not significantly affected by the lack of intracellular Ca in the target cell. Inhibition of DNA release is seen after 30 min and after 150 min of incubation. At higher killer/target ratios (100:1), DNA release, but not Cr release, is suppressed, confirming previous observations in NK cell-mediated DNA degradation (43).

Discussion

This study reports several new aspects in the induction of DNA degradation of target cells. The first important observation is the finding that purified pore-forming proteins in the absence of additional proteins can cause DNA degradation in susceptible target cells. The two pore formers studied, α-toxin and perforin, gave similar results. In P815 targets, the ratio of DNA/Cr release is >1, in L929 it is <1, and in YAC it is ~1 with either pore former. It is of interest to note that similar ratios

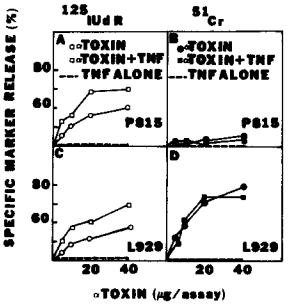

FIGURE 6. Enhancement of α-toxin-mediated DNA degradation by rTNF in P815 and L929. 10^4 labeled target cells in 0.2 ml were preincubated for 20 min with 2,000 U rTNF and then various amounts of α-toxin were added and further incubated for 3 h. (*A* and *C*) DNA release of TNF alone (*dashed line*) of α-toxin alone (*open circles*), and of toxin plus TNF (*open squares*). (*B* and *D*) Effect of the same combinations on Cr release. The spontaneous Cr release was 11% (P815) and 8% (L929); spontaneous DNA release was 15% (P815) and 13% (L929).

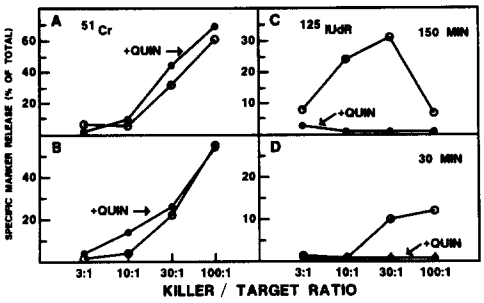

FIGURE 7. Inhibition of DNA-degradation mediated by LAK cells by intracellular Quin 2. Radiolabeled EL4 target cells were loaded with 1.7 mM intracellular Quin 2 (*closed circles*) or used without Quin 2 loading (*open circles*). Assays were done in the presence of normal concentrations of extracellular Ca (1.3 mM) at various killer/target ratios for 150 min (*A* and *C*) or 30 min (*B* and *D*). Cr release (*left panels*) and DNA release (*right panels*) are shown.

of DNA/Cr release were observed by Howell and Martz (9) when murine CTL were used as effectors of P815 and L929 lysis.

The finding that purified pore formers can cause DNA degradation in susceptible target cells addresses a controversial subject: several groups have found that the poreforming proteins of complement do not cause DNA degradation (3, 5, 10) in contrast to an earlier publication (12) describing single-strand breaks in targets attacked by complement. Similarly cytolytic granules were reported to cause DNA degradation (27) or to lack this activity (13). Studies with isolated pore-forming proteins such as perforin or α-toxin have not been reported to date. The distinction between isolated proteins and a protein system such as complement in serum or perforin in granules may be important. An increase in the density (clustering) of transmembrane channels delivered per unit surface area results from the concentrated action of protein systems when compared with single purified pore formers. Another important factor is the time span in which these hits are delivered and the repair potential of the target. All these factors can contribute to the divergent results reported previously (3, 5, 10, 12, 13, 27).

For the target cell to initiate the internal program of DNA degradation, a certain (unknown) time will be required. If the target membrane is irreparably damaged before this critical time, no DNA degradation will occur because essentially all soluble components will leak out of the target cell. When isolated granules attack a membrane, a package of highly concentrated perforin will perforate the membrane many times in a confined area. This reaction takes place in a very short time span of <1 min. It is probably difficult for the cell to repair such a cluster of pores over a confined area. If the same number of pores had been distributed over the total cell surface area, the cell conceivably could have repaired the damage by endocytosis. Both granules and complement will create clustered membrane pores. In contrast, α-toxin or purified perforin are not clustered and thus will randomly insert into target cells and create predominantly small incomplete ring lesions. In this situation, chances for a longer survival of the target are more favorable, permitting the initiation of the program for DNA degradation, presumably through increased and sustained intracellular Ca levels. It is possible even that the original membrane lesion is repaired, however the target cell goes on to die because the program of DNA degradation has been initiated and cannot be turned off.

In CTL lysis, granules may also be delivered to the target cell yet highly efficient DNA degradation is usually observed except at high killer/target ratio (13, 38) (Fig. 7). This may suggest the secretory process of CTL is substantially slower than the addition of isolated granules to target cells. Only at a high killer/target ratio, do

the secretory events approach the efficiency of isolated granules and DNA degradation is decreased due to early and extensive membrane damage.

The second insight from this study concerns the role of Ca ions in DNA degradation supporting similar conclusions by Allbritton et al. (27). These authors also found that the potassium ionophore valinomycin could trigger DNA degradation. The pore formers used in the present study also affect the distribution of potassium across the membrane and this effect could contribute to DNA degradation. Intracellular Ca, however, is absolutely required for triggering of DNA release. Treatment of target cells with EGTA alone could not completely eliminate DNA release in active DNA degraders, such as P815. Intracellular Quin 2 on the other hand totally eliminated any DNA degradation by α-toxin and by LAK cells. This would suggest that treatment of cells with EGTA alone, which is membrane impermeable, is not sufficient to completely deplete intracellular Ca (35). The absence of Ca ions increases Cr release even though it abolishes DNA degradation. We attribute the increased sensitivity of cells to lysis by pore formers in the absence of Ca to an impaired repair mechanism. As has been shown by Shin and Carney (44), transmembrane channels are removed from the cell surface of nucleated cells by endocytosis. Endocytosis is triggered by the influx of Ca ions through transmembrane channels. In the absence of Ca, the channels persist, and cells lyse owing to the lack of repair.

The importance of an acidic compartment in DNA degradation points to a role for the lysosomes in the process of triggering the nuclear break down. It is not clear at the present time how the Ca signal and the lysosome participation are linked.

The observation of cell lysis in the absence of DNA degradation raises the question of the contribution of DNA degradation to cell death. Lysosomotropic agents that significantly reduce DNA degradation do not affect Cr release. Thus, in the short-term assays used here, DNA degradation does not seem to contribute to cell lysis. In longer term assays (Fig. 1 *B*) it is clear, however, that cell lysis as measured by Cr release will ensue to the same extent as the original DNA release.

The next point of interest is the finding that pore formers and other proteins can cooperate in the triggering of DNA degradation. We used in this study TNF that is believed to act similar to lymphotoxin in triggering DNA degradation. The action of TNF in DNA degradation is also dependent on Ca ions and is inhibited by lysosomotropic agents (not shown). Due to its molecular size, it is highly unlikely that TNF could penetrate the transmembrane channel of α-toxin and thereby gain entry into the target cell. Rather, it is more probable that it is taken up by pinocytosis during endocytotic membrane repair. It has been shown previously that stimulation of pinocytosis in the presence of lymphotoxin causes rapid DNA degradation (42). However, other interpretations are possible and further clarification will be needed on this point.

Finally, the studies with perforin confirm that purified pore formers can mediate DNA degradation. Human LAK cells that have been shown to contain perforin (31) are active in triggering DNA release from murine target cells. DNA release is blocked by loading target cells with Quin 2, whereas the Cr release is unaffected.

We have shown that pore formers alone, and in cooperation with other factors, can induce DNA degradation in target cells. These findings may be taken into account in attempts to interpret the molecular mechanism of DNA degradation induced by CTL and NK cells. It appears that lysis by purified pore formers has many properties that are quite similar to the properties of CTL- and NK-mediated lysis.

Summary

This study investigates the effect of the purified membrane pore formers, staphylococcal α-toxin and CTL perforin, on target cell lysis as measured by ^{51}Cr release and on nuclear damage as measured by DNA degradation and $^{125}IUdR$ release. Both pore formers cause dose-dependent cell lysis, which is accompanied by DNA release. The ratio of DNA/Cr release depends on the nature of target cell and shows the same pattern as the ratio of release of the two markers reported for CTL-mediated lysis of the same targets. DNA degradation is dependent on the presence of intracellular Ca in the target cell and is totally blocked if Ca is chelated by Quin 2 intracellularly and EGTA extracellularly. DNA degradation, in addition, is inhibited by the lysosomotropic agents NH_4Cl, chloroquine, and monensin. rTNF doubles the degree of DNA degradation mediated by α-toxin in 3-h assays. We conclude that pore formers alone can mediate DNA degradation. In addition, they may promote the uptake of other factors and thereby accelerate their time course of action. DNA degradation by pore formers requires active target participation in a pathway that is dependent on intracellular Ca and lysosomes. These aspects of target lysis resemble CTL- and NK cell-mediated cytolysis.

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