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Endogenous endonuclease-induced DNA fragmentation: An early event in cell-mediated cytolysis

(cytotoxic T lymphocytes/cell death/calcium/zinc)

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ABSTRACT **Within minutes of exposure of target cells to cytotoxic T lymphocytes, their nuclear DNA begins to be fragmented. This phenomenon precedes ^{51}Cr release by at least an hour. DNA fragmentation occurs only when appropriately sensitized cytotoxic T cells are used and is not merely a result of cell death because killing of target cells by heating, freeze/thawing, or lysing with antibody and complement did not yield DNA fragments. Agarose gel electrophoresis of target cell DNA showed discrete multiples of an approximately 200-base-pair subunit, suggesting that fragmentation was the result of activation of a specific endonuclease. A similar pattern of DNA fragments is observed during glucocorticoid-induced killing of mouse thymocytes. The endonuclease in that case is inhibited by zinc ions, and we find that Zn^{2+} also inhibits DNA fragmentation and ^{51}Cr release induced by cytotoxic T cells, suggesting a final common biochemical pathway for both types of cell death.**

The mechanism by which cytotoxic T lymphocytes (CTL) induce lysis of target cells has been widely studied but for the most part remains unresolved. Cell-mediated cytolysis has been reported to require calcium and magnesium ions (1, 2) and takes place in at least three distinguishable stages (2–5). The first step requires Mg^{2+} or Ca^{2+} and involves cell recognition and establishment of stable cell contact. The second step is strictly calcium dependent and constitutes the "lethal-hit" stage, during which the target cell is irreversibly "programmed for lysis" (2, 5–7). The third step involves effector cell-independent target cell disintegration, wherein homeostasis breaks down and cytoplasmic macromolecules are released into the incubation medium. All of these steps appear to be independent of synthesis of protein (8), RNA (3), and DNA (1).

One of the most interesting hypotheses concerning the mechanism of specific cell-mediated cytolysis has been suggested by the work of Wyllie *et al.* (9) and involves apoptosis, the so-called "programmed cell death," characterized by chromatin condensation in the nuclei of the condemned cells. Microcinematography and electron microscopic studies have indicated that target cells being acted upon by CTL undergo apoptosis (10–13). These observations strongly support the suggestion of Russell's group (14) that CTL-mediated killing is the result of "internal disintegration" rather than osmotic lysis.

Wyllie, in a study of programmed cell death in thymocytes (15), showed that these cells can be induced to become apoptotic by glucocorticoids. DNA fragmentation accompanied the morphological appearance of apoptosis: the fragments obtained were discrete multiples of an approximately 180-base-pair subunit. Work in our laboratory has confirmed these observations and has shown that the endonuclease is present in isolated nuclei of normal thymocytes; is activated by incubating nuclei with Ca^{2+} and Mg^{2+}; and is inhibited by Zn^{2+} (16).

In the present study we have investigated the condition of DNA in either P815 tumor cells or concanavalin A (Con A)-stimulated lymphoblasts after their interaction with *in vitro* generated allogeneic CTL. We find that DNA fragmentation in the target cell nuclei precedes ^{51}Cr release, appears to be mediated by a specific endonuclease, and is inhibited by zinc ions but not by inhibitors of protein synthesis. DNA fragmentation in target cells is specifically related to cell-mediated cytolysis in that freeze/thawing, heating, and lysing of cells with antibody and complement did not yield DNA fragments. The fragments of DNA obtained appear to be identical to those seen in glucocorticoid-treated thymocytes.

MATERIALS AND METHODS

Animals. BALB/c ($H\text{-}2^d$ haplotype) and CBA/J ($H\text{-}2^k$) male mice, 8–15 weeks old, were used as spleen donors. Mice were purchased from The Jackson Laboratory.

Tissue Culture Media. RPMI 1640 culture medium (Flow Laboratories) supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (50 μM), and L-glutamine (2 mM), was used as tissue culture medium (TCM) for the *in vitro* generation of alloreactive CTL and Con A-stimulated T cell blasts (see below).

Cells. Mice were killed by cervical dislocation. Spleens were obtained under aseptic conditions and placed in sterile Hanks' balanced salt solution (HBSS) on ice. Single-cell suspensions were prepared by gently pressing these organs through wire mesh screens followed by two passages through a 25-gauge hypodermic needle. Viability of cell preparations was assessed by eosin exclusion. P815 murine mastocytoma cells (DBA/2 origin; $H\text{-}2^d$) were maintained in our laboratory in 10 ml of Eagle's medium supplemented with 5% heat-inactivated fetal calf serum, L-glutamine, penicillin, and streptomycin. Cells were grown in 25-cm^2 plastic culture flasks (Falcon).

Con A-Stimulated T-Cell Blasts. Con A-stimulated T-cell blasts for use as target cells were prepared by incubation of freshly obtained spleen cells (3×10^6/ml) in 10 ml of TCM containing Con A at 2.5 μg/ml for 2–3 days in 25-cm^2 plastic culture flasks.

Effector Cells. Responder spleen cells (7×10^6/ml) and irradiated (3,000 rads; 1 rad = 0.01 gray) allogeneic stimulator spleen cells (1×10^6/ml) were cultured together in 24-well tissue culture plates (Costar, Cambridge, MA) in 2 ml of TCM per well. After 5 days, the cells were harvested by vigorous pipetting and centrifugation (200 × *g*; 10 min). Effector cells were resuspended in TCM; viable cells were counted and diluted appropriately for use in CTL assays.

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Abbreviations: Con A, concanavalin A; CTL, cytotoxic T lymphocyte(s); E:T, effector-to-target cell ratio.

^{51}Cr Labeling of Target Cells. Cells to be used as targets in ^{51}Cr release assays were suspended at 5×10^6 in 100 μl of RPMI 1640 medium containing 1% heat-inactivated fetal calf serum to which 100 μCi (1 Ci = 3.7×10^{10} Bq) of ^{51}Cr (sodium chromate, New England Nuclear) were added. The cells were then incubated for 90 min at 37°C, washed twice in HBSS, resuspended in 10 ml of HBSS, and incubated on ice for 30 min. They were washed once more, their viabilities were determined, and they were diluted in TCM for use.

Radiolabeling Target Cell DNA. P815 tumor cells (2×10^6/flask), subcultured 2 days previously, or 2-day Con A-stimulated T cell blasts (see above) were incubated with either 10–20 μCi of [^{3}H]thymidine ([^{3}H]dThd; New England Nuclear) or 1–15 μCi of ^{125}I-labeled 5-iododeoxyuridine ([^{125}I]IdUrd; New England Nuclear) per flask for 6–24 hr at 37°C. The cells were washed extensively in TCM, incubated 60 min on ice, and washed once before use. Viabilities were determined and appropriate dilutions for assay prepared in TCM.

^{51}Cr Release Assay. ^{51}Cr-Labeled target cells (10^4 cells in 100 μl) were placed in individual wells of 96-well round-bottomed microtiter plates (Linbro) that contained various concentrations of effector cells in triplicate in 100 μl of TCM. The plates were centrifuged (50 × g; 5 min) to establish cell contact and incubated at 37°C for various times prior to harvest. At the end of the incubation period, the plates were centrifuged at 200 × g for 10 min and 100 μl of cell-free supernatant was collected from each well. Radioactivities in supernatants were measured in a gamma counter. Percent specific release was calculated by the following formula:

$$\% \text{ specific } ^{51}\text{Cr release} = \frac{\text{cpm}_{exp} - \text{cpm}_{spont}}{\text{cpm}_{max} - \text{cpm}_{spont}} \times 100.$$

Maximal release (cpm_{max}) was determined from supernatants of cells that were subjected to three rounds of freezing (−70°C) and thawing (37°C). Spontaneous release (cpm_{spont}) was determined from target cells incubated without added effector cells.

DNA Fragment Assay. DNA fragmentation in target cells was determined by a modification of the method of Wyllie (15). One hundred thousand [^{3}H]dThd- or [^{125}I]IdUrd-labeled target cells were placed in 15 ml Corex tubes (Corning) and various concentrations of effector cells were added for a final volume of 1.0 ml. The tubes were centrifuged at 50 × g for 5 min to establish cell contact and incubated at 37°C for various times. The cells were then harvested by centrifugation at 200 × g for 10 min and the incubation medium was carefully withdrawn and saved. In later experiments, 2×10^6 freshly obtained, unlabeled spleen cells were added to tubes containing fewer than 10^6 cells prior to harvesting to provide adequate cell numbers for efficient separation of incubation medium from labeled target cells. The pelleted cells were then lysed with 25 mM sodium acetate buffer, pH 6.6, and the lysates were subjected to centrifugation (27,000 × g; 15 min) to separate intact chromatin from fragmented DNA. The radioactivities of target cell DNA in the incubation medium, in the 27,000 × g supernatant, and in the 27,000 × g pellet were determined in either a beta or a gamma counter, depending on the labeled nucleoside employed. Specific DNA fragmentation was calculated by the following formula:

$$\% \text{ specific DNA fragments} = \frac{\text{cpm}_{frags,\ exp} - \text{cpm}_{frags,\ spont}}{\text{cpm}_{total} - \text{cpm}_{frags,\ spont}} \times 100,$$

in which cpm_{frags} = the cpm in the incubation medium plus the cpm in the 27,000 × g supernatant, and cpm_{total} = cpm_{frags} plus the cpm in the 27,000 × g pellet. Spontaneous fragment production ($cpm_{frags,\ spont}$) was calculated by using target cells incubated without added effector cells.

DNA Preparation. In addition to quantitation of percent DNA fragmentation, the size distribution of the DNA in target cell nuclei, and of the DNA released into the medium, was directly investigated. Nuclei were prepared by the method of Miovic and Pizer (17). In brief, cells were harvested by centrifugation (200 × g; 10 min) and resuspended in 2.5 ml of buffer containing 150 mM NaCl, 10 mM $MgCl_2$, and 10 mM Tris at pH 7.6. Triton X-100 (final concentration, 0.2%) and dithiothreitol (final concentration, 0.2 mM) were added, and the cells were homogenized in a Dounce homogenizer (Bellco, pestle B) and layered over 2 ml of 25% (vol/vol) glycerol containing 10 mM Tris at pH 7.6, 5 mM $MgCl_2$, and 0.2 mM dithiothreitol. The tubes were centrifuged at 200 × g for 10 min and the nuclear pellet so obtained was lysed immediately by the addition of 2 ml of buffer containing 100 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.5% Nonidet P-40, and 0.5% $NaDodSO_4$, pH 7.4. DNA from lysed nuclei, as well as from incubation medium, was extracted twice with an equal volume of phenol for 3 hr followed by a single extraction with an equal volume of chloroform for 10 min. After each extraction, DNA was recovered in the aqueous phase after centrifugation at 750 × g for 5 min. The DNA was dialyzed exhaustively against a buffer containing 10 mM Tris and 1 mM EDTA, pH 7.6, and concentrated to 100 μl.

Agarose Gel Electrophoresis. Horizontal electrophoresis of DNA was performed for 12 hr (4°C) at 70 V in 0.75% agarose. The running buffer contained 90 mM Tris, 90 mM boric acid, and 1.5 mM EDTA, pH 8.4. Samples were mixed with a stop buffer to give a final concentration of 1.5 mM EDTA, 0.2% $NaDodSO_4$, 5% (vol/vol) glycerol, and 0.1% bromphenol blue and heated (65°C; 10 min) prior to electrophoresis. [^{125}I]IdUrd-labeled target cell DNA was visualized by autoradiography using Kodak XAR film.

RESULTS

CTL Induction of DNA Fragmentation. To investigate the changes in target cell DNA during cell-mediated cytolysis, P815 murine mastocytoma cells labeled cytoplasmically with ^{51}Cr or in their DNA with [^{3}H]dThd were mixed with CBA anti-BALB/c or BALB/c anti-CBA cytotoxic cells at various effector-to-target (E:T) ratios and incubated at 37°C in 5% CO_2. Specific DNA fragmentation was determined after 90 min of incubation and specific ^{51}Cr release after 4 hr. The results of a representative experiment are summarized in Table 1. With increasing numbers of the appropriate effector cells (CBA anti-BALB/c) added to a constant number of labeled target cells, there was a concomitant increase both in the specific DNA fragments obtained

Table 1. DNA fragmentation and ^{51}Cr release from P815 cells

Effector	E:T ratio	% specific DNA fragments	% specific ^{51}Cr release
CBA anti-BALB/c	1.25:1	1	13
	2.5:1	4	24
	5:1	21	40
	10:1	46	71
	20:1	63	84
BALB/c anti-CBA	40:1	20	27

P815 cells labeled with either [^{3}H]dThd or ^{51}Cr were incubated with either CBA anti-BALB/c or BALB/c anti-CBA cytotoxic T lymphocytes at various E:T ratios. Specific DNA fragmentation was determined after 90 min and ^{51}Cr release after 4 hr. Spontaneous DNA fragmentation was 38–43%, while ^{51}Cr release was 24%. Each point represents the mean of triplicate measurements.

and in the specific ^{51}Cr released from the targets. Only low crossreactivity was observed when the inappropriate effector cells (BALB/c anti-CBA) were added. Similar results were obtained when Con A-stimulated T cell blasts were used as targets (data not shown).

To establish that the DNA fragmentation observed was associated with specific cell-mediated cytolysis and was not simply a nonspecific sequel of cell death, the experiments summarized in Table 2 were done. Heat-killing, freeze/thawing, and lysis with anti-H-2^d antibody and complement, while causing significant ^{51}Cr release (77–90%), did not cause measurable DNA fragmentation in the target cells. It appeared from these results that the DNA fragmentation observed when P815 cells were incubated with CBA anti-BALB/c CTL was induced by the appropriate effector–target cell interaction and was not merely the result of cell death.

Kinetics of DNA Fragmentation vs. ^{51}Cr Release. Having found that specific DNA fragmentation after 90 min was quantitatively similar to specific ^{51}Cr release after 4-hr incubation of target cells with CTL generated *in vitro*, we performed experiments to examine the time sequence of both measures of cell-mediated cytolysis. [^{125}I]IdUrd-labeled Con A-stimulated BALB/c T cell blasts (10^5 cells) were incubated for various times with either CBA anti-BALB/c or BALB/c anti-CBA CTL (Fig. 1). Again, DNA fragmentation was observed only when the appropriate cytotoxic T cells were used. These results indicate that DNA fragmentation is an early event in cell-mediated cytolysis, preceding ^{51}Cr release by at least 60 min.

Electrophoresis of Target Cell DNA. After interaction with CTL, DNA in target cells is fragmented, as evidenced by its failure to sediment upon centrifugation at 27,000 × *g* for 15 min. To ascertain the size of the DNA fragments, nuclear DNA was isolated from target cells after incubation (90 min; 37°C) alone or with CTL and examined by electrophoresis in 0.75% agarose (Fig. 2*A*). Only intact DNA was observed when [^{125}I]-IdUrd-labeled P815 cells were incubated alone (lane 1) or with inappropriate effector cells (lane 8), whereas a decreasing amount of intact DNA was seen with increasing numbers of CBA anti-BALB/c CTL added (lanes 2–7). When these appropriate effector cells were used, much of the target cell DNA migrated as discrete bands which, by comparison to DNA markers, were multiples of an approximately 200-base-pair subunit.

An unusual observation was made when CBA anti-BALB/c CTL were incubated with P815 cells at an E:T ratio of 40:1. Even though there was a significant decrease in the amount of target cell DNA migrating as intact chromosomal DNA, there appeared to be a relative lack of fragmented DNA in target cell nuclei as compared to that seen at lower E:T ratios. In an attempt to discover the fate of the target cell DNA at this E:T ratio, as well as in other conditions, labeled DNA found in the

Table 2. Comparison of DNA fragmentation and ^{51}Cr release induced by various cytotoxic treatments

Treatment	% DNA fragments	% ^{51}Cr release
None	0.5	11
Heating	0.7	77
Freeze/thawing	0.9	90
Antibody + complement	0.6	90

One hundred thousand P815 cells labeled with either [^{125}I]IdUrd or ^{51}Cr were subjected to various treatments: Heating, cells were heated to 56°C for 30 min; freeze/thawing, cells were subjected to three rounds of freezing (−70°C) and thawing (37°C); and antibody + complement, cells were treated with C57BL/10 anti-H-2^d serum for 30 min (4°C) followed by incubation at 37°C for 30 min in the presence of rabbit complement. DNA fragmentation and ^{51}Cr release were assessed immediately after the various treatments.

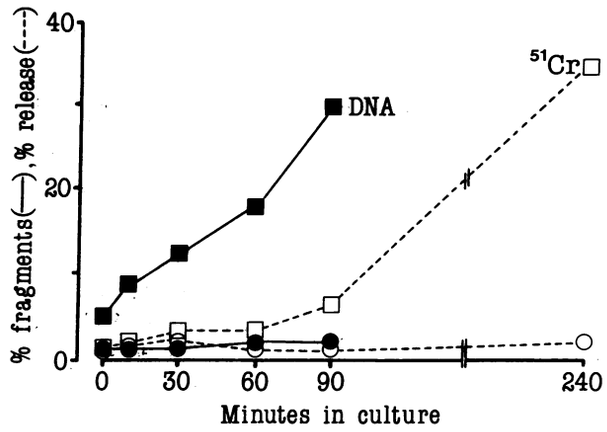

FIG. 1. Comparison of specific DNA fragmentation and ^{51}Cr release from Con A-stimulated T cell blasts. BALB/c Con A-stimulated T cell blast cells labeled with either [^{125}I]IdUrd or ^{51}Cr were incubated with either CBA anti-BALB/c (■, □) or BALB/c anti-CBA (●, ○) CTL at an E:T ratio of 20:1. Specific DNA fragmentation (——) and ^{51}Cr release (----) were determined at various times after incubation at 37°C. The spontaneous DNA fragmentation was 10–15% and spontaneous ^{51}Cr release was 5–20%.

culture medium after incubation of target cells with effector cells for 90 min was examined by electrophoresis (Fig. 2*B*). Very little [^{125}I]IdUrd-labeled DNA was found in the incubation medium when P815 cells were incubated alone (lane 9) or with BALB/c anti-CBA CTL (lane 16). However, when the appropriate CTL were used (lanes 10–15), fragmented DNA was found in the medium. This result indicates that the fragmented DNA missing from the nuclear preparations at the higher E:T ratios appears in the incubation medium and, in addition, validates our inclusion of DNA found in the incubation medium as fragmented DNA in calculating specific DNA fragmentation. The results in Fig. 2 are consistent with the fragmentation observed in target cell nuclear DNA being the result of activation of a specific endonuclease after interaction with cytotoxic T cells. This endonuclease causes the excision of 200-base-pair subunits of target cell DNA.

Inhibition of CTL Activity by Zinc Ions. In earlier experiments (unpublished data) we had found that a similar endonuclease activity present in mouse thymocyte nuclei was inhibited by zinc ions. The experiments reported in Table 3 were done to evaluate the effect of Zn^{2+} on cell-mediated cytolysis. Zinc inhibited both DNA fragmentation and ^{51}Cr release in a dose-dependent fashion, with 800 μM $ZnSO_4$ inhibiting both measures of CTL activity completely. Zinc ions were not lethal to either effector or target cells, because the viabilities of either population were the same after separate incubation with or without 800 μM $ZnSO_4$ in TCM for 4 hr.

Protein Synthesis Is Not Required for CTL Activity. Inhibition of protein synthesis prevented DNA fragmentation and cell death in mouse thymocytes incubated with glucocorticoids (16), presumably by interfering with the activation of endogenous endonuclease in these cells. To investigate the activation of endonuclease in target cells, CTL assays were carried out in the presence or absence of the protein synthesis inhibitors cycloheximide or emetine·HCl (Table 4). Neither cycloheximide nor emetine blocked CTL activity as measured by either DNA fragmentation or ^{51}Cr release, under conditions in which protein synthesis was totally inhibited in glucocorticoid-treated thymocytes. These results indicate that protein synthesis is not required for DNA fragmentation or ^{51}Cr release in target cells after interaction with cytotoxic T cells.

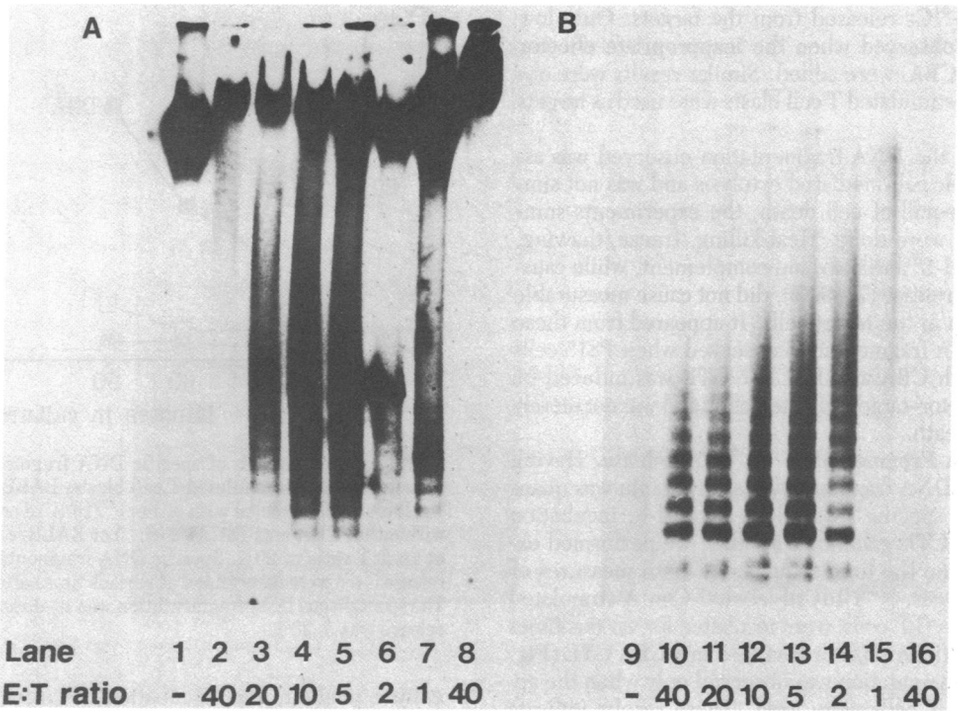

FIG. 2. Agarose gel electrophoresis of target cell DNA. Electrophoresis of DNA isolated from nuclei (*A*) or from incubation media (*B*) of 10^5 [^{125}I]IdUrd-labeled P815 cells after incubation alone (lanes 1 and 9), with appropriate CBA anti-BALB/c effector cells (lanes 2–7 and 10–15), or with inappropriate BALB/c anti-CBA effector cells (lanes 8 and 16). Target cells were incubated with effector cells at various E:T ratios for 90 min at 37°C. Electrophoresis in 0.75% agarose was for 12 hr at 70 V (4°C). DNA was visualized by autoradiography.

DISCUSSION

The results presented in this report strongly suggest that the appearance of a specific endonuclease activity in target cell nuclei is an early event in cytolysis mediated by CTL. Fragmentation of target cell DNA was observed within 10 min of establishment of cell contact and was induced only when CTL of appropriate specificity were used (Fig. 1). Killing of target cells by heating, freeze/thawing, or lysing with antibody and complement (Table 2) did not cause activation of this endonuclease activity. Thus DNA fragmentation is a specific consequence of effector–target cell interaction and not merely a result of cell death. These results support those of Russell and co-workers (14, 18), who have found that a "nuclear lesion" involving degradation of target cell DNA occurs when these cells are lysed by CTL but not by antibody and complement.

Table 3. Effect of zinc ions on CTL activity

Zn^{2+} added, μM	% specific DNA fragments	% inhibition	% specific ^{51}Cr release	% inhibition
None	17	—	20	—
50	15	12	20	0
100	9	47	17	15
200	4	76	8	61
400	0.2	99	4	78
800	0.4	98	0.2	99

CBA anti-BALB/c CTL and P815 cells labeled with either ^{51}Cr or [^{125}I]IdUrd (E:T ratio 50:1) were incubated in the presence or absence of various concentrations of $ZnSO_4$. Specific DNA fragmentation and ^{51}Cr release were determined after 4 hr. The spontaneous DNA fragmentation was 2–3% and spontaneous ^{51}Cr release was 19–22%. Viabilities of either cell population incubated alone in the presence of 800 μM $ZnSO_4$ were identical with those of control cells incubated in TCM alone for 4 hr.

Electrophoretic examination of DNA isolated from target cell nuclei after interaction with CTL revealed discrete bands whose sizes were integral multiples of an approximately 200-base-pair subunit (Fig. 2*A*). Increasing amounts of DNA fragments were observed when higher E:T ratios or longer incubation times were used. Only intact chromosomal DNA was found in the nuclei of target cells incubated alone or with inappropriate effector cells. After 60 min in culture, and before demonstrable ^{51}Cr release, target cell DNA appeared in the incubation medium. This DNA showed the same pattern of degradation as the DNA found in target cell nuclei except that little intact chromosomal DNA was observed (Fig. 2*B*). The mechanism by which DNA fragments are released from target cells, prior to ^{51}Cr release, is not yet known.

Chromatin condensation in the nuclei of target cells has been

Table 4. Effect of protein synthesis inhibitors on CTL activity

Inhibitor	% specific DNA fragments	% inhibition	% specific ^{51}Cr release	% inhibition
	Experiment 1			
None	17	—	20	—
Cycloheximide	13	24	18	10
	Experiment 2			
None	19	—	55	—
Emetine·HCl	22	—	52	5

CBA anti-BALB/c CTL and P815 cells labeled with either [^{125}I]IdUrd or ^{51}Cr at an E:T ratio of 50:1 (Exp. 1) or CBA anti-BALB/c CTL and Con A-stimulated BALB/c spleen cells labeled with either [^{3}H]dThd or ^{51}Cr at an E:T ratio of 20:1 (Exp. 2) were incubated in the presence or absence of either cycloheximide (50 μg/ml) or emetine·HCl (0.1 μM). Specific DNA fragments and ^{51}Cr release were determined after 4 hr in Exp. 1 or after 90 min (DNA) and 4 hr (^{51}Cr) in Exp. 2. The spontaneous DNA fragmentation was 3–6% and spontaneous ^{51}Cr release was 27–32%.

associated with cytolysis mediated by CTL (10–13). This process has been termed "apoptosis" and has also been observed in numerous other cells that are programmed to die (reviewed in ref. 19). Of interest here is the finding that rat thymocytes incubated with glucocorticoids undergo apoptosis and that this involves activation of an endogenous endonuclease which causes the excision of multiples of 180-base-pair subunits of DNA (15). The association between cell death and activation of an endogenous endonuclease has been observed in a number of studies (20–26) which show that DNA fragmentation is a typical event during normal differentiation and morphogenesis. Work in our laboratory has confirmed the results of Wyllie (15), using mouse thymocytes and glucocorticoids. In addition, we have found that the enzyme may be activated in isolated nuclei of thymocytes, spleen cells, and lymph node cells by incubation with Ca^{2+} and Mg^{2+} (16). A similar Ca^{2+}/Mg^{2+}-dependent endonuclease activity has also been described in the nuclei of rat liver cells (20, 21), calf thymocytes (27), and mouse gut epithelial cells (23).

The results presented here, together with the observations of others, suggest that cytotoxic T lymphocytes activate a "death program," which involves the appearance of endonuclease activity, which in turn leads to irreversible damage to the target cell DNA. That the endonuclease activity observed in target cells is the same as that observed in other systems is suggested by the following comparisons: (*i*) the "lethal hit" stage of cell-mediated cytolysis requires Ca^{2+} (2–5, 7) and there is a requirement for calcium both in glucocorticoid-mediated thymocyte killing (28) and in activation of the endogenous endonuclease in isolated nuclei (20, 21, 23, 27); (*ii*) Zn^{2+} inhibits DNA fragmentation in target cells (Table 3) and Zn^{2+} inhibits the endonuclease activity in thymocytes incubated with glucocorticoids and in isolated thymocyte nuclei incubated with Ca^{2+} (16); and (*iii*) DNA fragments found in target cells consist of multiples of approximately 200-base-pair subunits (Fig. 2) and similarly sized fragments are found in thymocytes incubated with glucocorticoids (15, 26) and in isolated nuclei of several cell types incubated with Ca^{2+} and Mg^{2+} (20, 21, 23, 27).

Although activation of an endonuclease-mediated death program may occur in target cells, the mechanism of activation appears to be different from that induced in thymocytes by glucocorticoids. Protein synthesis was required for the activation of the endogenous endonuclease in thymocytes (16); however, inhibitors of protein synthesis had no effect on DNA fragmentation observed in target cells incubated with CTL (Table 4). While an endogenous endonuclease activity may be observed in isolated thymocyte nuclei, no such activity is demonstrable in target or effector cell nuclei prior to their mutual interaction (unpublished data). Further studies are required to localize the source of the endonuclease activity (whether from target or effector cells) observed in target cells after interaction with cytotoxic effector cells. Such studies will provide valuable insights into the mechanisms of cell-mediated cytolysis and possibly other forms of programmed cell death.

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