susceptible to TCR-initiated cell death, the implication is that there may be a specific role for ζη-containing TCRs in the deletion of anti-self thymic T cells. In such a model, the biological responses of T cells at different stages of development might depend on the composition of their TCRs, or there may be stage-specific variation in the cellular response to ζη-TCR occupancy because of altered receptor coupling or cellular receptivity to these signals. It may be possible to test this speculation by quantitating ζ_2 and ζη expression in developing thymocytes.

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Second Cytotoxic Pathway of Diphtheria Toxin Suggested by Nuclease Activity

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Diphtheria toxin (DTx) provokes extensive internucleosomal degradation of DNA before cell lysis. The possibility that DNA cleavage stems from direct chromosomal attack by intracellular toxin molecules was tested by in vitro assays for a DTx-associated nuclease activity. DTx incubated with DNA in solution or in a DNA-gel assay showed Ca^{2+}- and Mg^{2+}-stimulated nuclease activity. This activity proved susceptible to inhibition by specific antitoxin and migrated with fragment A of the toxin. Assays in which supercoiled double-stranded DNA was used revealed rapid endonucleolytic attack. Discovery of a DTx-associated nuclease activity lends support to the model that DTx-induced cell lysis is not a simple consequence of protein synthesis inhibition.

THE KILLING MECHANISM OF DIPHtheria toxin (DTx) is widely accepted as being exclusively linked to its ability to inhibit protein synthesis in eukaryotic cells via adenosine diphosphate (ADP)–ribosylation of elongation factor 2 (*1*). The ADP-ribosyl (ADPr) transferase activity is ascribed to the A domain of cleaved, reduced toxin; the B or receptor-binding domain has no known enzymatic activity. We had identified two relatively early markers for DTx-triggered cytolysis, the appearance of widespread membrane blebbing and extensive prelytic internucleosomal DNA cleavage, and showed that these properties are not associated with protein synthesis inhibition per se but with DTx treatment (*2*). On the basis of these observations, we proposed that DTx might activate an endogenous nuclease-dependent suicide pathway akin to cytotoxic models established for glucocorticoid hormones (*3*), cytotoxic effector cells (*4*), ionizing radiation (*5*), and the compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*6*). We have now investigated the possibility that DTx might itself catalyze target cell DNA degradation rather than serve to activate an endogenous nuclease.

Human U937 target cells begin to show evidence of DNA fragmentation about 5 hours after exposure to DTx (Fig. 1A, lane 4). The characteristic ladder pattern of DNA breakdown is indicative of internucleosomal DNA degradation (*7*), which precedes DTx-triggered cytolysis by 2 to 3 hours (*2*). The early triggering of DNA fragmentation is not observed with cycloheximide (Fig. 1A, lane 5) or sodium azide plus 2-deoxyglucose (Fig. 1A, lane 6), even though these agents lead to rapid and prolonged inhibition of translation activity in U937 cells (*2*). Addition of DTx to DNA extracted from untreated cells also provoked extensive cleavage (Fig. 1A, lane 1). The degradation profile suggests progressive destruction of DNA into small fragments. Sham-treated DNA displayed no loss of integrity (Fig. 1A, lane 2). Since studies of cell suicide processes revealed that endogenous nuclease activation requires Ca^{2+} and Mg^{2+}, the effect of adding these cations was tested. The presence of both cations caused a very marked enhancement of toxin-associated deoxyribonuclease (DNase) activity (Fig. 1B). Detection of nuclease activity in the presence of 1 m*M* EDTA (Fig. 1A, lane 1) required extended incubation.

DTx incubated with lambda phage DNA provoked marked DNA degradation (Fig. 1C, lane 3), whereas buffer fractions (containing no DTx) from the same column elicited no DNA degradation (Fig. 1C, lane 7). Moreover, addition of DTx-specific antibody completely blocked the nuclease activity of the DTx (Fig. 1C, lane 4). The antibody also inhibited purified monomer preparations of DTx. Nonspecific antibodies had no effect on the DTx-associated nuclease activity (Fig. 1C, lane 6).

To discount the presence of nuclease contaminants that might be sensitive to antitoxin inhibition, we subjected samples of "intact" DTx (*8*) and Arg-C–cleaved DTx (*9*) to electrophoresis under reducing conditions on SDS-polyacrylamide gels that contained embedded DNA (Fig. 2). After being washed to remove SDS, the gels were incubated for 24 hours in 40 m*M* tris buffer (*p*H 7.6) with $MgCl_2$ and NaN_3. The absence of ethidium bromide staining was used to detect nuclease activity. As expected (*10*), pancreatic DNase I showed no activity with $MgCl_2$ (Fig. 2, lane c). However, nuclease activity was observed in the cleaved toxin sample at a position corresponding to DTx fragment A (Fig. 2, lane b at 24 hours). After a second 24-hour incubation in buffer containing $CaCl_2$ plus $MgCl_2$, DNase I gave the predicted positive response (Fig. 2, lane d), and an activity corresponding to the A domain of "intact" DTx appeared (Fig. 2, lane a at 48 hours). At this time, the size of the DNA-negative area associated with the A domain of the nicked DTx increased significantly (Fig. 2, lane b at 48 hours). Indeed, the observation that the nuclease

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activity migrating with fragment A increases after toxin proteolysis (Fig. 2) is strong evidence that the activity does not originate from a contaminant.

Serial 24-hour incubations in Ca^{2+} and Mg^{2+} led to progressive increases in the size of the DNA-negative bands. After 11 days, the only DNase-active bands observed were those at a position corresponding to the A domain of DTx (Fig. 2, lanes a and b at 264 hours). The possibility that DNA was being masked (*10*) in such a way as to prevent ethidium bromide intercalation was eliminated as described (*11*).

Labeled DNA was used in subsequent DNA-gel experiments (Fig. 3). Here, gels were incubated in Ca^{2+}- and Mg^{2+}-containing buffer immediately after being washed in tris. Again, toxin that was nicked with endoproteinase Arg-C (Fig. 3, lane n at 24 hours) showed higher levels of fragment A–associated nuclease activity than did intact DTx (Fig. 3, lane u at 24 hours). Progressive destruction of labeled DNA was observed by incubating the gels in fresh Ca^{2+}- and Mg^{2+}-containing buffer after fluorographic analysis at −85°C (Fig. 3). After incubation for 72 hours, a faint band of activity was observed at the position of unnicked whole toxin, and unnicked toxin samples showed more nuclease activity at this position than nicked toxin samples. The activity observed at the position of intact DTx may be low because the B domain of DTx has a propensity to aggregate irreversibly on denaturation, and the whole toxin may be prevented from renaturating on SDS removal. A second faint band was observed in the vicinity of fragment B. With prolonged incubation, this band increased very little in size compared to the activities associated with whole DTx and fragment A. A band of this molecular weight also appears with trypsin-cleaved DTx; its precise identity is unknown. When electrophoresis was conducted in the absence of detergent (where A and B fragments do not separate), the nuclease activity comigrated with DTx (*12*).

Since acquisition of nuclease activity in these assays depends on renaturation, the relative specific activities of the two proteins were assessed by more conventional assays of enzymatic activity. Endonuclease activity was assayed by incubating toxin with closed circular double-stranded DNA (dsDNA). Intact DTx (*8*) at 0.1 μg exhibits significant endonuclease activity in the presence of Ca^{2+} and Mg^{2+}, as demonstrated by the rapid endonucleolytic cleavage of the supercoiled, closed circular form of dsDNA and the simultaneous appearance of unit-sized linear dsDNA (Fig. 4A). In this assay system, further digestion of the unit-sized linear dsDNA can be seen within 1 min of toxin addition. These results, and those obtained with ^{32}P-labeled DNA, establish that DTx-catalyzed DNA degradation occurs by internucleotidyl cleavage and not by the removal of bases. The activity of 0.01 μg of DNase I is shown under identical conditions in Fig. 4B. Comparisons of the activities of DTx and DNase I indicate that in the presence of Ca^{2+} and Mg^{2+}, 1 mol of DNase I is equivalent to 4 mol of DTx (intact or fully cleaved) with this substrate (average of five experiments conducted with our monomeric and commercial DTx preparations; ratios ranged from 1:3 to 1:7, commercial toxin being less active than DTx monomer prepa-

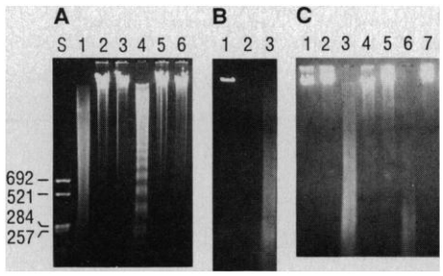

Fig. 1. (**A**) DTx-dependent DNA degradation of cellular DNA in vivo and in vitro. Human U937 cells (ATCC) (1×10^6 per milliliter) were distributed in 96-well microtiter plates (200 μl per well) and incubated in the presence or the absence of "intact" DTx [300 ng/ml; List Biologicals; (*8*)], cycloheximide (1 μg/ml), or NaN_3 (2.5 m*M*) plus 2-deoxyglucose (5 m*M*) for 18 hours at 37°C under specified cell culture conditions (*2*). U937 cell DNA was extracted as previously described (*2*) and separated by electrophoresis for 3 hours at 50 V in a 1.2% agarose gel. DNA was visualized by ethidium bromide staining. In vitro studies were performed with extracted cell DNA (2 μg of DNA in 375 μl of 10 m*M* tris and 1 m*M* EDTA, *p*H 8.0) incubated with or without intact monomer DTx [1 μg; (*8*)] for 18 hours at 37°C before concentration by evaporative centrifugation and electrophoresis (*2*). Lane 1, DTx incubated with extracted DNA; lane 2, extracted DNA control; lane 3, untreated cells; lane 4, DTx incubated with cells; lane 5, cycloheximide-treated cells; and lane 6, cells treated with NaN_3 plus 2-deoxyglucose. Lane S contains molecular weight markers shown in base pairs. (**B**) DTx-associated nuclease activity is stimulated in the presence of Ca^{2+} and Mg^{2+}. Lambda phage DNA (2 μg) was incubated with intact monomer DTx (1 μg) in 10 m*M* tris (200 μl), *p*H 7.6, with or without 5 m*M* each of $CaCl_2$ and $MgCl_2$ for 25 min at 37°C. Lane 1, control DNA; lane 2, DTx with cations; and lane 3, DTx without cations. (**C**) Inhibition of DTx-associated nuclease activity by DTx antitoxin. Lambda phage DNA (5 μg) was incubated in the presence or the absence of DTx (1 μg; List Biologicals) with either equine DTx antitoxin (5 μg; Connaught Laboratories) or equine antiserum to human thymocyte globulin (5 μg; Upjohn) for 25 min at 37°C in 10 m*M* tris (200 μl) supplemented with 5 m*M* $CaCl_2$ and Mg^{2+}, *p*H 7.6. Lane 1, control DNA; lane 2, DTx antitoxin; lane 3, DTx; lane 4, DTx in the presence of DTx antitoxin; lane 5, control antibody; lane 6, DTx in the presence of control antibody; and lane 7, column buffer fraction. The nuclease activity of intact monomer DTx (*8*) was also subject to antitoxin inhibition (data not shown).

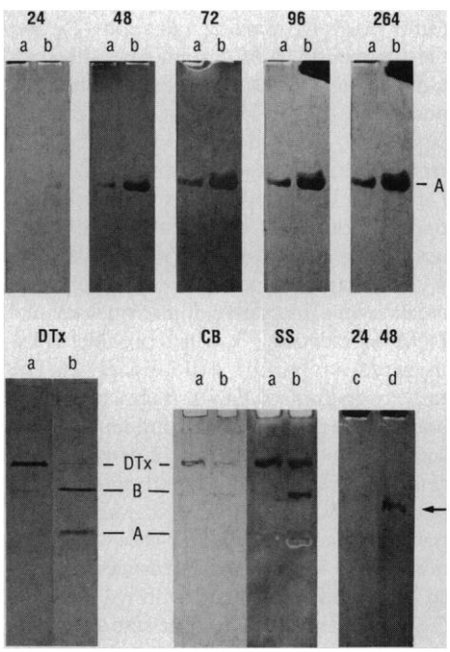

Fig. 2. The nuclease activity of DTx is associated with fragment A. (**Top**) Proteins were solubilized in SDS-sample buffer containing 0.01% β-mercaptoethanol and separated by electrophoresis on a 12.5% polyacrylamide gel (*18*) containing heat-denatured calf thymus DNA (20 μg/ml). DNA gels (*19*) were poured without stacks and run for 1.5 hours at 170 V before loading protein samples [DTx monomer and Arg-C–cleaved DTx monomer, 4 μg each, and bovine pancreatic DNase I (Sigma), 1 μg]. After electrophoresis, the DNase I lane was separated from the toxin lanes, and all gel pieces were washed (three times for 60 min each time) in 40 m*M* tris, *p*H 7.6, before a 24-hour incubation in 2 m*M* $MgCl_2$, 40 m*M* tris, and 0.02% NaN_3, *p*H 7.6, at 30°C. Subsequently, the gels were incubated in buffer containing 2 m*M* $CaCl_2$ and 2 m*M* $MgCl_2$. Every 24 hours the gels were examined for DNase activity after ethidium bromide staining (1 μg/ml; 1 hour) and destaining (1 hour) in 40 m*M* tris, *p*H 7.6. Lanes labeled a contain 4 μg of intact DTx; lanes labeled b contain 4 μg of cleaved DTx. Fragment A nuclease activity is labeled A. (**Bottom**) Before incubation, toxin standards (4 μg intact and 4 μg cleaved DTx) were run on a 12.5% polyacrylamide gel with a 4.5% stack (*18*) and stained with Coomassie blue (DTx). Shown are the 264-hour DNA gel after Coomassie blue (CB) and silver staining (SS), and DNase I (1 μg) activity at 24 hours (c) and at 48 hours (d). The low levels of fragment A observed after prolonged incubation at 30°C reflect unequal leaching from washed gels. The DTx standards under reducing conditions show the relative levels of fragment A present before incubation. The propensity of fragment B to aggregate irreversibly probably retards leaching.

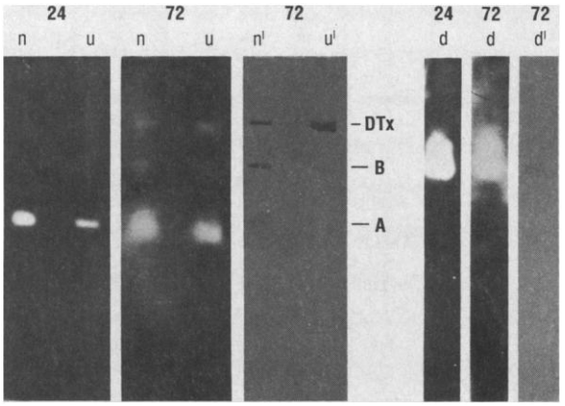

Fig. 3. DNase activity in a ^{32}P-labeled DNA gel. Samples were run on a 12.5% polyacrylamide gel prepared as described in Fig. 2, but with ^{32}P-labeled DNA [2.5 ng per 30-ml gel; sonicated salmon sperm DNA (16,000 cpm/ng) was labeled by the primer-extension method (*20*)]. After the DNase I lane was separated from the toxin lanes, the gel pieces were washed (legend to Fig. 2) and incubated in 40 m*M* tris, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, and 0.04% NaN_3, *p*H 7.6, for 24 hours at 30°C, before autoradiography (−85°C, Kodak XAR film, Dupont Cronex screen). The gel was then thawed, incubated for an additional 48 hours in $CaCl_2$ and $MgCl_2$ buffer, and reexamined for nuclease activity. The DNase activities of DTx monomer (u), cleaved DTx monomer (n), and DNase I (d) are shown after 24 hours or 72 hours. The corresponding lanes after Coomassie blue staining are designated n′, u′, and d′. The DNase I sample was run unreduced in this experiment.

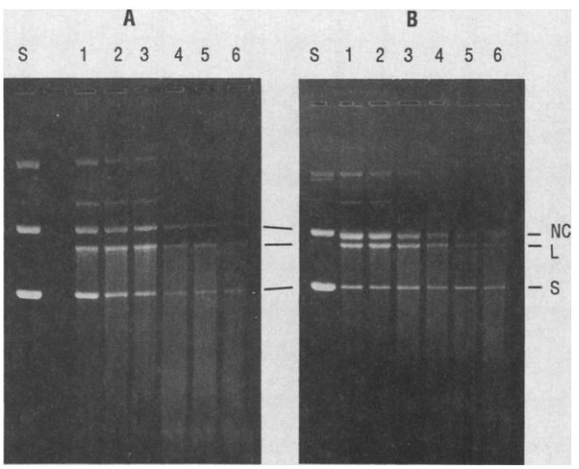

Fig. 4. Endonucleolytic attack of supercoiled DNA. (**A**) At time zero, intact DTx [0.1 μg; (*8*), List Biologicals] was added to pBluescript II DNA (1 μg; Stratagene) in a total volume of 100 μl of 10 m*M* tris supplemented with 2.5 m*M* each of $CaCl_2$ and $MgCl_2$ (*p*H 7.6) at 23°C. At designated times, the digestion was stopped by adding an equal volume of a mixture of chloroform and phenol (50:50), followed by DNA precipitation (*2*) and agarose gel electrophoresis. Lanes 1 to 6 represent incubation times of 1, 5, 8, 10, 15, and 20 min, respectively. Lane S represents an untreated DNA sample. (**B**) As in (A), but with 0.01 μg pancreatic DNase I instead of DTx. NC, nicked circular monomer; L, linear monomer; S, supercoiled pBluescript II DNA.

rations). Inclusion of 0.01% β-mercaptoethanol (*p*H 7.6) had no effect (*12*). Hence, we conclude that cleavage and reduction of DTx are not required for expression of nuclease activity. When the rates of cleavage of ^{32}P-labeled salmon sperm DNA were measured as production of acid-soluble material, equivalent nuclease activity occurred at a 1 to 5.4 molar ratio of DNase I to intact DTx monomer (*13*).

The DNA gel assay results indicate that the nuclease activity of DTx is in the A domain. Thus, DTx cleavage with trypsin or endopeptidase Arg-C always led to higher levels of A-associated nuclease activity (Figs. 2 and 3), as did reduction (10 m*M* dithiothreitol or 0.01% β-mercaptoethanol, *p*H 7.6). Inclusion of 10 m*M* nicotinamide adenine dinucleotide (NAD) in the incubation buffer (*p*H 7.6) had little effect on the activity of DTx in the DNA gel assay. The presence of Ca^{2+} and Mg^{2+} (2 to 2.5 m*M* each) stimulated nuclease activity; EDTA (0.1 to 1.0 m*M*) was inhibitory.

Whether DTx is responsible for all of the internucleosomal cleavage observed in toxin-treated cells (Fig. 1A) is still unclear. It is possible that the chromosomal damage inflicted by DTx is not particularly extensive, but that it exceeds the capabilities of the target cell's DNA repair system. Data obtained with a mutant form of DTx that has no ability to bind NAD indicate that the nuclease active site is not coincident with the ADPr-transferase site (*12*). *Pseudomonas* exotoxin A (PTx), a homolog of the phage-encoded DTx (*14, 15*), also exhibits nuclease activity in the presence of Ca^{2+} and Mg^{2+} (*16*). Domain III of PTx, the counterpart of the A domain of DTx (*14, 15*), contains a sequence (RGYVFVGY) that is similar to the nucleic acid–binding consensus region of heterologous nuclear RNA (hnRNA) binding proteins (*17*). Although the three-dimensional structure of DTx is not known, the structure of the A domain of DTx has been predicted by adjusting the coordinates of domain III of PTx in the context of identified primary sequence homologies between PTx-III and DTx-A (*15*). Even though our preliminary results suggest that the NAD binding site and the nuclease active site are distinct, we do not discount the possibility of some overlap.

Since a strict correlation does not exist between protein synthesis inhibition and cytolysis (*2*), discovery of a DTx-associated nuclease activity lends credence to our hypothesis that DTx-induced cell lysis is inextricably linked to chromosomal degradation. This model deviates from the general model for apoptosis or programmed cell death (*4*) in that the capacity of DTx to participate directly in DNA destruction would bypass the need for an endogenous nuclease–activation mechanism.

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9. Nicked toxin was produced by treating monomeric DTx (105 μg) with 0.5 units of endoproteinase Arg-C (Sigma) in 485 μl of 15 m*M* tris, 150 m*M* NaCl, and 1 m*M* EDTA, *p*H 7.4 (30 min, 37°C).
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13. Ten microliters of DNase I (0.1 mg/ml stock) or DTx (2.1 mg/ml stock) in 150 m*M* NaCl, 50 m*M* tris, and 1 m*M* EDTA, *p*H 7.8, were added to 560 μl of a mixture containing labeled [6250 cpm/μg; (*20*)] sonicated salmon sperm DNA (0.05 μg/μl), 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, and 40 m*M* tris, *p*H 7.6, at 22°C. Portions (50 μl) were taken at 1-min intervals and mixed with 20 μl of calf thymus DNA (2 mg/ml) in 40 m*M* tris, *p*H 7.6, and 70 μl of ice-cold 10% trichloroacetic acid. After 15 min on ice, the samples were spun for 15 min at 18,000*g* in a microcentrifuge. Two portions (50 μl) from each supernatant were counted and averaged. The rate constants expressed per micromolar protein were 0.014 ± 0.004 per min for DTx and 0.081 ± 0.027 per min for DNase I ($n = 4$). Equivalent nuclease activities occurred at DNase I: DTx molar ratios of 1:5.0, 1:5.3, 1:6.0, and 1:6.2.
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" Our portions come in three sizes: bit, byte and megabyte."