18. PCR on reverse-transcribed RNA was as described (*5, 19*). Control reactions in which reverse transcriptase was omitted were done for each sample. Because of the presence of closed circular EBV episomes in some samples, reverse transcription was preceded by treatment with ribonuclease-free deoxyribonuclease [F. A. W. Fuqua, S. A. Fitzgerald, W. L. McGuire, *Biotechniques* **9**, 206 (1990)]. Under these conditions, BCRF1 RNA was detected in the JDV and NPR (H. Yssel, H. Gascan, P. Schneider, H. Spits, J. E. de Vries, in preparation), Daudi, and RPMI8866 cell lines.
19. S. O. Gollnick *et al.*, *J. Immunol.* **144**, 1974 (1990).
20. J. Dillner and B. Kallin, *Adv. Cancer Res.* **50**, 95 (1988).
21. C. A. Smith *et al.*, *Science* **248**, 1019 (1990).
22. PCR was done as described (*19*) with the following primers: 5′-ACAGGAATTCGATGGAGCGAAGGTTAGTGGTCACTCTGCAG-3′ (sense) and 5′-GTAGGAATTCCTCACCCTGGCTTTAATTGTCATGTATGCTTCTAT-3′ (antisense), where the Eco RI sites for cloning are indicated by underlining and the translation start and stop sites are bold. The PCR product was extracted with phenol and ethanol precipitated, and subjected to a second round of amplification (40 cycles) under the same conditions. After phenol extraction and ethanol precipitation, the amplified fragment was digested with Eco RI to generate cohesive ends for cloning. After purification by agarose gel electrophoresis, the BCRF1 fragment was cloned into a modified form of the pcDSRα296 plasmid (*8*).
23. Rats were immunized with 50-fold concentrated, serum-free COS-7–BCRF1 transfection supernatant in complete Freund's adjuvant, then boosted 1 month later with the same immunogen in incomplete Freund's adjuvant. Sera were collected before the first immunization and 10 days after the second. COS-7 cells on day 3 after transfection were cultured in methionine-free medium supplemented with ^{35}S-methionine at 1 mCi/ml (Amersham) for 4 hours at 37°C. Solid-phase radioimmunoabsorption was done (*9*) with the following modifications in cases where rat antisera were used. Each well of a round-bottom 96-well microtiter plate was coated with 20 μl of goat antibody to rat immunoglobulin G (100 μg/ml) (Jackson Immunoresearch) overnight at 4°C. After blocking in 10% fetal calf serum, (FCS), the plates were washed, incubated with immune or control rat serum (1%) for 1 hour at 37°C, and washed again. A mixture of 50% of a 3-day mock COS-7 transfection supernatant and a 1:4 dilution of ^{35}S-methionine-labeled transfection supernatant in 5% FCS, 0.02% Tween-20 was then incubated in each well for 1 hour at 37°C. After subsequent washing, samples were processed as described (*9*) for SDS-PAGE and autoradiography for 1 to 10 days.
24. Human PBMC were isolated from buffy coats from healthy donors by centrifugation over Ficoll-Hypaque and cultured at 10^6per milliliter with varying amounts of COS-7–BCRF1 or COS-7 (mock) supernatants and PHA (0.1 μg/ml) for 3 days, or with recombinant IL-2 (20 unit/ml) for 5 days in Yssel's medium with 1% human AB$^+$ serum [H. Yssel, J. E. de Vries, M. Koken, W. van Blitterswijk, H. Spits, *J. Immunol. Methods* **72**, 219 (1984)]. Cultures were done in triplicate in 96-well plates, 200 μl per well. IFN-γ in culture supernatants was measured by enzyme-linked immunosorbant assay (ELISA) [C. Favre *et al.*, *Mol. Immunol.* **26**, 17 (1989)]. The limit of sensitivity of the ELISA was 0.3 ng/ml. Because COS-7 supernatant may contain transforming growth factor–β (TGF-β), which can inhibit IFN-γ production (*1*), experiments were done with a neutralizing monoclonal antibody to TGF-β (anti–TGF-β) (10 μg/ml) [J. R. Dasch, D. R. Pace, W. Waegell, D. Inenaga, L. Ellingsworth, *J. Immunol.* **142**, 1536 (1989)] included in the cultures. The profile of inhibition was unchanged in the presence of anti–TGF-β, showing that inhibition of IFN-γ synthesis in the cultures was primarily the effect of BCRF1.
25. PBMC (*24*) were cultured in the presence of the anti-CD3 monoclonal antibody SPV-T3b [H. Spits *et al.*, *Hybridoma* **2**, 423 (1983)] at a 1:3000 dilution of ascites fluid. Where indicated, COS-7–BCRF1 and COS-7 (mock) supernatants were included at 10% of culture volume. Cells were harvested after 24 hours and total RNA was prepared as described [H. Yssel, J. P. Aubrey, R. de Waal Malefyt, J. E. de Vries, H. Spits, *J. Immunol.* **139**, 2850 (1987)]. RNA blot analysis was performed with 10 μg per lane. The 1.1-kb Pst I–Hinc II fragment of pcD-H-IFN-γ [T. Yokota *et al.*, *Lymphokines* **13**, 1 (1987)] and a mouse β-actin cDNA were used as probes. Hybridization conditions were substantially as described (*5, 19*).

26. The authors thank D. Hayward, W. Sugden, and E. Kieff for the Bam HI C fragment subclone used in this study, H Yssel for EBV-transformed cell lines and EBV-containing supernatant from the B95-8 cell line, F. Vega and D. Robison for synthetic oligonucleotides, and E. Mocarski, E. Kieff, J. Sixbey, J. Banchereau, and J. Luka for helpful discussions.

4 May 1990; accepted 7 September 1990

Technical Comments

Does Diphtheria Toxin Have Nuclease Activity?

The report by M. P. Chang *et al.* (*1*) proposing a "second pathway" of cell killing by diphtheria toxin (DT) by means of a putative toxin nuclease activity contradicts genetic evidence concerning the toxin killing mechanism. Biochemical studies (*2*) demonstrate that DT inhibits cellular protein synthesis by adenosine diphosphate (ADP)–ribosylating and thus inactivating protein synthesis elongation factor 2 (EF-2). Mutations in the EF-2 gene producing amino acid substitutions near the site of ADP-ribosylation of the factor render the cell resistant to the biologic effects of the toxin (*3*). Cellular resistance to DT is also caused by mutations in the pathway of enzymes that synthesize diphthamide, the unique posttranslational histidine derivative in EF-2 that is ADP-ribosylated (*4*). Cells bearing these mutations are unaffected by the intracellular production of otherwise lethal levels of the catalytic DT fragment A (*5*). In these mutant cells toxin resistance results from alterations in EF-2 that prevent its ADP-ribosylation by toxin. Conversely, a mutation producing a single amino acid substitution in DT abolishes its ability to ADP-ribosylate EF-2 and yields a nontoxic molecule (*6*). Thus, cell killing by DT requires ADP-ribosylation of EF-2, and failure to ADP-ribosylate EF-2 prevents killing. The genetic evidence therefore demonstrates that there is only one biologically relevant pathway of cell killing by DT.

JAMES W. BODLEY
Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455

REFERENCES

1. M. P. Chang, R. L. Baldwin, C. Bruce, B. J. Wisnieski, *Science* **246**, 1165 (1989).
2. A. M. Pappenheimer, Jr., *Ann. Rev. Biochem.* **46**, 69 (1977).
3. J. M. Moehring and T. J. Moehring, *Somatic Cell Genet.* **5**, 453 (1979); R. S. Gupta and L. Siminovich, *ibid.* **6**, 361 (1980); K. Kohno, T. Uchida, M. Mekada, Y. Okada, *Somatic Cell Mol. Genet.* **11**, 421 (1985); K. Kohno and T. Uchida, *J. Biol. Chem.* **262**, 12298 (1987).
4. B. G. Van Ness, J. B. Howard, J. W. Bodley, *J. Biol. Chem.* **255**, 10710 (1980); J. M. Moehring, T. J. Moehring, D. E. Danley, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1010 (1980); J.-Y. Chen, J. W. Bodley, D. M. Livingston, *Mol. Cell. Biol.* **5**, 3357 (1985); J. M. Moehring and T. J. Moehring, *J. Biol. Chem.* **263**, 3840 (1988); J.-C. Chen and J. W. Bodley, *ibid.*, p. 11692.
5. J. P. Perentesis *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8386 (1988).
6. T. Uchida, A. M. Pappenheimer, Jr., A. A. Harper, *Science* **175**, 901 (1972); T. Uchida, A. M. Pappenheimer, Jr., R. Greany, *J. Biol. Chem.* **248**, 3838 (1973).

29 January 1990; accepted 6 July 1990

The potent toxicity of diphtheria toxin (DT) is widely attributed to its ability to catalyze the adenosine diphosphate (ADP)–ribosylation of elongation factor 2 (EF-2) resulting in the inhibition of protein synthesis (*1*). Recently, M. P. Chang *et al.* (*2*) proposed a second cytotoxic pathway for DT. They reported that DT exhibits a nuclease activity that is stimulated by Ca^{2+} and Mg^{2+}, is susceptible to inhibition by antitoxin, and migrates with the A subunit of the toxin. They further suggest that DT-induced cell lysis is not simply a consequence of protein synthesis inhibition, but may instead involve direct chromosomal attack by intracellular toxin molecules. While this is an intriguing proposal, it does not explain why cells that cannot be ADP-ribosylated because of mutations in EF-2 are resistant to DT (*3*).

It was observed that the DT used to make DT-based immunotoxins contained nuclease activity, whereas the purified immunotoxins had no detectable nuclease activity. Since DT-based immunotoxins are subjected to purification by high-performance liquid chromatography (HPLC) gel filtration, the loss of nuclease activity could be the result of either the chemical linkage with an antibody or the HPLC purification step. To test this hypothesis, we obtained DT from one of the same sources used by Chang *et al.* (*2*) and chromatographed it on a TSK-3000 gel filtration HPLC column, collected the DT peak, and evaluated the nuclease activi-

ty. The original commercial preparation exhibited significant nuclease activity, whereas the HPLC-purified DT had no detectable nuclease activity (Fig. 1A). However, the ability of the purified toxin to inhibit protein synthesis by the ADP-ribosylation of EF-2 was unaltered by the gel filtration step, demonstrating that, even though the purified toxin lost nuclease activity, it retained full toxicity (Fig. 1B).

These results raise the possibility that the nuclease activity associated with DT represents a contaminant. To investigate this possibility, we subjected nicked DT (Calbiochem) to HPLC gel filtration and analyzed individual fractions for nuclease activity. As shown in Fig. 2A, HPLC of the sample yielded one major peak (fraction 33/34) and several minor peaks. Analysis of these peaks for nuclease activity showed that the major peak contained little or no activity (Fig. 2B, lanes 4 and 5) compared with the starting material, while one of the minor peaks, fraction 37, had nuclease activity that was at least ten times higher in specific activity than was the original material (Fig. 2B, lanes 6 and 7).

We also examined nuclease activity using DNA-containing gels (Fig. 2C) similar to those used by Chang *et al.* (*2*). These gels further demonstrate that DT subjected to additional purification by HPLC gel filtration lacks nuclease activity and that the nuclease activity can be separated and recovered independently from DT. In addition, these gels indicate that the nuclease activity does not co-migrate with the DT A subunit, but instead appears to migrate with a molecular mass (M_r) smaller than that of DT fragment A (arrow, Fig. 2C).

The nuclease activity therefore appears to be a contaminant that associates with DT during purification but which can be removed by HPLC gel filtration. Although Chang *et al.* (*2*) demonstrate that nuclease activity can be inhibited by antibody to DT, the possibility exists that the antibody was raised against a DT preparation containing contaminating nuclease. In this case, a portion of the antiserum may be against the nuclease itself and not DT and could account for the inhibition of the nuclease activity by the antitoxin.

The ability to separate the nuclease activity from the ADP-ribosylation activity of DT raises doubts concerning not only the relevancy of the nuclease activity as a proposed

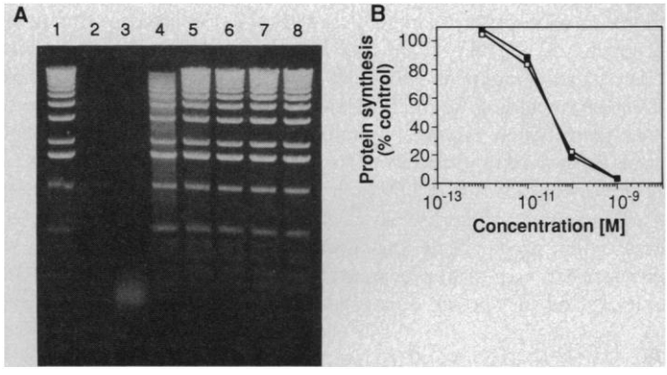

Fig. 1. Nuclease activity of DT removed by HPLC gel filtation. (**A**) DT (List Biologicals) was further purified by gel filtration with a TSK-3000 HPLC column. The major peak with approximate M_r 58,000 was collected, concentrated with a Centricon-10, and quantified by Lowry protein determination (*4*) with bovine serum albumin used as a standard. DNA (2 μg of 1-kilobase standard, Life Technologies, Inc.) was incubated in 10 mM tris, pH 7.5, containing 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, and varying amounts of DT for 1 hour at 37°C. At the end of the digestion period, sample buffer was added, and the DNA was separated in 1% agarose gels and visualized by ethidium bromide staining. Lanes 1 and 8, undigested DNA control; lanes 2, 3, and 4, digested with 10 μg, 1 μg, and 0.1 μg, respectively, of DT before HPLC gel filtration; lanes 5, 6, and 7, digested with 10 μg, 1 μg, and 0.1 μg, respectively, of DT after gel filtration. (**B**) Protein synthesis inhibition was assayed in 10^4 Vero cells in 100 μl of leucine-free RPMI medium containing 2% fetal calf serum in 96-well microtiter plates. DT before (■) and after (□) HPLC gel filtration was added and cells were incubated with the toxin for 5 hours followed by a 1-hour pulse with [^{14}C]leucine. The results are expressed as a percentage of the [^{14}C]leucine incorporated in mock-treated control cultures.

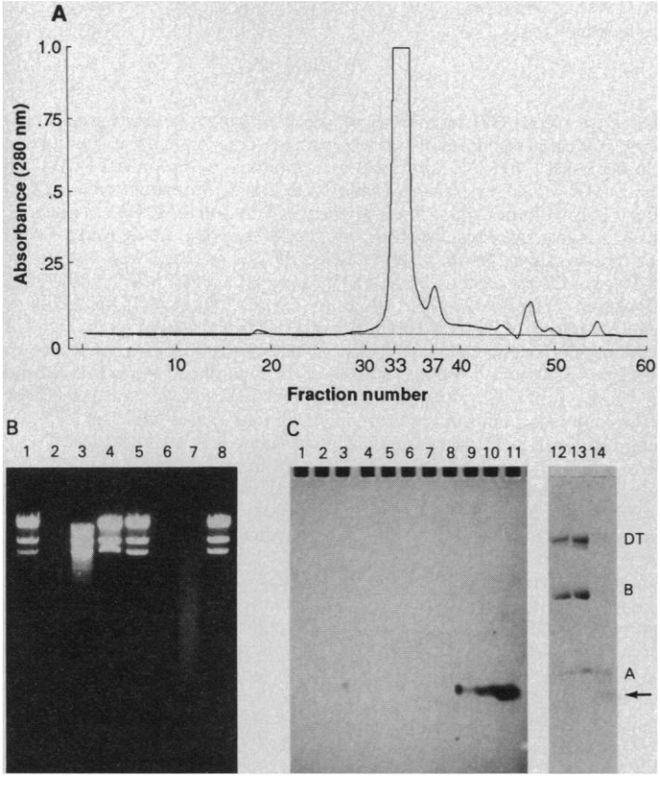

Fig. 2. (**A**) HPLC purification profile of DT. Nicked DT (Calbiochem) was applied to a TSK-3000 HPLC column and chromatographed with 0.1 M phosphate buffer, pH 7.2. The major peak (fractions at 33 and 34) corresponds to DT with ~M_r 58,000. (**B**) Nuclease activity of DT after HPLC chromatography. Fractions at 33 and 34 minutes and at 37 minutes were isolated and concentrated, and the protein was quantified (*4*). A λDNA/Hind III fragment (0.7 μg) was digested as described in Fig. 1 with 10 μg and 1 μg of nonpurified DT (lanes 2 and 3, respectively), 10 μg and 1 μg of purified DT (fraction 33/34) (lanes 4 and 5, respectively), and 1 μg and 0.1 μg of fraction 37 (lanes 6 and 7, respectively). Lanes 1 and 8 show nondigested control DNA. (**C**) Nuclease activity of HPLC fractions. Proteins were solubilized in SDS–sample buffer containing 0.05% β-mercaptoethanol and separated by electrophoresis in 12% polyacrylamide containing heat-denatured calf thymus DNA (20 μg/ml) (*2, 5*). These gels were poured without stacks and prerun at 170 V for 1.5 hours before protein samples were loaded. After electrophoresis, the nuclease portion of the gel was renatured by washing in 40 mM tris, pH 7.5 (three washes, 1 hour each), and then in 40 mM tris (pH 7.5) containing 2 mM $MgCl_2$ (24 hours at 30°C). The gels were subsequently incubated in 40 mM tris containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$ and examined for deoxyribonuclease activity after ethidium bromide staining (1 μg/ml, 1 hour). Nuclease activity was apparent after 8 hours and reached a maximum at 72 hours. Lanes 1, 2, and 3 contain 5, 10, and 15 μg, respectively, of nicked DT before HPLC gel filtration; lanes 5, 6, and 7 contain 5, 10, and 15 μg, respectively, of HPLC-purified DT (fraction 33/34); lanes 9, 10, and 11 contain 5, 10, and 15 μg, respectively, of fraction 37; lanes 4 and 8 are empty. Part of the gel was separated after electrophoresis and stained with Coomassie blue: lanes 12, 13, and 14 contain 10 μg of nicked DT before HPLC purification, HPLC fraction 33/34, and HPLC fraction 37, respectively. A, DT fragment A; B, DT fragment B.

second pathway of toxicity but also concerning the more basic issue of whether the nuclease activity can be attributed to the toxin itself.

VIRGINIA GRAY JOHNSON
Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892

REFERENCES

1. T. Honjo, Y. Nishizuka, O. Hayaishi, *J. Biol. Chem.* **243**, 3553 (1968); M. Gill, A. M. Pappenheimer, R. Brown, J. Kurnick, *J. Exp. Med.* **129**, 1 (1969); R. J. Collier, *Bact. Rev.* **39**, 54 (1975); A. M. Pappenheimer, *Ann. Rev. Biochem.* **46**, 69 (1977).
2. M. P. Chang, R. L. Baldwin, C. Bruce, B. J. Wisnieski, *Science* **246**, 1165 (1989).
3. T. J. Moehring and J. M. Moehring, *Cell* **11**, 447 (1977); R. K. Draper, D. Chin, D. Eurey-Owens, I. E. Scheffler, M. I. Simon, *J. Cell Biol.* **83**, 116 (1979).
4. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
5. A. L. Rosenthal and S. A. Lacks, *Anal. Biochem.* **80**, 76 (1977).

1 March 1990; accepted 21 June 1990

Recently, a number of papers (*1–3*) have alleged that the catalytic A fragment of diphtheria toxin (DT) has strong deoxyribonuclease (DNase) activity. The well-characterized adenosine diphosphate (ADP)–ribosylation of elongation factor 2 (EF-2), which blocks protein synthesis, has been generally assumed to be the sole enzymic activity of DT and to account entirely for the toxin's lethal effects. It was initially reported by Chang *et al.* (*4*) that several hours after it inhibited protein synthesis, DT induced extensive internucleosomal degradation of cellular DNA, followed by cytolysis. In contrast, DNA fragmentation and cytolysis were not observed when protein synthesis was inhibited by alternative means, such as by addition of cycloheximide, an antibiotic that reversibly inhibits polypeptide chain elongation in mammalian cell cultures (*5*). Subsequent communications (*1–3*) asserted that nuclease activity is a property of the toxin molecule itself and that this activity resides specifically within the amino-terminal A fragment, in a region distinct from the active site for ADP-ribosylation. The authors further proposed (*3*) "a model for cell killing in which toxin acts as a double-edged sword, using apparently nonoverlapping sites to effect translation inhibition and chromosomal cleavage."

It has long been known that even highly purified preparations of DT may show DNase activity (*6*), and a close correlation between secretion of DNase by strains of *Corynebacterium diphtheriae* and toxigenicity has been reported (*7*). Earlier workers assumed (*6*), and presented evidence (*8, 9*), that nuclease was present as an impurity and was not part of the toxin molecule itself . We have reinvestigated the question of whether the nuclease activity is an integral property of the DT molecule. Our results confirm those of previous workers: DNase activity present in most preparations of DT is a contaminant that can be chromatographically separated from the toxin or its A fragment.

We have confirmed that even relatively pure preparations of DT, as well as fragments or mutant forms of the toxin produced by *C. diphtheriae*, possess Ca^{+2}- and Mg^{+2}-dependent DNase activity that comigrates on SDS-polyacrylamide gels with DT fragment A (DTA) (Fig. 1). However, cloned toxin fragments produced in *Escherichia coli*, including fragment A and each of four toxin fusion proteins containing fragment A, showed no detectable DNase activity (less than 10^{-8} that of DNase I; Table 1). Furthermore, we found the level of nuclease activity in preparations produced by *C. diphtheriae* to vary from preparation to preparation, and the specific activity was consistently many orders of magnitude lower than that of bovine pancreatic DNase I (Table 1). In contrast, Chang *et al.* (*2*) reported toxin having specific DNase activities on the same order of magnitude as DNase I.

The DNase activity in fragment A preparations from *C. diphtheriae* could be readily separated from fragment A protein (and its associated ADP-ribosylation activity) by anion-exchange chromatography on a Mono Q FPLC column (Fig. 1A). Separation of DNase from whole toxin could also be effected in this manner. After this purification step, fragment A no longer showed DNase activity, as determined by three separate assays: release of radiolabel from [^{3}H]thymidine-labeled plasmid DNA (Fig. 1A); degradation of plasmid DNA as analyzed by electrophoresis on agarose gels (Table 1); or degradation of salmon sperm DNA embedded in SDS-polyacrylamide gels (Fig. 1B). Also, we found that mild trypsinization of intact DT leads to destruc-

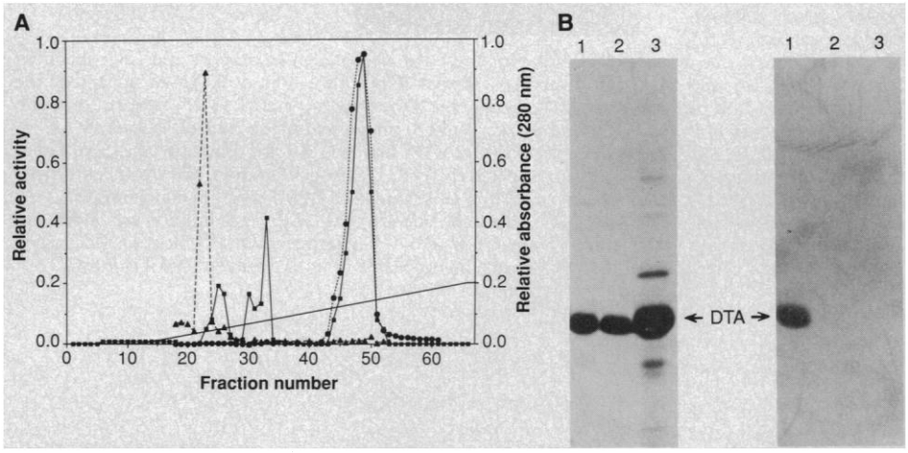

Fig. 1. Resolution of DNase activity from DT fragment A by anion-exchange chromatography. (**A**) Partially purified DTA (100 μg), prepared (*17*) from nicked whole toxin (Connaught), was equilibrated with buffer A (200 μl, 10 mM tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 0.2 mM EDTA) and chromatographed on a Mono-Q HR 5/5 anion-exchange fast protein liquid chromatography (FPLC) column (Pharmacia; 5 by 50 mm, bed volume) with a linear gradient of 0 to 200 mM NaCl in buffer A. Column fractions (0.5 ml) were monitored for relative absorbance at 280 nm (■) and assayed for both DNase (▲) and ADP-ribosyltransferase (ADPRT) (●) activity. DNase activity was assayed by incubating portions (1 μl) in 10 μl of DNase reaction buffer (10 mM tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$) containing [*thymidine*-^{3}H]pBR322 DNA (0.23 μg; 20 μCi/mg) at 25°C for 24 hours. The reactions were terminated by addition of 20 μl of 100 mM EDTA, 20 μl of salmon sperm DNA (1 μg/μl), and 500 μl of cold 10% trichloroacetic acid (TCA); after 30 minutes at 0°C, the precipitate was centrifuged at 16,000*g* and 4°C for 15 minutes. Portions (500 μl) of the supernatant were removed and the radioactivity was counted in 3 ml of ACS Fluor (Amersham). ADPRT activity was assayed by incubating 1-μl portions of the column fractions in reaction buffer (50 mM Hepes-KOH, pH 7.5, 20 mM dithiothreitol) containing [*adenine*-2,8-^{3}H]nicotinamide adenine dinucleotide (NAD) (0.68 μM, 29.3 mCi/μmol) and partially purified wheat germ EF-2 (0.2 μM) in a total volume of 50 μl at 25°C for 16 minutes. Portions (45 μl) were removed and applied to 3 MM (Whatman) paper saturated with trichloroacetic acid (TCA), washed with 5% TCA and methanol, air-dried, and counted in 3 ml of ACS Fluor (Amersham). (**B**) Samples of DTA (11 μg) before (lane 1) and after (lane 2) FPLC purification and a sample of cloned DTA (20 μg) (lane 3), obtained from *E. coli* periplasmic extracts, were solubilized in 1% SDS–sample buffer containing 20 mM β-mercaptoethanol and separated by electrophoresis on a 12.5% polyacrylamide gel that was polymerized in the presence of heat-denatured salmon sperm DNA (200 μg/ml). The DNA-containing gel was first run for 1.5 hours at 150 V before a 4.5% stacking gel was poured, the protein samples were loaded in duplicate, and the gel was run at 70 V. After electrophoresis, half of the gel (left) was stained with Coomassie blue. The other half of the gel (right) was washed three times for 60 minutes each time in buffer (50 mM tris-HCl, pH 7.5) and incubated in DNase reaction buffer containing 0.02% NaN_3 at room temperature for 48 hours; it was then stained with ethidium bromide (1 μg/ml) for 1 hour, destained in wash buffer for 1 hour, and examined under ultraviolet light.

tion of the DNase concurrent with generation of the enzymically active A fragment (Fig. 2). This, together with the fact that the DNase is thermolabile (>90% of activity lost after 5 minutes at 50°C or 2 minutes at 95°C), confirms that it is proteinaceous.

In parallel studies with a nontoxigenic strain of *C. diphtheriae* (*belfanti* 1030) we found a DNase in the culture medium that coelectrophoreses with fragment A on SDS-polyacrylamide gels and shows optimal conditions similar to or identical with those reported for the putative DT-associated DNase activity (*1, 2*). Thus production of a DNase similar or identical to that contaminating DT preparations is a property of a nontoxigenic *C. diphtheriae* and hence is genetically distinguishable from toxin production. This correlates with independent evidence showing that mutant *C. diphtheriae* strains that cannot synthesize a secretable DNase can still be fully toxigenic (*9*).

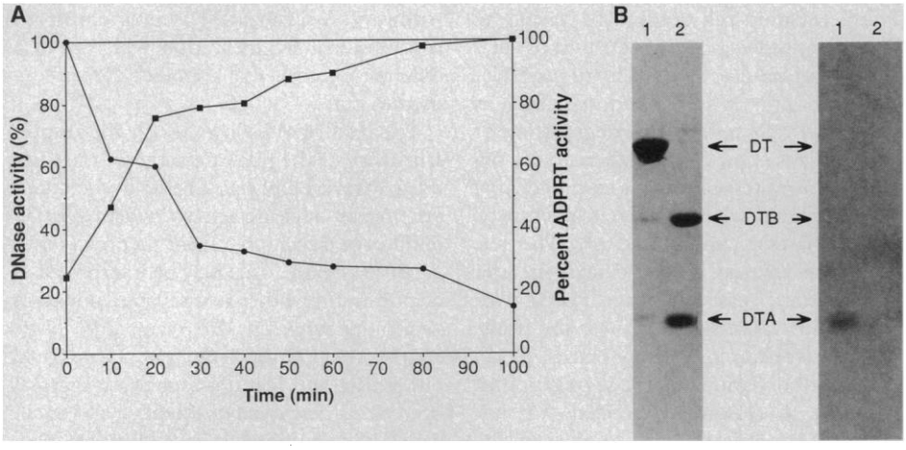

Fig. 2. Nuclease activity associated with intact DT is sensitive to trypsin treatment. (**A**) Intact DT (50 μg, predominantly unnicked, List Biologicals), containing nuclease activity, in nicking buffer (50 mM tris-HCl, pH 7.5, 30 mM DTT, 0.1 mM EDTA) was incubated at 25°C in a total volume of 100 μl with bovine pancreatic trypsin treated with L-1-tosylamide-2-phenylethyl-chloromethyl ketone (0.5%, Sigma), in the presence of 10 mM NAD (Boehringer/Mannheim) to protect the fragment A from proteolysis. At designated time intervals, 10-μl portions were removed, and proteolysis was terminated by addition of soybean trypsin inhibitor (10 μl, 5 μg/μl, Sigma). The samples were each assayed for both DNase (●) and ADPRT (■) activity as described in Fig. 1. (**B**) Nuclease activity of intact DT (8 μg) before (lane 1) and after (lane 2) trypsin treatment at 25°C for 60 minutes. Samples were run in duplicate on a DNA-embedded 12.5% SDS-polyacrylamide gel and either stained with Coomassie blue (left) or incubated first in DNase reaction buffer and then stained with ethidium bromide (right), as described in Fig. 1. DTB, DT fragment B.

Table 1. Nuclease activity associated with DT, or fragments or mutant forms thereof, produced in *C. diphtheriae* or *E. coli*. Toxin samples were incubated for 6 hours at 22°C with 0.2 μg of φX174 RFI plasmid DNA, in 10 mM tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, and nuclease-free bovine serum albumin (100 μg/ml) (Sigma). Incubations were terminated by addition of ethylenediaminetetracetic acid, and samples were electrophoresed on agarose gels in tris-borate buffer as described (*3, 16*). Degradation of DNA was estimated by visual inspection after staining with ethidium bromide as described in caption to Fig. 1. One DNase I unit was defined as the degree of degradation effected by 10 femtograms per milliliter of the bovine pancreatic DNase I. The values reported represent the average of at least duplicate assays.

Bacterial strain	Product	DNase activity (unit/μg protein)	Reference
	Bovine pancreatic DNase I	1×10^8	
C. diphtheriae			
PW8	Toxin (List)	2×10^4	(*18*)
PW8	Toxin (Connaught)	6×10^4	(*18*)
PW8	Purified, nucleotide-free toxin (Connaught)	6×10^0	(*17*)
PW8	Toxin A fragment	2×10^4	(*17*)
PW8	Toxin A fragment after purification (Fig. 1)	ND*	
$C7(\beta)^{CRM22}$	CRM22	2×10^5	(*17, 18*)
$C7(\beta)^{CRM45}$	CRM45	2×10^5	(*17, 18*)
$C7(\beta)^{CRM1001}$	CRM1001	2×10^5	(*17, 18*)
belfanti 1030^{tox-}	DNase, purified	$>5 \times 10^6$	(*9*)
E. coli	Toxin A fragment	ND*	(*19*)
E. coli	DAB_{389} α-MSH	ND*	(*10, 14*)
E. coli	DAB_{486} IL-2	ND*	(*11, 14*)
E. coli	DAB_{389} IL-4	ND*	(*12, 14*)
E. coli	DAB_{389} IL-6	ND*	(*13, 14*)

*Not detectable; less than one unit per microgram of protein.

We may summarize the evidence that the DNase activity that is often found in preparations of DT and its A fragment resides on a separate contaminating molecule as follows.

1) DNase activity can be separated from DT or fragment A by chromatography, as demonstrated here, or by specific precipitation with antitoxin at antibody equivalence (*5, 8*). In the latter case, the nuclease remains in the supernatant fraction.

2) DNase activity declined as DT was "nicked" by trypsin to generate the enzymically active fragment A.

3) DNase activity has not been found in cloned fragment A or fragment A–containing fusion gene products (Table 1) isolated from *E. coli* (*10-14*).

4) Mutants of sensitive mammalian cell lines that are unable to synthesize the diphthamide residue, a posttranslationally modified histidine of EF-2 that is the site of ADP-ribosylation, are resistant to even very high levels of the toxin [$>10^5$ cytotoxic doses; (*15*)].

5) Mutant *C. diphtheriae* strains that cannot synthesize a secretable DNase can still be fully toxigenic (*9*), and certain strains of nontoxigenic *C. diphtheriae* secrete DNase into the culture medium. In the case of the *C. diphtheriae belfanti* 1030 strain, the DNase showed chromatographic and enzymatic properties similar to those found in toxin preparations.

Given that DT apparently has no intrinsic nuclease activity, the DNA fragmentation and subsequent cytolysis observed in DT-treated cells remain interesting consequences of toxin action that warrant further exploration.

BRENDA A. WILSON
STEVEN R. BLANKE
Harvard Medical School and
Shipley Institute of Medicine,
Boston, MA 02115
JOHN R. MURPHY
Evans Department of Clinical Research and
Department of Medicine,
Boston University Medical Center,
Boston, MA 02118
A. M. PAPPENHEIMER, JR.
Department of Cellular and Molecular Biology,
Harvard University, Cambridge, MA 02138
R. JOHN COLLIER
Harvard Medical School and
Shipley Institute of Medicine

REFERENCES

1. M. P. Chang, R. L. Baldwin, C. Bruce, B. J. Wisnieski, *Science* **246**, 1165 (1989).
2. L. T. Nakamura and B. J. Wisnieski, *J. Biol. Chem.* **265**, 5237 (1990).
3. C. Bruce, R. L. Baldwin, S. L. Lessnick, B. J. Wisnieski, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2995 (1990).
4. M. P. Chang, J. Bramhall, S. Graves, B. Bonavida, B. J. Wisnieski, *J. Biol. Chem.* **264**, 15261 (1989).
5. R. S. Goor, A. M. Pappenheimer, E. Ames, *J. Exp. Med.* **126**, 923 (1967); A. M. Pappenheimer, R. J. Collier, P. A. Miller, in *Cell Culture in the Study of Bacterial Disease*, M. Solotorovsky, Ed. (Rutgers Univ. Press, New Brunswick, NJ, 1963).
6. C. G. Pope, *Brit. J. Exp. Pathol.* **38**, 212 (1957).
7. O. V. Messinova, D. V. Yusupova, N. S. Shamsuldinov, *Mikrobiologiya* **1**, 20 (1963); D. V. Yusupova, O. V. Messinova, N. S. Shamsuldinov, *Laboratornoe Delo* **6**, 46 (1963).
8. R. J. Collier, thesis, Harvard University (1964).
9. N. B. Groman and C. K. Dean, *Infect. Immun.* **8**, 442 (1973).
10. J. R. Murphy *et al.*, *Proc. Natl. Acad. Sci., U.S.A.* **83**, 8258 (1986).
11. D. P. Williams *et al.*, *Protein Eng.* **1**, 493 (1987).
12. F. Lakkis, A. Steele, T. B. Strom, J. R. Murphy, in preparation.
13. L.-F. Jean, T. B. Strom, J. R. Murphy, in preparation.
14. D. P. Williams, C. E. Snider, T. B. Strom, J. R. Murphy, *J. Biol. Chem.*, in press.
15. T. J. Moehring and J. M. Moehring, *Cell* **11**, 447 (1977); R. S. Gupta and L. Siminovich, *Somatic Cell Genet.* **6**, 361 (1980); J.-Y. C. Chen, J. W. Bodley, D. M. Livingston, *Mol. Cell. Biol.* **5**, 3357 (1985).
16. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), p. 156.
17. S. F. Carroll, J. T. Barbieri, R. J. Collier, *Methods Enzymol.* **165**, 68 (1988).
18. A. M. Pappenheimer, Jr., T. Uchida, A. A. Harper, *Immunochemistry* **9**, 891 (1972).
19. B. A. Wilson, V. E. Escuyer, K. A. Reich, R. J. Collier, unpublished results.

1 June 1990; accepted 21 June 1990

Response: Diptheria toxin (DTx)–induced cell death has been an intriguing research problem for decades. Our demonstration that target cell lysis is not a simple consequence of protein synthesis inhibition together with the enigmatic observation of a DTx nuclease activity has now sparked a lively debate as to whether the observed nuclease activity is intrinsic to the DTx molecule itself or is attributable to a specific contaminating protein. We can only speculate as to the relevance of the experiments that focus on target cell mutations and transfections with DTx-resistant elongation factor 2 (EF-2) genes that render cells insensitive to DTx. First, it is possible that translation inhibition is a prerequisite for extensive DTx-induced chromosomal degradation, because it would preclude the synthesis-expression of DNA repair enzymes at a time when their levels are crucial to cell survival. Second, to discount (as does Bodley) the role of DTx nuclease activity on the basis of an experiment (*1*) in which an EF-2^R gene from mutagenized hamster cells was transfected into mouse cells that were then tested for *Pseudomonas* toxin (PTx) resistance (since mouse cells are "naturally" resistant to DTx) is to assume that PTx displays a nuclease activity as high as that of DTx. It does not (*2*). The question is, What would have happened if the EF-2^R gene had been transferred back into unselected, unmutagenized hamster cells, and these cells had then been tested for DTx resistance (including assays for chromosomal and membrane integrity)? Third, equating cell death with having an adenosine diphosphate (ADP)–ribosylatable form of EF-2 or an inability to use [^{3}H]-leucine for protein synthesis does not tell us how many cells suffer DNA degradation or cytolysis later. One cannot expect to discover how many resistance loci exist and how they segregate when only one phenotype (EF-2^R) is being monitored (*3*). The sole phenotype assayed by tetrad analysis after DTx-resistant yeast cells were mated with DTx-sensitive yeast cells (*3*) was the resistance of cytoplasmic extracts (that is, EF-2) to ADP ribosylation. As the segregation of resistance to DTx-induced *killing* was not addressed by Bodley and his co-workers, the possibility remains that the mutagenized, resistance-selected haploid cells carried more than one mutation. As long as killing is assumed to be equal only to low translation levels or to EF-2 sensitivity to ADP-ribosylation, or both, then the way in which DTx provokes other effects on eukaryotic cells will remain an enigma. Have Wilson *et al.* characterized the sequellae of DTx treatment on yeast cells? Are yeast the ideal cell system in which to assess the mechanism by which DTx causes the cytolysis of mammalian cells? Now that a second enzymic activity has been observed for DTx, the possibility is raised that there might be a second cytotoxic pathway. Assessing DTx involvement in chromosomal fragmentation will require a different experimental approach from those used to date.

The data of Wilson *et al.* on the trypsin sensitivity of the nuclease activity (their figure 2A) reveal biphasic kinetics in the disappearance of nuclease activity, where after 30 minutes of trypsin treatment there is a residual 30% activity. This may be interpreted as demonstrating either two separate nucleases or a single nuclease that retains 30% of its activity after trypsin treatment. How could one isolate and identify a nuclease that has been either destroyed or modified and attenuated with trypsin? It would seem more reasonable to use argC-cleaved preparations (the form of DTx we used) in any attempts to refute our conclusions. Since Wilson *et al.* do not show where their nuclease-active fragment runs on an SDS gel that contains DNA, there is no evidence that they isolated

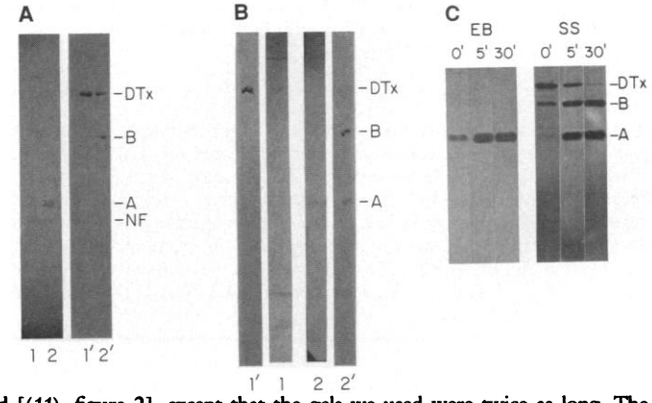

Fig. 1. Nuclease activity of DTx. (**A**) Endoproteinase argC-cleaved DTx showing nuclease activity in a DNA gel assay (*11*). Toxin 3 μg; ~90% intact monomer) was cleaved and then denatured in the absence (lane 1) and presence (lane 2) of 0.01% mercaptoethanol. Lanes 1′ and 2′ show the same gel piece after staining with Coomassie blue. The assay for nuclease activity in DNA-containing gels was performed as described [(*11*), figure 2], except that the gels we used were twice as long. The positions of DTx and the B and A subunits of DTx are indicated; NF refers to a nuclease active fragment. (**B**) Nuclease-active bands associated with unreduced ~95% intact DTx monomer (lane 1) and reduced argC-cleaved DTx (lane 2). Lanes 1′ and 2′ correspond to samples in lanes 1 and 2 silver-stained immediately after electrophoresis. The three cracks across lane 1 occurred during gel handling. (**C**) DTx fragment A–associated nuclease activity increased by argC treatment. Intact (~95%) monomeric DTx and proteinase argC (at 0.22 μg/μl and 0.022 μg/μl, respectively) were incubated together in 150 mM NaCl and 20 mM tris (pH 7.6) for 5 minutes or 30 minutes at 37°C; portions were then removed, mixed with reducing SDS loading buffer (0.5% β-ME) and boiled immediately for 2 minutes. Samples were stored at −20°C, thawed, and then boiled again for 1 minute before loading onto a gel. The SDS/12.5% polyacrylamide gel contained denatured calf thymus DNA (20 μg/ml, Sigma) and 2 mM EDTA. The stacking gel contained 2 mM EDTA, but no DNA; the running buffer contained 2 mM EDTA. The gel was prerun at 170 V for 1.5 hours before the samples were loaded. After the gel was run, it was washed three times for 40 minutes in 300 ml of 20 mM tris (pH 7.6), 0.04% NaN_3, at room temperature and then incubated for 14 hours in 300 ml of tris (pH 7.6), 2mM $CaCl_2$, 2 mM $MgCl_2$, and 0.04% NaN_3 at 30°C. It was then stained in 0.5 μg of ethidium bromide per milliliter for 1.5 hours and destained in 20 mM tris (pH 7.6) and 0.04% NaN_3 for 45 minutes. The gel was photographed under ultraviolet illumination and then silver-stained. EB, ethidium bromide-stained gel; SS, silver-stained gel; lane 0′ contains 4 μg of untreated whole DTx; lanes 5′ and 30′ contain 3 μg of DTx after argC cleavage for 5 minutes and 30 minutes; DTx, B, and A mark the positions of whole DTx, fragment B, and fragment A, respectively.

Fig. 2. HPLC profiles of intact DTx dimer (top panel) and monomer (bottom panel) preparations. The middle panel shows a mixture of the two preparations. The arrow indicates the injection point; elution positions of molecular weight standards (in kilodaltons) are indicated. HPLC was performed as described (*14*).

the same nuclease-active band that comigrates with fragment A of DTx [that is, a single protein band (size ~24,000 kD)]. It is possible that they isolated an unrelated contaminant or generated a new fragment A that exhibits nuclease activity but no ADPrT activity. To check for molecular weight consistencies, one should run argC-cleaved DTx next to several trypsin-cleaved DTx and purified A fragment preparations (in figure 2B of the comment by Wilson *et al.*, trypsin-generated fragment B appears to be identical in size to fragment B in their untreated preparation, while trypsin-generated fragment A appears to be slightly smaller than fragment A in their untreated preparation).

The use of ADP-ribosylation activity as a marker for fragment A is problematic. While trypsin treatment of DTx generates fragment A that is ADPrT-active but nucleolytically-inactive (as shown by Wilson *et al.*), argC treatment generates fragment A that is active in both respects. Because fragment A contains many lysines (10%) and few arginines, the cleavage products generated by trypsin treatment of DTx vary in size and quantity from preparation to preparation (*5*). In contrast, argC cleavage of DTx, even if allowed to proceed for hours, leads to an A band of consistent size and amino terminus (there are no arginines in the first 124 amino acids of fragment A). It is possible that argC treatment produces more fragment A–associated activity, while trypsin treatment destroys DTx nuclease activity because the amino terminus of A is crucial for DNA binding-cleavage (note that trypsin-sensitive lysines occur at positions 10 and 24, among others). Furthermore, toxin fusion products and cloned fragment A (the sequence identity of which is not stated by Wilson *et al.*) may exhibit conformations that are inconsistent with DNA binding or nuclease activity, or both. Wilson *et al.* are in an excellent position to test how argC-cleaved and -uncleaved preparations of CRM22, CRM45, and other mutant forms behave on SDS gels that contain DNA. Do any of the truncated forms of DTx renature and display nuclease activity? Do any of these truncated forms display comigrating nuclease activity after electrophoresis under nondenaturing conditions, as we have seen with both DTx and CRM197 (*4*)?

Bands of nuclease activity can be seen in Fig. 1A that are associated with unreduced (lane 1) and reduced (lane 2) argC-cleaved DTx. This particular preparation of monomeric DTx contains a second active peptide (labeled NF), similar in size to the one seen by Johnson (lanes 9 through 11 of her figure 2C). In addition, a nuclease-active band that comigrates precisely with the A subunit of DTx appears in our reduced sample (Fig. 1A, lane 2) and in Wilson *et al.*'s reduced DTx and DTA samples (their figures 1B and 2B). The appearance of subunit A–associated nuclease activity depends on the peptide's ability to renature after SDS-PAGE. Too much reducing agent or too much protein per sample, or both, can lead to compacted protein bands that show little or no nuclease activity. Contaminants in SDS as well as residual persulfate and other potentially deleterious chemicals in the gel matrix compound the problem of optimizing renaturation conditions (*6*). Add to this the potential for protein leaching from the gel and one has a situation in which the ability to demonstrate subunit A–associated activity requires not only strict adherence to published procedures but also some trial and error testing.

Our preparation of DTx monomer displays only whole DTx-associated activity (Fig. 1B, lane 1) in an uncleaved, unreduced sample and only A-associated activity after argC cleavage and reduction (Fig 1B, lane 2). Preparations of DTx purchased from List Biologicals and Calbiochem gave results similar to those shown in Fig. 1B; the cytolytic activities of the three toxin preparations were also similar. Wilson *et al.*'s toxin samples (their figures 1B and 2B) also exhibit only DTA-associated activity. The band marked by an arrow in Johnson's figure 2C may represent a fragment of the A domain that was able to refold and express nuclease activity more effectively than the A domain itself. We have seen nuclease active bands smaller than A in some trypsin-cleaved and naturally cleaved toxin preparations (for example, Fig. 1A). As discussed above, the generation of active peptides by trypsin cleavage of DTx is less reproducible than such generation by argC cleavage. How

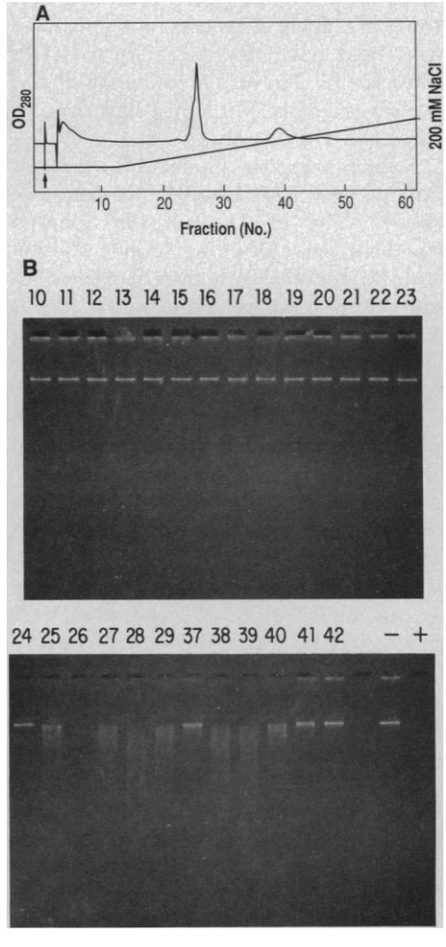

Fig. 3. Nuclease activity comigrating with fragment A of CRM197 after FLPC on a Mono-Q column. (**A**) Analysis of CRM197 by FPLC anion exchange chromatography. CRM197 (100 μg; Swiss Serum and Vaccine) was loaded onto a Mono-Q HR 5/5 anion-exchange FPLC column (Pharmacia, 5 × 50 mm bed volume) equilibrated with 10 mM tris-Cl (pH 7.5) and 0.2 mM EDTA (buffer A). After the sample (1 ml) was loaded (arrow), the column was washed with buffer A for 10 minutes; a linear elution gradient was then developed in 50 minutes from 0 to 200 mM NaCl in buffer A. The flow rate was 1 ml/min (1 ml per fraction), 280 nm detection at 0.2 absorbance units (**B**) Nuclease activity of FPLC fractions. Portions (10 μl) of fractions 10 to 29 and 37 to 42 were mixed with DNA digestion buffer to give final concentrations of 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM tris (pH 7.6) in 40 μl total volume, with 0.1 μg of phage lambda DNA. Samples were incubated for 2 hours at 37°C, and activity was stopped by addition of 5 μl of loading buffer (containing 25 mM EDTA) to 20 μl of the sample. Samples were electrophoresed on a 1% agarose gel as described (*15*) and viewed by ultraviolet illumination after ethidium bromide staining. Lanes − and + correspond to digestion assays carried out with 10 μl of buffer A, and 4 μg of CRM 197 in 10 μl of buffer A, respectively.

Johnson's DTx was cleaved is not stated.

The fact that we see a strict correlation between the level of subunit A–associated nuclease activity and the extent of argC cleavage of DTx (Fig. 1C) makes it unlikely that we are dealing with a nuclease contaminant. The discovery (*4*) that nuclease activity also comigrates with the A subunit of the CRM197 mutant form of DTx, even though CRM197's A subunit runs with a distinctively higher molecular weight, strengthens the argument for an A-associated nuclease activity. As we have observed inhibition of DTx nuclease activity with the use of antibody from two different sources and DTx from three different sources, it appears that this nuclease is a ubiquitous "contaminant." This so-called "contaminant" exhibits nuclease activity at molecular weights corresponding to those of both whole DTx and the A subunit (Fig. 1).

It would be valuable to know the sequences of the band marked by an arrow in lane 14 of Johnson's figure 2C and the nuclease-active fast protein liquid chromatography (FPLC) fraction of Wilson *et al.* If they are proteolytic fragments of the A domain of DTx, it would expain why Johnson and Wilson *et al.* were able to separate the ADPrT activity from the nuclease activity of the toxin; however, fraction 37 (in Johnson's figure 2A) does not appear to have been tested for its ability to ADP-ribosylate EF-2 in vitro or to shut off cellular protein synthesis. Without a B domain, it could not bind to cells.

Sodium phosphate inhibits DTx nuclease activity (by 50% at 0.01 M; data not shown). This may explain why Johnson could not detect DNA digestion in solution with DTx purified by high-performance liquid chromatography (HPLC) (her figures 1A and 2B). Although DTx expresses nuclease activity in the presence of Mg^{2+} alone (peaking at 1 mM Mg^{2+}), 250 μM of the Ca^{2+}-chelator EGTA abolishes this activity; thus we conclude that the Mg^{2+}-stimulated activity of DTx depends on some Ca^{2+} impurities (*7*). Johnson's use of 0.1 M phosphate (counterion not indicated) during HPLC would inhibit DNA degradation in solution because of its Ca^{2+}-precipitating activity and because K^+ and Na^+ both inhibit DTx nuclease activity (*7*). Her methods are not detailed enought to ascertain the phosphate content of the nuclease reaction buffer containing fraction 37 versus that containing fractions 33/34. Alternatively, her two HPLC peaks may well represent DTx monomer and dimer rather than DTx monomer and a smaller peptide (her fraction 37). HPLC profiles of our intact DTx monomer and dimer preparations (Fig. 2) show no evidence of a peak corresponding to Johnson's fraction 37 (that is, after our monomer peak). Although our intact dimer preparations (Fig. 2) exhibited no nuclease activity in solution, some nuclease activity was recovered after treatment with DMSO, a reagent that elicits dimer dissociation (*8*).

In light of the reported ability of Wilson *et al.* to separate the nuclease activity from whole DTx by chromatography (data not shown), we note that, when we subjected intact CRM197 to FPLC on a mono-Q column (Fig. 3A) and then treated the fraction with argC, fragment A_{197}–associated nuclease activity was detected in fraction 26 (eluting at 64 mM NaCl). Our activity gels contained 20 μg of DNA/ml (compared with 200 μg/ml used by Wilson *et al.*) and, because of the small sample size, they were incubated with divalent cations for several days and monitored daily. When we assayed for nuclease activity in solution (a much more sensitive assay that does not depend on protein renaturation), only two regions of nuclease activity were observed (Fig. 3B), namely fractions 25 through 29 and 37 through 40. Both sets of fractions contained CRM197-derived protein. Although CRM197 was used initially because of its higher specific activity, similar results were obtained with DTx (*9*). We did not detect any evidence of nuclease activity in fractions corresponding to those in which Wilson *et al.* observed a nuclease-active peak (that is, eluting at 47 mM NaCl).

Collier's data showing that antitoxin precipitates the ADPrT but not the nuclease activity of DTx (*10*) seems to indicate that the two enzymes in his preparation do not associate with each other. However, our data demonstrate that nuclease activity comigrates with whole DTx and CRM197, and with fragments A and A_{197} during electrophoresis under denaturing as well as in native conditions [Fig. 1; (*4*, *11*)]. Collier does not appear to have tested his antibody-precipitated DTx for nuclease activity, nor is it clear that his reaction buffer was suitable for detecting DTx-nuclease activity. Moreover, the specificity of his antibody preparation is not stated. Since the antibodies, as employed *10*, did not neutralize the ADPrT activity of DTx, it is difficult to interpret the observation that they did not destroy the nuclease activity in the supernatant (indeed, it increased by 50%). There is no evidence that all DTx-related peptides were precipitated; indeed, many antibody preparations actually contain nuclease-active material.

The reason for the large discrepancy between the enzymic activity values obtained by Wilson *et al.* and those we reported may be due to the fact that Wilson *et al.* measured extents of cleavage after 6 hours (the source of their DNase standard is not stated), while we measured initial rates (typically after less than 10 minutes) and used the historically accepted rate assay of Kunitz to establish rate constants. Interestingly, Collier previously found (*10*) that the nuclease activity associated with his purified DTx was 1/50 that of DNase on a weight to weight basis. This value, based on rates, is much closer to the one we reported.

The DNase test agar (Difco) used by Groman and Dean (*12*) contains ~100 mM NaCl and less than optimal concentrations of divalent cations. We have shown that 100 mM NaCl completely inhibits DTx nuclease activity (*7*). Hence, the nuclease activity that they (*12*) were assaying may not be the same nuclease activity that co-purifies with DTx. The test conditions used by Messinova *et al.* (who found a 1-to-1 correspondence between toxigenicity and nuclease activity) were better suited to the nuclease activity requirements of DTx (*13*). At present, there is no reason to believe that *C. diphtheriae* secrete a single nuclease.

In short, we do not believe the interpretation of our data is invalidated by the evidence presented by Bodley, Johnson, and Wilson *et al.* If we are dealing with a nuclease contaminant, it is one that enigmatically but consistently behaves like an integral component of both DTx and CRM197. Until the points raised above are satisfactorily addressed, we consider it premature to dismiss our original conclusion.

S. L. Lessnick, C. Bruce,
R. L. Baldwin, M. P. Chang,
L. T. Nakamura, B. J. Wisnieski
Department of Microbiology and Molecular Genetics, University of California, Los Angeles, CA 90024

REFERENCES

1. K. Kohno and T. Uchida, *J. Biol Chem.* **262**, 12298 (1987).
2. C. Bruce *et al.*, in preparation.
3. J-Y. C. Chen, J. W. Bodley, D. M. Livingston, *Mol. Cell. Biol.* **5**, 3357 (1985).
4. C. Bruce *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2995 (1990).
5. W. Cieplak, C. Hasemann, L. Eidels, *Biochem. Biophys. Res. Commun.* **157**, 747 (1988).
6. A. Blank *et al.*, *Anal. Biochem.* **120**, 267 (1981); A. Blank, J. R. Sibler, M. P. Thelen, C. A. Dekker, *ibid.* **135**, 423 (1983); A. Blank and T. A. McKeon, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3169 (1989).
7. L. T. Nakamura and B. J. Wisnieski, *J. Biol. Chem.* **265**, 5237 (1990).
8. S. F. Carroll *et al.*, *Biochemistry* **25**, 2425 (1986).
9. S. L. Lessnick *et al.*, in preparation.
10. R. J. Collier, thesis, Harvard University (1964).
11. M. P. Chang *et al.*, *Science* **246**, 1165 (1989).
12. N. B. Groman and C. K. Dean, *Infect. Immun.* **8**, 442 (1973).
13. O. V. Messinova *et al.*, *Zh Mikrobiol. Epidemiol. Immunobiol.* **40**, 20 (1963).
14. S. F. Carroll, J. T. Barbieri, R. J. Collier, *Methods Enzymol.* **165**, 68 (1988).
15. M. P. Chang *et al.*, *J. Biol. Chem.* **264**, 15261 (1989).

11 July 1990; accepted 25 September 1990