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Inhibition of Protein Synthesis in Intact HeLa Cells by *Shigella dysenteriae* 1 Toxin

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Shiga toxin purified to near homogeneity from cell lysates of *Shigella dysenteriae* 1 inhibited protein and deoxyribonucleic acid syntheses in intact HeLa cells. Inhibition was dependent on toxin concentration and time of incubation. A minimal latent period of 30 min was observed with saturating doses of toxin. Ribonucleic acid synthesis, uptake of α-aminoisobutyric acid, and maintenance of intracellular K^+ concentrations were not affected until well after maximal inhibition of protein and deoxyribonucleic acid syntheses. The inhibitory effect of toxin was sensitive to heat inactivation and was prevented by antibody neutralization. Several cytotoxic components were separated by polyacrylamide gel electrophoresis of the purified toxin preparation; all inhibited protein and deoxyribonucleic acid syntheses equally.

Shigella dysenteriae 1 produces activities which are lethal to rabbits (23) and mice (28), enterotoxic in rabbit experimental models (3, 12), and cytotoxic to several mammalian cell lines (15, 29; M. K. Gentry and J. M. Dalrymple, submitted for publication). The role of the toxin in shigellosis has not been established, but involvement has been suggested in bacterial invasion of the colonic mucosa, in intestinal cell death (9), and in the initial diarrheal phase of the disease (12). The demonstration of toxin in *Shigella flexneri* (14, 21) and *Shigella sonnei* (14) and the report of a similar toxin in *Escherichia coli* RDEC-1 (A. D. O'Brien, M. R. Thompson, J. R. Cantey, and S. B. Formal, Abstr. Annu. Am. Meet. Soc. Microbiol. 1977, B103, p. 32) suggest a broad involvement in diarrheal disease.

Experiments using crude or partially purified preparations have failed to delineate the pathophysiological basis of this toxin. Cavanagh et al. (3) observed vascular damage in intestines after intravenous injection. Inoculation of ligated ileal loops with toxin produces morphological damage (13) and fluid secretion, although the fluid composition is different from that produced after choleragen exposure (12). In contrast to cholera enterotoxin-induced intestinal secretion, several studies suggest that cyclic adenosine monophosphate does not mediate Shiga toxin stimulation of intestinal fluid and electrolyte secretion (7, 8, 26). Increased activity of adenylate cyclase from toxin-treated mucosa is observed when a high adenosine triphosphate (ATP) concentration is employed in the assay system (4). Exposing ileal mucosa to toxin results in an impairment of both sugar transport and amino acid transport, suggesting cytotoxic effects (2).

Preliminary studies have addressed the possible molecular mechanism by which Shiga toxin functions. We have reported previously that Shiga toxin inhibits protein synthesis in intact HeLa cells (J. E. Brown and S. W. Rothman, Fed. Proc. **38**:3213, 1979). In addition, toxin has been shown to inhibit mammalian cell-free protein synthesis (27) and cell-free polypeptide chain synthesis in ribosome-enzyme preparations from *E. coli* and *S. dysenteriae* (22). At the moment, studies of cytotoxic properties of Shiga toxin provide the best opportunity for relating molecular events to pathophysiological activities. An understanding of the biochemical mode of action causing cytotoxicity may provide a useful avenue for delineating the role of the toxin in disease. To elucidate the basis of its cytotoxicity, we now investigated the effects of a highly purified Shiga toxin preparation on both macromolecular synthesis and membrane functions in HeLa cells. The results suggest that inhibition of protein synthesis is the primary cytotoxic effect of Shiga toxin on intact HeLa cells.

MATERIALS AND METHODS

Establishment of HeLa cell monolayers. HeLa cell line CCL2 (Flow Laboratories, Rockville, Md.) was adapted at this institute to growth in medium supplemented with fetal bovine serum. This complete growth medium consisted of Eagle minimum essential medium with Earle salts supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 180 U of penicillin per ml, and 0.18 mg of streptomycin per ml. Fetal bovine serum and Eagle minimum essential medium with Earle salts were from HEM Research, Rockville, Md. Cultures were maintained at 35°C and 5% CO_2 in 32-ounce (960-ml) glass bottles. To establish monolayers, freshly trypsinized cells were suspended

to a concentration of 1.6×10^5 cells per ml in complete medium, and 0.10-ml portions were pipetted into 96-well microtiter dishes (Costar, Cambridge, Mass.). Cells were allowed to attach for 18 to 20 h before experimental use.

Cytotoxicity assay. The extent of HeLa cell detachment was employed as an indicator of cytotoxicity. This was measured by staining with crystal violet dye after overnight incubation of serially diluted toxin with HeLa cell monolayers in 96-well microtiter dishes (Gentry and Dalrymple, submitted for publication). Then the stained monolayers were dissolved in 50% ethanol–1% sodium dodecyl sulfate. The absorbance at 595 nm of the retained dye was determined either with a spectrophotometer or with a microtiter dish colorimeter constructed by the Division of Instrumentation, Walter Reed Army Institute of Research, from a design provided by Robert Yolken, Johns Hopkins University, Baltimore, Md. A logarithmic plot of absorbance versus dilution of toxin allowed determination of the dilution yielding 50% cell detachment (CD_{50}). With this assay, we defined 1 U of cytotoxic activity (1 CD_{50}) as the amount of toxin resulting in a 50% reduction of the dye retained in a microtiter well. Protein concentrations were determined by the method of Lowry et al. (20).

Macromolecular synthesis. Two series of experiments were designed to investigate whether toxin inhibits macromolecular synthesis in intact HeLa cells. In one set of experiments, the effect of toxin on protein and deoxyribonucleic acid (DNA) syntheses was determined by using [^{14}C]leucine and [^{3}H]thymidine as precursors. In the second set, the effect of toxin on protein and ribonucleic acid (RNA) syntheses was determined by using [^{14}C]leucine and [^{3}H]uridine. At the end of the incubation period, radioactivity in the trichloroacetic acid-precipitable fraction was measured to indicate the extent of macromolecular synthesis.

For protein and DNA syntheses, monolayers were established in microtiter dishes as described above. Immediately before the experiment, complete medium was replaced with 0.10 ml of incorporation medium (Eagle minimum essential medium with Earle salts without leucine [GIBCO Laboratories, Grand Island, N.Y.] but with 1 mg of bovine serum albumin per ml). At appropriate times, we added an additional 0.10 ml of incorporation medium containing toxin, 0.5 μCi of [*U*-^{14}C]leucine (355 Ci/mmol) per well, and 1 μCi of [*methyl*-^{3}H]thymidine (20 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per well. Incubation was stopped by rapidly cooling the plate on an ice and water slurry, immediately removing the radioactive medium, and washing the cells three times with chilled complete medium. The last wash contained no measurable radioactivity.

Cells were solubilized for 30 min at room temperature by placing the microtiter dish on a vibrating platform after the addition of 0.10 ml of 0.5 M KOH per well. After the addition of 0.025 ml of 0.15% bovine serum albumin as carrier protein, 0.10 ml of solubilized cells was withdrawn from each well and precipitated with 0.90 ml of cold 5% trichloroacetic acid. Trichloroacetic acid precipitates were collected at 4°C on glass fiber filters by using a MASH II cell harvester (Microbiological Associates, Bethesda, Md.) and washed with 5% trichloroacetic acid. Precipitates on the filter disks were treated with NCS solubilizer (Amersham/Searle, Arlington Heights, Ill.). Radioactivity was determined with a Searle Mark III liquid scintillation counter, using Liquifluor scintillation cocktail (New England Nuclear Corp.).

For protein and RNA synthesis studies, cells were treated as described above, except that the incorporation medium contained toxin, [^{14}C]leucine, and 1 μCi of [5-^{3}H]uridine (27 Ci/mmol; New England Nuclear Corp.) per well. After the cells were washed at the end of the incubation period, they were detached by trypsinization (10 min at 35°C), collected on glass fiber filters, and washed with phosphate-buffered saline. After precipitation and washing with cold 5% trichloroacetic acid, precipitates were treated as described above. Recovery of ^{14}C radioactivity was identical by both procedures.

Amino acid uptake. Uptake of α-aminoisobutyric acid (α-AIB) by monolayers was determined in 96-well microtiter dishes. These experiments were carried out as described above for macromolecular synthesis. After a 2-h incubation in the presence of [^{14}C]leucine and toxin, 1 μCi of *methyl*-^{3}H-labeled α-AIB (25 Ci/mol; New England Nuclear Corp.) per well in 0.050 ml of incorporation medium was added at 1-min intervals up to 10 min. The plates were chilled on an ice and water slurry, the radioactive medium was aspirated, and the cells were washed three times with ice-cold complete medium. The cells were solubilized by incubation for 30 min at room temperature in 0.10 ml of 0.5 M KOH. After the addition of 0.025 ml of 1% bovine serum albumin, 0.050-ml amounts were withdrawn for measurement of [^{14}C]leucine incorporation into protein as described above. Additional 0.050-ml amounts were withdrawn from each well, placed in scintillation vials, and treated with NCS solubilizer, and radioactivity was measured in order to determine the extent of ^{3}H-labeled α-AIB uptake by cells.

Intracellular K^+ content. Maintenance of high intracellular K^+ concentrations was determined after exposure of cells to toxin for different time periods. About 1.4×10^6 HeLa cells were inoculated into Falcon tissue culture dishes (60 by 15 mm) and allowed to attach overnight. Toxin (10^4 CD_{50}/ml; 8.8 ml/dish) was added, and cells were incubated for varying lengths of time. We removed the medium and washed the cell monolayers three times with cold K^+-free phosphate-buffered saline; the pooled medium and washes contained no detectable detached cells. We lysed the cells with 1.0 ml of distilled water, followed by freezing and thawing. The DNA in the lysates was hydrolyzed by treatment with deoxyribonuclease I. The lysates were dried overnight at 70°C and then dissolved in 2.0 ml of flame photometer diluent; K^+ concentrations were measured with an Instrumentation Laboratories model 343 flame photometer (Instrumentation Laboratories, Inc., Lexington, Mass.).

Electrophoresis. Discontinuous buffer electrophoresis in polyacrylamide gel rods was performed by the method of Davis (6). Gel rods were stained with Coomassie brilliant blue R250 for protein (30) or with fast green (CI 42053) for densitometry (10). Polyacrylamide electrophoresis with sodium dodecyl sulfate was

performed in gel rods by the method of Laemmli (17) after the samples were boiled for 5 minutes in the presence of 1% sodium dodecyl sulfate–1% 2-mercaptoethanol. Gels were stained with 0.04% Coomassie brilliant blue G250 in 3.5% perchloric acid.

RESULTS

Purification of Shiga toxin. The toxin used in this study was purified from cell lysates of *S. dysenteriae* 1 strain 3818-0 (J. E. Brown, M. K. Gentry, D. E. Griffin, S. W. Rothman, W. J. Cahillane, B. P. Doctor, and M. R. Thompson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, B42, p. 22). Bacteria were grown in 500 liters of modified syncase medium (21) and disrupted in a Manton Gaullin press (New England Enzyme Center, Boston, Mass.). The lysate was then processed through the following stages: overnight centrifugation, ammonium sulfate fractionation, ion-exchange chromatography on diethylaminoethyl cellulose (DEAE-Sephacel) and carboxymethyl cellulose (CM 52), concentration by ultrafiltration, gel filtration on Sephacryl S-200, preparative sucrose gradient isoelectric focusing (Ampholine [LKB Instruments, Rockville, Md.]; pH 5 to 8), ampholyte removal by Sephadex G-25 chromatography, and final concentration by PM-10 ultrafiltration. Yields ranged between 1 and 10 mg of toxin protein.

The final toxin preparation had a cytotoxic activity of 2.2×10^8 CD_{50}/mg of protein (1 CD_{50} = 4.5 pg), as determined by the microtiter cytotoxicity assay. This represented a purification of about 8,000-fold from the clarified cell lysate. The preparation induced fluid secretion in rabbit ileal loops and was lethal to mice in microgram amounts. The electrophoretic pattern on polyacrylamide gels after discontinuous buffer electrophoresis is shown in Fig. 1A. When gel electrophoresis progressed until the dye front reached the bottom of the gel (Fig. 1A, gels 1 and 3), two major protein bands and one faint intermediate minor protein band were observed. When electrophoresis was allowed to continue for twice this time (Fig. 1A, gels 2 and 4), the slow major band was separated into four components. A gel from a preparation such as gel 2 or 4 was sliced, and the toxin from these slices was eluted with 20 mM Tris-hydrochloride–100 mM NaCl–0.02% sodium azide (pH 8.0) at 4°C and assayed for cytotoxicity. All four bands contained cytotoxic activity proportional to their staining intensity. Analysis of densitometric tracings of such gels indicated that these active toxin bands represented 93% of the total protein stain. The band shown in Fig. 1A, gel 3, at the dye front is an artifact from the final ultrafiltration step. After polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol, the preparation was resolved into only two major components and one minor component. Details on purification and characterization will be published elsewhere (J. E. Brown, D. E. Griffin, S. W. Rothman, R. V. Lewis, M. K. Gentry, and B. P. Doctor, manuscript in preparation).

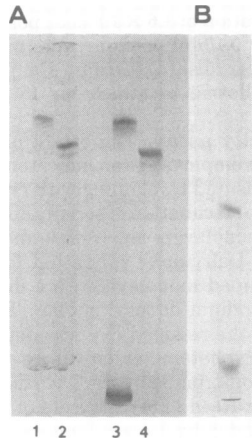

FIG. 1. *Polyacrylamide gel electrophoresis of purified Shiga toxin. (A) Discontinuous buffer electrophoresis was carried out until the dye band reached the bottom of the gel (gels 1 and 3) or for twice that time (gels 2 and 4). A 40-μg amount of protein was applied to each gel. Gels 1 and 2 were stained with Coomassie brilliant blue R250; gels 3 and 4 were stained with fast green. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 40 μg of protein. The dye front was marked by injecting the gel with India ink. Electrophoresis was performed as described in the text.*

Inhibition of macromolecular synthesis. Intact HeLa cell monolayers were incubated with varying concentrations of Shiga toxin, and the effects on protein, DNA, and RNA syntheses in intact HeLa cell monolayers were measured. After a minimal latent period of approximately 30 min, there was an inhibition of [^{14}C]leucine and [^{3}H]thymidine incorporations which was proportional to both toxin concentration and time of incubation (Fig. 2 and 3). Higher toxin concentrations (10^5 CD_{50}/well) did not shorten the lag period. With 50 ng of toxin (10^4 CD_{50}), [^{14}C]leucine incorporation was completely stopped after 45 min, whereas [^{3}H]thymidine incorporation continued at a very slow rate. No effect of toxin on [^{3}H]uridine incorporation was observed for 90 min (Fig. 4). Our finding that [^{3}H]uridine incorporation continued in a linear fashion between 30 and 90 min of incubation, while [^{14}C]leucine and [^{3}H]thymidine incorporations were inhibited, implies that the delayed effect of toxin on RNA synthesis is secondary to the inhibition of protein or DNA synthesis. As

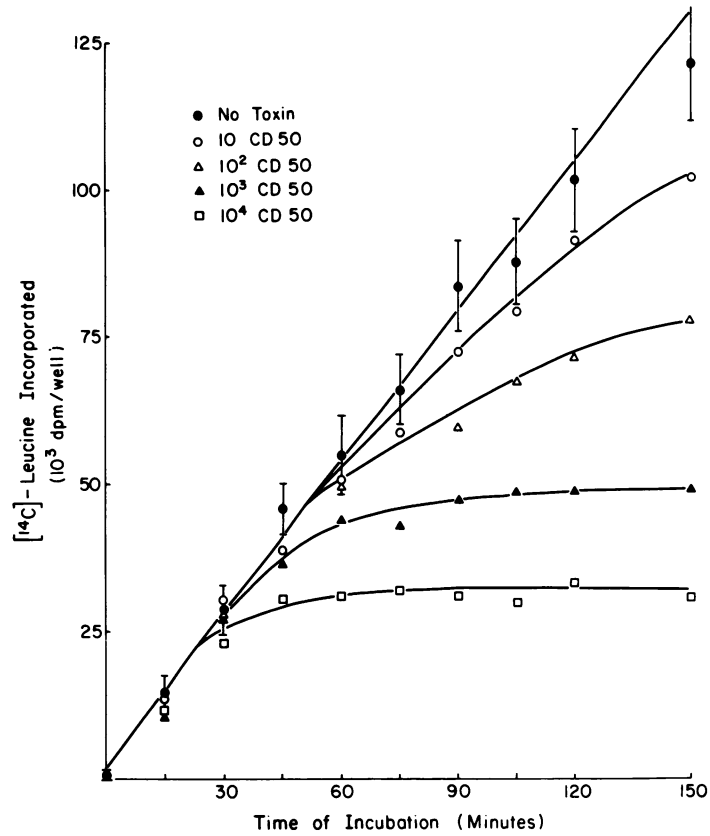

FIG. 2. *Shiga toxin inhibition of [^{14}C]leucine incorporation by HeLa cells into trichloroacetic acid-precipitable material. The amount of toxin in each well is given on the figure. Each control data point represents the mean of eight determinations. Toxin-treated points are averages of four values. Standard deviations are given for only control data samples for visual clarity.*

expected, all macromolecular synthesis continued in a linear fashion in the absence of toxin.

Uptake of α-AIB and maintenance of intracellular K^+ by HeLa cells in the presence of toxin. The observed inhibition of macromolecular synthesis by toxin could have been due to (i) inhibition of amino acid uptake, (ii) loss of cellular K^+ or amino acid pools due to gross membrane damage, or (iii) depletion of ATP. To rule out these possibilities, we examined the uptake of α-AIB and the maintenance of K^+ in toxin-treated HeLa cells. The rate of ^{3}H-labeled α-AIB uptake was determined in cells preincubated for 120 min with [^{14}C]leucine and toxin (10^3 CD_{50}/well). From the results (Fig. 5) it is evident that although protein synthesis was completely stopped after 90 min of incubation, α-AIB uptake was not interrupted by toxin. In a separate experiment cell monolayers were exposed to toxin for varying times, and then ^{3}H-labeled α-AIB was added for a 5-min pulse. No significant difference in accumulated ^{3}H-labeled α-AIB was observed from 0 to 150 min of toxin preincubation. Since a high intracellular potassium concentration is maintained by the sodium-potassium adenosine triphosphatase, changes in K^+ concentration can provide a sensitive indication of alterations in metabolic conditions, such as depletion of ATP due to gross membrane damage or inhibition of oxidative phosphorylation. As Table 1 shows, intracellular K^+ levels remained constant for at least 120 min after the addition of toxin, well after protein synthesis had completely stopped. These results indicate that Shiga toxin did not cause gross membrane damage or exhaust ATP supplies and that inhibition was not due to loss of precursor pools or interference in the uptake of precursors.

Cytotoxicity and inhibition of macromolecular synthesis are attributable to the same proteins. If a toxin preparation pretreated by methods which destroyed cytotoxicity were able to inhibit macromolecular synthesis, it would indicate that the molecular and cytotoxic activities of this preparation could not be attributed to the same protein. Cytotoxic

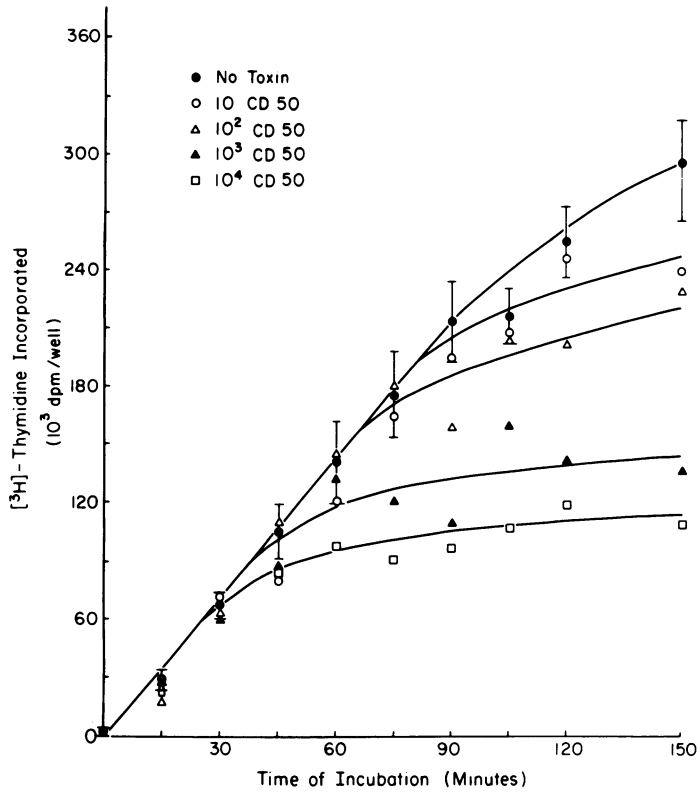

FIG. 3. *Shiga toxin inhibition of [^{3}H]thymidine incorporation by HeLa cells into trichloroacetic acid-precipitable material. The amount of toxin in each well is given on the figure. Control points represent means of four values ± standard deviations. Toxin-treated points are averages of two values.*

activity can be inactivated by incubation at 75°C for 30 min (Brown et al., manuscript in preparation). Purified toxin samples were incubated at varying temperatures for 30 min, cooled, and diluted with medium. Using these samples, an experiment similar to the one shown in Fig. 3 was carried out for 120 min. Toxin samples heated at 60 and 70°C were capable of inhibiting [^{3}H]thymidine incorporation to the same extent as unheated toxin. However, with toxin samples heated at 80, 90, and 100°C, incorporation proceeded at 82 to 85% of control levels. Cytotoxicity can also be abolished by neutralization with rabbit antiserum (Gentry and Dalrymple, submitted for publication). Purified toxin samples (10^3 CD_{50}/well) were neutralized by preincubation for 30 min at 37°C with serially diluted rabbit antiserum. This antiserum, which was produced against toxin eluted from a single polyacrylamide gel slice, formed a single precipitin line of identity when tested against a crude van Heyningen extract of *S. dysenteriae* 60 R and a partially purified preparation from *S. dysenteriae* 3818 T (A. D. O'Brien, G. D. LaVeck, D. E. Griffin, and M. R. Thompson, submitted for publication). These samples were then used in experiments similar to the one shown in Fig. 2. The cells were incubated for 90 min in the presence of [^{14}C]leucine and neutralized toxin. It is evident that the ability of toxin to inhibit [^{14}C]leucine incorporation was blocked by neutralization with specific antiserum (Fig. 6).

To demonstrate that all four toxin bands in our preparation (Fig. 1A, gels 2 and 4) showed cytotoxicity as well as inhibition of protein and DNA syntheses, the following experiment was carried out. A 40-μg amount of toxin was subjected to polyacrylamide gel electrophoresis for twice as long as required for the dye marker to reach the bottom of the gel. The gel was sliced into 1-mm slices, and the toxin was eluted from individual slices. Eluants tested for cytotoxicity were further tested for their ability to inhibit incorporation of [^{14}C]leucine and [^{3}H]thymidine. As Fig. 7 shows, incorporation of both substrates was inhibited simultaneously by all four cytotoxic peaks. The inhibitory activity correlated with the position of the protein-stained bands,

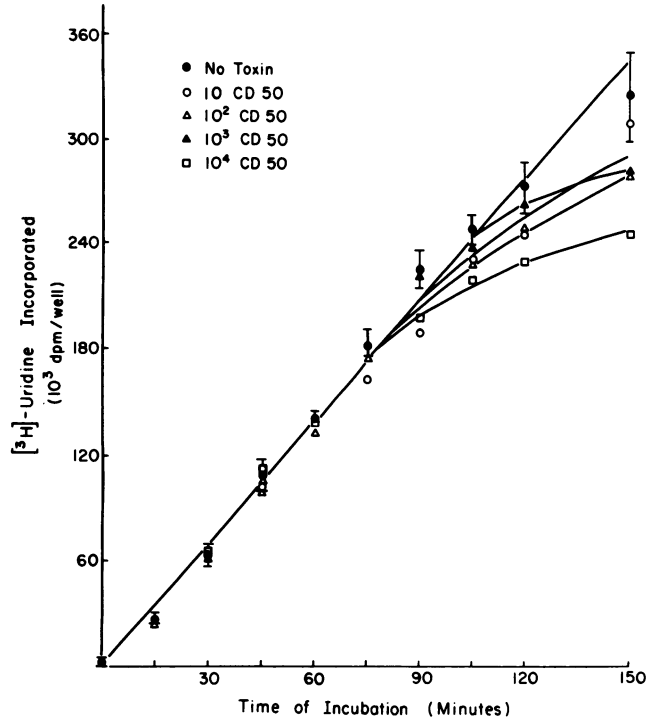

FIG. 4. *Shiga toxin inhibition of [³H]uridine incorporation by HeLa cells into trichloroacetic acid-precipitable material. See legend to Fig. 3 for details.*

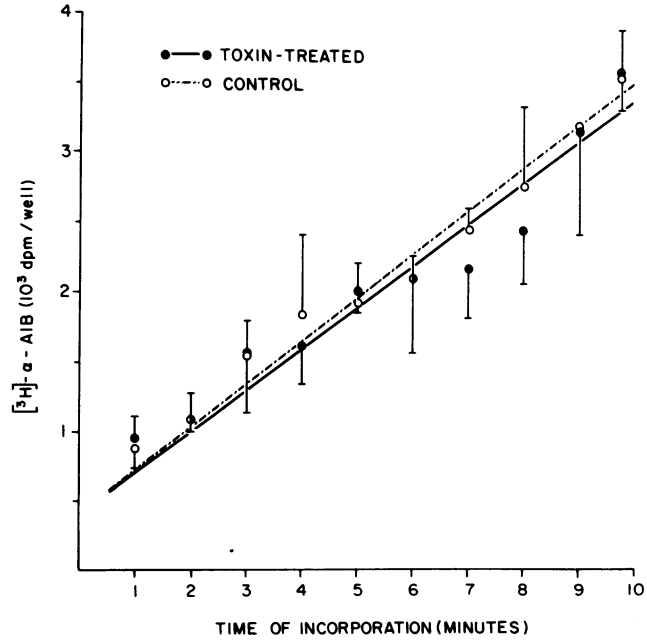

FIG. 5. *³H-labeled α-AIB uptake by HeLa cells. Toxin-treated cells were exposed to 10^4 CD_{50}/well for 120 min. Each datum point represents the mean of three determinations ± standard deviation. The curves were derived from a least-squares linear regression analysis.*

and the degree of inhibition was proportional to the staining intensity. No other region of the gel displayed cytotoxicity in the cell detachment assay. These results demonstrate that cytotoxicity and the ability to inhibit macromolecular synthesis exist in the same protein moiety.

TABLE 1. *Maintenance of intracellular K^+ levels after exposure to Shiga toxin*

Time of incubation (min)	Intracellular K^+ content (μmol of K^+/60-mm dish)[a]	
	Control	With toxin
0	1.16 ± 0.04	1.08 ± 0.07
60	1.07 ± 0.01	1.06 ± 0.03
120	1.17 ± 0.06	1.04 ± 0.05
180	1.13 ± 0.01	0.81 ± 0.03
240	1.18 ± 0.04	0.52 ± 0.02
480	1.31 ± 0.02	0.19 ± 0.02
960	1.92 ± 0.09	0.09 ± 0.01

[a] Values represent means of three determinations ± standard deviation.

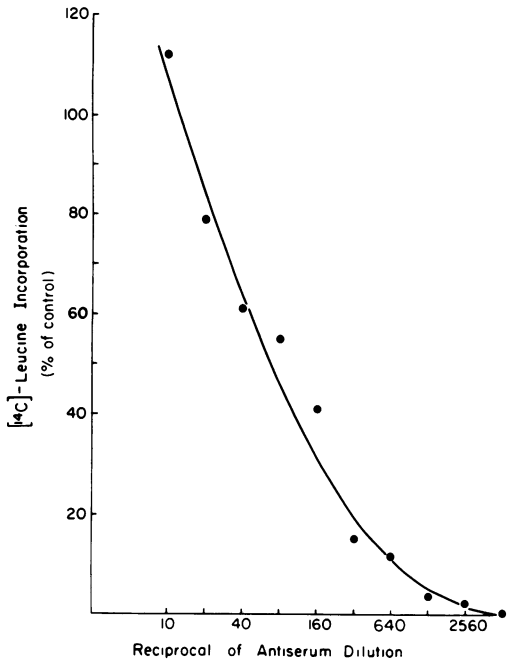

FIG. 6. *Protection by toxin-specific antiserum from inhibition of protein synthesis in HeLa cells. Diluted toxin was mixed with an equal volume of serially diluted antitoxin. The mixture was preincubated for 30 min at 37°C before addition to the wells. Incorporation of [^{14}C]leucine proceeded for 90 min. The amount of toxin used was 10^3 CD_{50}/well before neutralization. Toxin-negative controls containing antiserum diluted 1:20 incorporated 9,180 ± 360 dpm/well (n = 6). Toxin-treated controls without antiserum incorporated 3,070 ± 70 dpm/well (n = 6). Percentages were calculated by using the difference between these control values as 100%. Each point represents three determinations.*

DISCUSSION

The purpose of this investigation was to elucidate the basis of the cytotoxicity expressed by Shiga toxin in intact HeLa cells. The sequence of some events that may cause cytotoxicity (cell death) was studied. We observed that toxin inhibition of macromolecular synthesis followed a definite sequence. Although inhibition of protein synthesis and inhibition of DNA synthesis appeared to be concurrent, the primary effect of toxin was most likely on protein synthesis. Our data do not clearly resolve the relationship between inhibition of protein synthesis and inhibition of DNA synthesis; however, at high doses a slight residual incorporation of [^{3}H]thymidine was observed at extended time intervals, whereas [^{14}C]leucine incorporation ceased completely. At a toxin dose of 10^3 CD_{50}/well, cumulative [^{3}H]thymidine incorporation at 150 min was 6% greater compared with controls than [^{14}C]leucine incorporation; at 10^4 CD_{50}/well, the difference was 11%. Similar concurrent inhibition of protein and DNA syntheses has been observed for abrin (18), ricin (19), and emetine (11). All are known to inhibit specifically protein synthesis, although recent work indicates that ricin can also inhibit DNA polymerase both in whole cells and in a cell-free system (1). No inhibition of RNA synthesis by toxin was observed, even with the high concentration of toxin used, up to 75 to 90 min; this was well after protein and DNA syntheses had ceased in these cells. It is clear, therefore, that inhibition of protein synthesis by toxin cannot be attributed to inhibition of RNA synthesis. We suspect that this inhibition of RNA synthesis may result from inhibition of protein or DNA synthesis.

The decreased incorporation of [^{14}C]leucine that we observed could have occurred from depletion of intracellular amino acid pools or from inhibition of cellular energy metabolism. Because no change in ^{3}H-labeled α-AIB uptake was observed even after complete cessation of protein synthesis, amino acid depletion was unlikely. Shiga toxin did not have a direct effect on cellular energy metabolism. This conclusion is supported by the findings from our study of intracellular K^+ levels in toxin-treated cells. Since high intracellular K^+ levels are maintained by the Na^+K^+-adenosine triphosphatase system, any depletion of cellular ATP would result in a rapid decrease in intracellular K^+ concentration. Intracellular K^+ levels were unchanged until after complete cessation of protein synthesis, suggesting that neither inhibition of ATP pro-

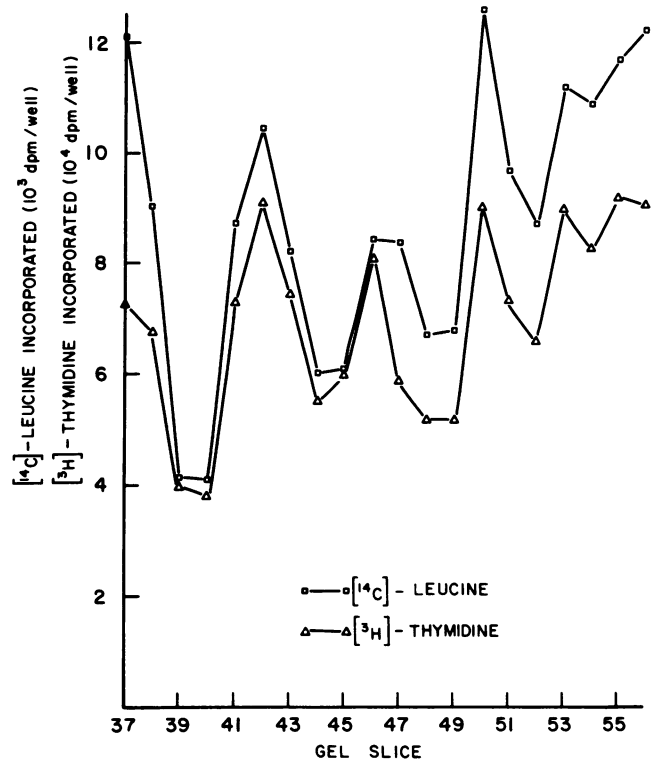

FIG. 7. *Inhibition of [^{14}C]leucine and [^{3}H]thymidine incorporations by toxin. After electrophoresis (40 μg/gel) for twice the time required for the dye band to reach the bottom of the gel, the gel rod was sliced into 1-mm slices, and the slices were eluted. Slices showing cytotoxic activity were assayed. Cells were incubated with 1:25 dilutions of eluant for 150 min; this was followed by a 60-min pulse of [^{14}C]leucine and [^{3}H]-thymidine. Each datum point is the mean of three values.*

duction (i.e., oxidative phosphorylation) nor increased hydrolysis of ATP can account for the early inhibition of protein and DNA syntheses. Preferential inhibition of protein synthesis has also been observed in human intestinal epithelial cells by using partially purified *S. dysenteriae* toxin (M. S. Osata, T. A. Brawner, and D. J. Hentges, Am. J. Clin. Nutr. **32**:268, 1979).

Shiga toxin has been shown to inhibit protein synthesis in both mammalian and bacterial cell-free systems. Using nearly homogeneous toxin in a rat liver cell-free system, Thompson et al. (27) showed inhibition of amino acid transfer from amino acyl-transfer RNA complexes to ribosome-polypeptide chain complexes for both polyuridylic acid-directed ribosomes and intact polysomes. No effect on aminoacylation of transfer RNA was observed, and exogenous oxidized nicotinamide adenine dinucleotide was not required for toxin activity. Olenick and Wolfe (22) were able to show inhibition of [^{14}C]polyphenylalanine synthesis by using polyuridylic acid-directed ribosome-enzyme preparations from either *E. coli* or *S. dysenteriae* 1. Inhibition of bacterial polypeptide synthesis occurs only when ribosomes are preincubated with toxin before the addition of polyuridylic acid. Once [^{14}C]phenylalanine incorporation is initiated, the toxin has no effect. This has been attributed to an inability of the polyuridylic acid messenger to attach to ribosomes in the presence of toxin. These cell-free studies support the conclusion that inhibition of protein synthesis in intact cells is a primary cytotoxic effect.

Shiga toxin does not appear to act as a cytolytic toxin. No effect on either amino acid uptake (and retention) or intracellular K^+ levels was observed until well after complete inhibition of protein synthesis. Loss of both K^+ and the nonmetabolizable amino acid α-AIB would have quickly followed any gross changes in membrane permeability. In addition, an initial lag phase was observed at all toxin concentrations. Exposure to increasing levels of toxin shortened the lag period before the onset of inhibition, but the minimum latent period was 30 min. Finally, the differential inhibition of macromolecular synthesis (protein and DNA versus RNA) supports

an intracellular role for toxin.

Our data suggest that Shiga toxin inhibits protein synthesis in whole cells in an enzymatic manner. Assuming a molecular weight of 70,000 (24) and an estimate of 5×10^6 ribosomes per HeLa cell (11), the cytotoxic activity of 2.2×10^8 CD_{50}/mg of protein for the purified toxin indicates a ratio of at least 1,000 ribosomes per toxin molecule. Protein toxins inhibiting protein synthesis seem to fall into two general categories. Diphtheria toxin and *Pseudomonas* toxin A inactivate elongation factor 2 by transfer of adenosine diphosphate-ribose from oxidized nicotinamide adenine dinucleotide (5). Abrin, ricin, and modeccin inhibit protein synthesis by inactivation of the 60S ribosomal subunit (25). The mechanism of action of Shiga toxin has not been resolved, but early studies showed that the addition of exogenous oxidized nicotinamide adenine dinucleotide to cell-free lysates containing Shiga toxin causes no additional inhibition (27). In recent experiments with wheat germ extracts, no adenosine diphosphate ribosylation activity was detected using purified toxin (7 μg/ml), either untreated or after pretreatment with trypsin or urea and dithiothreitol (J. E. Brown, S. H. Leppla, and S. W. Rothman, unpublished data).

The data indicate that inhibition of protein and DNA syntheses is the basis of the cytotoxic activity of Shiga toxin. Interpretations of the reported pathophysiological effects of toxin must now take into account these molecular phenomena. The role of Shiga toxin in bacillary dysentery, for which bacterial invasion is the recognized hallmark of the disease (16), remains unclear. However, cytotoxicity at the subfemtogram level of toxin per mammalian cell implies that Shiga toxin could be an effective virulence factor during the disease process.

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