AD_____

MODE OF ACTION OF SHIGELLA TOXIN: EFFECTS ON RIBOSOME STRUCTURE AND FUNCTION

Final Report

Tom G. Obrig, Ph.D.

May, 1988

Supported by

U.S. Army Medical Research and Development Command

Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6065

Albany Medical College
Albany, New York 12208

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188 Exp. Date: Jun 30, 1986

1a REPORT SECURITY CLASSIFICATION: Unclassified	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; Distribution unlimited.
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION: Albany Medical College	6b OFFICE SYMBOL (If applicable)
6c ADDRESS (City, State, and ZIP Code): 47 New Scotland Avenue, Albany, New York 12208	7b ADDRESS (City, State, and ZIP Code)
8a NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Res. & Dev. Command	8b OFFICE SYMBOL (If applicable)
8c ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5012	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-86-C-6065

10 SOURCE OF FUNDING NUMBERS			
PROGRAM ELEMENT NO: 61102A	PROJECT NO: 3M1-61102BS10	TASK NO: AE	WORK UNIT ACCESSION NO: 041

11 TITLE (Include Security Classification)

MODE OF ACTION SHIGELLA TOXIN: EFFECTS OF RIBOSOME STRUCTURE AND FUNCTION

12 PERSONAL AUTHOR(S)

Tom G. Obrig, Ph.D.

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final	FROM 10/1/85 TO 3/15/87	1988 May	31

16 SUPPLEMENTARY NOTATION

17 COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	03	

18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

Keywords: Shigella, Shiga toxin, Shigellosis, protein synthesis, ribosomes, dysentery, RA 1

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The goal of this contract research project was to describe, in biochemical terms, the detailed mechanism of Shiga toxin inhibition of eukaryotic protein synthesis. It was previously determined that Shiga toxin preferentially inhibited peptide elongation, but the exact steps of peptide elongation representing the toxin target had not been described in detail. In the present study, purified components for protein synthesis were utilized to measure individual steps of peptide elongation. Our results clearly indicate that Shiga toxin is a primary inhibitor or eukaryotic elongation factor-1 (eEF·1) dependent binding of aminoacyl-tRNA to ribosomes. In addition, Shiga toxin was shown to be a less potent inhibitor of eEF.2-dependent translocation of aminoacyl-tRNA on reticulocyte ribosomes. Excess eEF·1 protein could not reverse the toxin-related effects on aminoacyl-tRNA binding whereas excess exogenous eEF·2 protein could easily overcome the

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	Unclassified

22a NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Mrs. Virginia M. Miller	301/663-7325	SGRD-RMI-S

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

Abstract (continued)

toxin-induced inhibitions of peptidyl-tRNA translocation.

An additional discovery during this project was the observation that Shiga toxin exhibited a latent cytotoxic response towards human vascular endothelial cells, *in vitro*. This response was both toxin dose- and time-dependent. The significance of these results is that Shiga toxin action described in this study may explain the nature of hemolytic uremic syndrome (HUS), vascular disease of children. HUS occurs 3 to 4 days after a peak of bacillary dysentery. It is hypothesized that Shiga toxin enters the blood during a severe case of dysentery, binds to vascular endothelial cells and results in disseminated microvascular thrombosis, thrombocytopenia and kidney failure which are the characteristics of HUS.

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised, 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

TABLE OF CONTENTS

Abbreviations appearing include:

rRNA - ribosomal ribonucleic acid
rprotein - ribosomal protein
tRNA - transfer ribonucleic acid
mRNA - messenger ribonucleic acid
eEF 1 - eukaryotic elongation factor 1 which is the aminoacyl-transfer RNA binding factor
eEF 2 - eukaryotic elongation factor 2 which is the translocase factor
eIF - eukaryotic initiation factor
Met·tRNA$_f$ - initiator methionyl-tRNA
GTP - guanosine triphosphate
DTT - dithiothreitol, a reducing agent
polysome - a mRNA molecule with 3+ ribosomes attached
80S ribosome - ribosome comprised of 60S and 40S subunits
60S, 40S ribosome - large and small ribosomal subunits, respectively
DOC-KCl ribosomes - ribosomes treated with 1% Deoxycholate and 0.5M KCl
TCA - trichloroacetic acid
HUVEC - human umbilical vein endothelial cells
HUS - hemolytic uremic syndrome
DMEM - Dulbecco's modified Eagle's medium
PBS - 0.02M sodium phosphate, pH 7.0, 0.1M NaCl
FBS - fetal bovine serum
EGTA - ethyleneglycol-bis-(β-aminoethyl ether) N,N,N^1,N^1-tetraacetic acid
ID_{50} - concentration of toxin yielding 50% inhibition of test system.

STATEMENT OF THE PROBLEM

1. Shiga toxin, as a documented inhibitor of mammalian protein biosynthesis, may specifically affect individual functional steps in the overall process.

2. The toxin, known to inactivate large ribosomal subunits, may interact with and modify a protein or RNA component of the ribosome.

3. Toxin-induced structural modification of the ribosome may be responsible for the change in ribosome protein biosynthetic activity.

4. Because the process of eukaryotic cytoplasmic protein biosynthesis is virtually identical in all mammalian cells and tissues, it is likely that information obtained from this study will be pertinent to Shiga toxin mode of action in intestinal tissues invaded by toxigenic Shigella.

TECHNICAL OBJECTIVES

1. Describe, in biochemical terms, how purified Shiga toxin inhibits mammalian protein biosynthesis.

2. Identify the individual functional steps of protein synthesis which are inhibited by Shiga toxin.

3. Determine if the toxin-induced alteration in sub-cellular ribosome function is due to a structural modification of the ribosome.

4. Compare data from above with existing information on plant-derived toxins which resemble Shiga toxin mode of action at ribosome level.

BACKGROUND

It was established early on that a proteinaceous toxin is produced by the pathogen Shigella dysenteriae 1 (1). However, the relationship of the toxin and pathogenic properties of Shigella bacilli remains to be fully determined. During infection, Shigella penetrates the bowel epithelium and causes intestinal lesions (2). It has been suggested that a toxin produced by Shigella dysenteriae 1 is responsible for eliciting host responses to the pathogen (3,4). Host responses to Shigella include fluid production by ileal loops, in vitro, and diarrhea, in situ (4).

Moreover, a further understanding of Shiga action in the disease process would be aided by a series of investigations utilizing purified toxin. With partially purified toxin, several concepts pertinent to Shiga toxin function have become known. It appears that Shiga toxin is cytotoxic to several cell lines including HeLa human cervical carcinoma and WI-38 human fibroblasts (5-8). In addition, the relative activity of Shiga toxin in cell cultures is parallel to that in the rabbit ileum test system (6). From these data one might hypothesize that Shiga toxin is a non-selective agent which manifests its toxic properties on a wide array of cell and tissue types. However, recent reports indicate that toxin from S. Shigae exhibits high-affinity binding to a limited number of cell types (8,9). This strongly suggests that the toxin resembles other well-known microbial-derived toxins (i.e., diphtheria toxin, cholera toxin and Pseudomonas exotoxin A) which bind with high affinity ($Kd = 10^{-10}$ M) to receptors on cells (10).

All of the above mentioned toxins appear to be potent inhibitors of protein biosynthesis in eukaryotic cells. It is generally accepted that clinical symptoms associated with these toxin-producing bacteria are, in part, a result of their effect on protein synthesis. To go one step further, it has been suggested that Shiga toxin also elicits different physiological responses in the host target by virtue of its ability to efficiently inhibit protein biosynthesis (11). Indeed, there is ample evidence indicating that Shiga toxin has, as its primary action, the inhibition of protein biosynthesis in whole animals (12) cell cultures (8,11) and cell-free lysates (11,14,22). It seems clear that ribosomes are the primary target of Shiga toxin and more recent results indicate that the large ribosomal subunit of eukaryotic cells are specifically affected (15) At this time, little is known about the ribosomal component which is modified by Shiga toxin. Data from our laboratory indicates that the toxin may be a ribonuclease (20,24).

It is conceivable that such information would be used to advantage in design of a mode of therapy for Shiga toxin based on information regarding the ribosomal substrate. In addition, these studies should lead to implementation of a new molecular assay for Shiga toxin which would have great sensitivity and specificity.

Shiga toxin has been purified to apparent homogeneity from S. shigae (9,16) and S. dysenteriae 1 (17,18). The holotoxin from both sources has an approximate molecular weight of M_r = 70,000 (9,18). Structural analysis of S. shigae toxin (9) indicates its similarity to

cholera toxin; Shiga toxin has a single larger peptide of M_r = 30,000 and multiple copies of a smaller peptide (M_r = 5,000). It is also apparent that Shiga toxin resembles other multicomponent proteinaceous toxins (19) by having catalytic toxicity and cell binding specificity functions located on distinct peptides. That is, the larger peptide (subunit "A") is catalytic while smaller peptides (subunit "B"), appear to infer binding specificity properties of the holotoxin.

Recently, attention has turned to resolving the role of Shiga toxin in human disease by employing isolated target cell types, such as intestinal epithelia, which are purported to be damaged during natural progression of the infectious disease process (13,25).

In addition to the typical diarrhea and dysentery syndromes resulting from Shigella dysenteriae 1 infection (26-28), a small but significant number of cases were reported to progress towards development of disseminated vascular complications, and hemolytic uremic syndrome (HUS) (29-33). Pathology of HUS includes microangiopathic nephrotoxicity, disseminated microvascular thrombosis, anemia, and thrombocytopenia (34). Several reports point to the vascular endothelial lining as being a primary cell type affected in HUS (34-36). A role for bacterial endotoxin in HUS has been suggested, but has not been established (37). In contrast, parenteral presentation of Shiga toxin to animals suggested that systemic Shiga toxin may have a direct or indirect cytotoxic effect on endothelial cells (35,36).

METHODS

Activation of Shiga Toxin - Shiga toxin was purified from Shigella dysenteriae 1 strain 3818-0 as described by Brown, et al.(18) and activated, to increase in vitro potency by 70 to 100-fold (14), by adding 10 μg TPCK trypsin to a 1.0 ml solution containing Shiga toxin (0.42 mg/ml), 10 mM Tris-Cl, pH 8.0 and 100 mM NaCl. The mixture was incubated at 37°C/60 min. and 0.02 μg of phenylmethylsulfonyl fluoride was added to inactivate the trypsin. Urea and dithiothreitol were added to 8 M and 10 mM final concentrations, respectively, and incubated at 37°C/60 min. This sample was dialyzed vs. 3 x 100 vol of Type 1 water. Activated toxin was then lyophilized and stored at -70°C. Upon reconstitution in water, toxin prepared in this manner retained full activity as an inhibitor of cell-free protein synthesis.

Reticulocyte Lysates and Ribosomes - To obtain reticulocytes, New Zealand white rabbits (2-2.5 kg) were injected daily on days 1 through 4 with 0.25 ml/kg of 2.5% phenylhydrazine, pH 7.0 in 0.14 M NaCl. On the 7th day rabbits were sacrificed and blood drained from the heart into a freshly prepared ice-cold NKM (0.14 M NaCl, 0.03 M KCl, 0.002 M $MgCl_2$) solution containing 200 units of heparin/ml. Reticulocytes as measured with methylene blue staining were found to represent >90% of the total cell population. This whole blood was filtered through cheesecloth, centrifuged at 1,000 x g/10 min and serum removed along with an upper "buffy coat" layer of cells. Packed reticulocytes were gently resuspended in 20 vol of NKM solution and centrifuged as above. This washing procedure was repeated a total of three times.

Lysate was prepared with the addition of 1 vol of Type I water to packed cells followed by gently shaking (4°C/10 min) and centrifugation at 20,000 x g /15 min. Aliquots of the resultant supernatant were stored at -80°C for up to 1 year without loss of activity. When first employed, each batch of lysate was tested with varying concentrations of hemin and magnesium acetate to determine concentrations required for maximum protein synthetic activity. Rates of protein synthesis in the lysate system were very close to that of whole reticulocytes.

Ribosomes were prepared from reticulocytes by washing with deoxycholate, then with 0.5 M KCl and will be referred to as DOC-KCl ribosomes. Ribosomal subunits were obtained by dissociation of polysomal 80S monomers in the presence of 0.5 M KCl and 1 mM puromycin (23). Subunits were separated with 35 ml 10-30% sucrose gradients by centrifugation in a SW27 rotor (Beckman) at at 13,500 rpm/16 h/4°C and precipitated by addition of magnesium chloride to 10 mM and 0.7 vol. 95% pre-cooled (-20°C) ethanol. Precipitated ribosomes were collected by centrifugation at 6,000 x g/20 min/4°C, resuspended and stored at -70°C.

Purification of eEF-1 and eEF-2 from Rabbit Reticulocytes - eEF-1 and eEF-2 was isolated from the 100,000 x g supernatant of reticulocyte lysate. The purification procedure included sequential steps of ammonium sulfate fractionation, gel filtration, DEAE-cellulose, hydroxylapatite and Cm-cellulose chromatography. In some cases,

purified elongation factors from wheat germ supplied by Dr. J. Ravel (U. Texas at Austin) were utilized and found to be comparable in activity to the reticulocyte proteins.

[^{3}H] Phe-tRNA Synthesis - Aminoacylation of yeast tRNA with [^{3}H]Phe was carried out in 4.0 ml reaction containing: 100 mM Tris-Cl, pH 7.4, 10 mM $Mg(OAc)_2$, 10 mM dithiothreitol, 2 mM ATP, 2.6 mg creatine phosphate, 0.1 mg creatine phosphokinase, 200 μg of reticulocyte ribosomal 0.5 M KCl wash protein, 5 mg tRNA, and 100 μg [^{3}H]Phe (SA=2,000 Ci/mol). The reaction was incubated at 37°C/40 min and monitored for cold 10% trichloracetic acid-insoluble radioactivity precipitated onto glass fiber filters. [^{3}H]Phe-tRNA was extracted by addition of 2.0 M K(OAc), pH 5.0 to a final concentration of 0.1 M and 1 vol phenol saturated with 10 mM K(OAc), pH 5.0. Following agitation for 15 min/4°C and centrifugation, the aqueous layer was removed and [^{3}H]Phe-tRNA precipitated by addition of 2.5 vol of 95% ethanol at -20°C/2 h. The [^{3}H]Phe-tRNA pellet was washed with 95% ethanol and then with ethyl ether to remove residual phenol.

eEF-1 and eEF-2 GTPase Assay - The eEF-2 GTPase reaction (21) mixture was performed in a total volume of 50 μl containing, in order of addition: 25 mM Tris-Cl, pH 7.4, 100 mM KCl, 5 mM $Mg(OAc)_2$, 2.5 mM dithiothreitol, Shiga toxin as indicated, 1.0 A_{260} units DOC-KCl washed ribosomes, 0.2 μg eEF-2, and 5 μM [α-^{32}P]GTP (SA=1,000 Ci/mol). The reaction mixture was incubated at 37°C/10 min, stopped by addition of 0.25 ml 0.02 M silicotungstic acid in 0.02 N H_2SO_4, 0.5 ml 1 mM potassium phosphate, pH 6.8, and 0.25 ml of 5% (W/V) ammonium molybdate in 4 N H_2SO_4. The phosphomolybdate was extracted into 1.0 ml of isobutanol/benzene (1:1 v/v), centrifuged at 500 x g for 5 min and radioactivity of 0.5 ml of the aqueous phase was monitored in a scintillation counter.

Ribonuclease Assay Total rRNA, extracted from 60S ribosomes with phenol-chloroform, was separated into 28S, 5.8S and 5S species on preparative 5% polyacrylamide gels in the presence of 8M urea. The 5.8S or 5S rRNA were extracted from gels and rerun to obtain purified RNA samples. Throughout, caution was taken to utilize RNase-free labware, reagents and type I water.

Toxins were incubated with either 5S or 5.8S rRNA in water at 37°/10min. Concentrations of toxins are noted in each figure. Following incubation, rRNA was 3' end-labeled at 4°C/12hr. with [^{32}P-5']pCp, S.A.=2500 Ci/mmol, and T4 ligase as described by Peattie (13). The sample was precipitated with 70% ethanol and dissolved in electrophoresis sample buffer consisting of : 8M urea, 20 mM Tris-HCl, pH 7.4, 1 mM Na_2-EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue. Samples (5μl) were heated (65°C) and layered onto a 0.75mm thick 10% polyacrylamide gel containing 7M urea and electrophoresed at 300v/4.5hr/10°C in a Tris-borate, pH 8.3 buffer system containing EDTA. Autoradiography was performed using X-OMAT (Kodak) film.

Endothelial Cells - Human umbilical vein endothelial cells (HUVEC) were isolated using a method described previously (38). At the time of collection, the umbilical cord was cut from the placenta, the blood drained and the cord placed in a sterile container with 25ml of

Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5μg/ml Fungizone and 100μg/ml gentamicin sulfate. Under sterile conditions, the outside of the cord was wiped with a 10% povidone-iodine solution and the ends cut clean with a number 10 scalpel blade. Cannulas were inserted into each end and secured with braided umbilical tape. The cord was perfused with calcium and magnesium-free phosphate buffered saline (PBS) supplemented with 2.5μg/ml fungizone, and 100μg/ml gentamicin sulfate. The cord was then perfused with 500 units/ml CLS II collagenase in DMEM - RPMI 1640 medium supplemented with 2.5% bovine serum albumin containing 10% fetal bovine serum (FBS) and incubated at 37°C for 10min. The umbilical cord was gently massaged and then perfused with RPMI 1640 medium supplemented with 20% FBS. The resultant effluents were placed in a 15ml tube and centrifuged at 500 x g for 10min at 10°C. The supernate was then removed and the pellet resuspended in complete medium which consisted of RPMI 1640 supplemented with 20% FBS, 75μg/ml heparin and 6.7μg/ml retinal-derived growth factor (39). This cell suspension was placed in a 25 cm^2 tissue culture flask and incubated in a 5% CO_2 atmosphere at 37°C. Cells were fed every third day by exchanging existing media with new complete media. When confluent, cells were removed from the plate with a solution, containing in final concentrations: 0.05% trypsin; 0.02% EGTA; and 1% polyvinylpyrrolidone, 0.9% NaCl in HEPES buffer (40). Characteristic of endothelial cells, virtually all cells contained factor VIII related protein shown by a fluorescent double antibody test.

For each expt, cells were seeded into 24-well (16mm diam) plates at a cell density of 2.5 x 10^4 cells per well (non-confluent) or 1 x 10^5 cells per well (confluent) and incubated at 37°C for 12 to 24h prior to use. Each well contained 1ml of complete medium. All experimental components were prepared in RPMI-1640 medium, sterilized by filtration, and added in a total volume of ≤100μl. Control wells received an equal volume of RPMI-1640 medium only. Following an incubation period at 37°C in a 5% CO_2 atmosphere, medium was removed carefully from each well and the remaining substratum-attached cells were released from the plate with 250μl of a trypsin-EGTA solution containing 0.04% (w/v) trypan blue. The cells were enumerated with a hemocytometer or a Coulter counter. All experiments were performed with triplicate or quadruplicate wells for each treatment mode.

Antitoxin. Polyclonal antibody was produced in rabbits treated with purified Shiga toxin. The IgG fraction was partially purified from rabbit serum by protein-A affinity chromatography and preincubated (37°C/1h) with Shiga toxin where indicated before addition to cell cultures. Monoclonal antibody vs the β-subunit of Shiga toxin was prepared as described elsewhere (41).

Binding of [^{125}I] Shiga toxin to HUVEC. Purified Shiga toxin was iodinated to a specific activity of approx. 9.2μCi/μg protein using the immobilized lactoperoxidase method (42). Biological activity of mock iodinated Shiga toxin was unaltered. The binding assay was carried out as follows. To each well of a 24-well culture plate was added [^{125}I] Shiga toxin (5μl, 1 x 10^6cpm) in 0.5ml HUVEC complete medium, yielding a final concentration of approx. 10^{-9}M toxin. The cells

were incubated at 4°C or 37°C/60min in a 5% CO_2 atmosphere. To terminate the reaction, medium was removed from the wells, each well was washed with 3 x 1ml complete media. Total cell protein was solubilized by incubation in 0.5ml of 0.5M sodium hydroxide at 37°C/20min. Radioactivity present in this solution was measured in a gamma counter. In some cases, before addition to cell cultures, [^{125}I] Shiga toxin was preincubated (37°C/2h) with either a 100-fold excess of unlabeled Shiga toxin or with a 1:50 dilution of Shiga toxin anti- β-subunit monoclonal antibody.

Endotoxin. A Limulus amoebocyte lysate assay was used to determine bacterial endotoxin levels in cell culture components. The lower level of endotoxin detection was 0.01 ng/ml with this technique. A final estimate of endotoxin concentration in the complete cell culture assays was based on the dilution of each component in the assay.

RESULTS AND DISCUSSION

Effect of Shiga Toxin on eEF-1 Dependent Reactions - Enzymatic binding of [^{3}H]Phe-tRNA to DOC-KCl washed ribosomes was inhibited by Shiga toxin in a concentration dependent fashion (Fig. 1). A 50% inhibition of eEF-1-dependent [^{3}H]Phe-tRNA binding was observed at 7 nM Shiga toxin. Enzymatic Phe-tRNA binding remained maximally inhibited at 40% of control levels (Fig. 1). The toxin, activated by trypsin, urea, and dithiothreitol treatment, inhibited reticulocyte lysate endogenous protein synthesis by 50% at 6.4 nM concentration (Fig. 1), in close correlation to the effect on enzymatic binding of [^{3}H]Phe-tRNA.

Excess eEF-1 has been shown to partially reverse the inhibitory effect of ricin and phytolaccin on peptide elongation. Thus, we tested for a possible effect of excess eEF-1 on Shiga toxin inhibition of Phe-tRNA binding to ribosomes. In control reactions, maximum enzymatic binding of [^{3}H]Phe-tRNA was obtained at 60 ug eEF-1 protein/ml (Fig. 2A). In Shiga toxin-treated preparations, increasing eEF-1 concentrations up to 90 ug/ml did not overcome the inhibition of Phe-tRNA binding. In fact, in the presence of Shiga toxin Phe-tRNA binding was reduced further from 50% to 30% of control values as eEF-1 protein was increased (Fig. 2B). This may be due to the increased efficiency of Shiga toxin at higher rates of the reaction.

Phe-tRNA binding to DOC-KCl washed ribosomes was measured at $\geq$ 10 mM Mg^{2+} concentrations to test the effect of Shiga toxin on non-enzymatic binding. While eEF-1 dependent Phe-tRNA binding carried out 6 mM Mg^{2+} is sensitive to Shiga toxin (see above), non-enzymatic Phe-tRNA binding performed at $\geq$10 mM Mg^{2+} was refractory to toxin action (data not presented). These data suggest that Shiga toxin may not be capable of interacting with ribosomes at higher Mg^{2+} ion concentrations. Similar results have been obtained with phytolaccin and ricin A.

To further define the inhibition of eEF-1 associated reactions, the effect of Shiga toxin on eEF-1 dependent GTPase activity was examined. GTPase activity was measured during incubation of DOC-KCl washed ribosomes, Phe-tRNA and eEF-1 protein with different concentrations of activated toxin (Fig. 1). Shiga toxin exhibited a marked inhibitory effect on eEF-1 GTPase activity in a concentration-dependent fashion within the 2 to 130 nM toxin range. GTPase activity was decreased to 50% of control values by 130 nM Shiga toxin. No further increase in inhibition was observed at a 10-fold higher toxin concentration. Data presented in Figure 1 indicate that eEF-1 GTPase and enzymatic Phe-tRNA binding both become inhibited at Shiga toxin concentrations which inhibit reticulocyte total protein synthesis.

The effect of Shiga toxin on enzymatic [^{3}H]Phe-tRNA binding to ribosomes was compared to that of other toxins which also inhibit this reaction. Alpha sarcin and phytolaccin (previously referred to as PAP) inhibited [^{3}H]Phe-tRNA binding to ribosomes as did Shiga toxin; diphtheria toxin was without effect (Table 1). Thus, Shiga toxin, alpha sarcin and phytolaccin specifically inactivate 60S ribosomes and are also inhibitors of the eEF-1 dependent aminoacyl-tRNA binding reaction. In contrast, diphtheria toxin which inactivates eEF-2 protein by ADP-ribosylation had no effect on eEF-1 dependent [^{3}H]Phe-tRNA binding to ribosomes.

We also compared the inhibition of eEF-1 GTPase activity by Shiga toxin with the effect of alphasarcin, phytolaccin and diphtheria toxin on this reaction (Table 1). At saturating levels, Shiga toxin reduced GTPase activity to 49% of the control value. In contrast, diphtheria toxin had no effect on this eEF-1 dependent reaction while alpha sarcin completely inhibited and phytolaccin reduced the activity to 67% of control. Thus, Shiga toxin has in common with alpha sarcin and phytolaccin the ability to inhibit eEF-1 dependent GTPase activity.

Ribonuclease activity of Shiga toxin.

We have proposed that Shiga toxin exhibits RNase activity with rRNA substrate (20). Since our initial findings new and compelling evidence has come forth from Japan that Shiga toxin inactivates ribosomes by a deglycosylase rather than a RNase action. At this time, we agree with the Japanese that the deglycosylation mechanism is the correct, naturally occurring physiological means by which Shiga toxin inactivates ribosomes. If so, what is the RNase activity? Our data continue to support our original findings that Shiga toxin does possess RNase activity and further define the rather non-physiological assay conditions which must be utilized to detect the RNase. Firstly, the substrate to enzyme molar ratio must approach unity. Secondly, the substrate must have single-stranded, purine-rich regions such as encountered in hairpin loops of rRNA species. Thirdly, we have found that the RNase activity proceeds maximally in the absence of magnesium ions and is completely inhibited by 1 mM $MgCl_2$ or $Mg(OAc)_2$.

Shiga toxin effect on non-confluent HUVEC. To test for a direct cytotoxic effect of Shiga toxin on human umbilical vein endothelial cells (HUVEC), the toxin was added to wells containing confluent monlayers or non-confluent substratumattached cells. The number of remaining viable attached cells was then monitored at times between 24 and 96h. The results demonstrate that Shiga toxin added to actively dividing, nonconfluent HUVEC caused a time and dose-dependent reduction in the number of viable attached HUVEC (Figs. 3 & 4). Cells continued to grow and divide at a reduced rate for 48h in the presence of 0.1pM or 0.01nM Shiga toxin (Fig. 3). Thereafter, a constant cell number was maintained. Higher concentrations of Shiga toxin (1 and 100nM) resulted in an abrupt reduction in viable cell number within 24h (Fig. 3). Only in the absence of toxin did the non-confluent HUVEC continue to increase in number over the 4 day period. All concentrations of Shiga toxin examined yielded a steady-state number of HUVEC beyond 48h incubation with the toxin (Fig. 3).

Differential toxin-sensitivity of confluent vs non-confluent HUVEC. A clear distinction was observed between the effect of Shiga toxin on non-confluent and confluent vascular endothelial cells. Typically, a 24h incubation of toxin (1nM) with non-confluent HUVEC resulted in a 60% reduction of viable attached cells while a similar incubation of toxin with confluent HUVEC was without effect (Fig. 4). Shiga toxin was a less potent inhibitor of confluent than of non-confluent HUVEC at all incubation times examined. For example, toxin ID_{50} values for a 96h incubation with confluent cells and a 24h incubation with non-confluent HUVEC were approx 10 and 0.2nM, respectively. This 50-fold difference in toxin sensitivity suggests that specific growth phases of HUVEC may

recognize, process, or respond to Shiga toxin in a different manner.

Binding of [^{125}I] Shiga toxin to HUVEC. To determine if recognition of Shiga toxin by human vascular endothelial cells was specific and perhaps receptor-mediated, we measured the binding of [^{125}I] toxin to HUVEC. [^{125}I] Shiga toxin (1nM) bound efficiently at 4°C to both confluent and non-confluent cells. Toxin binding was saturable and specificity was indicated by the decrease in binding in the presence of excess unlabeled Shiga toxin. On a per cell basis, non-confluent HUVEC bound twice the amount of toxin as confluent HUVEC at 4°C. Our results indicate toxin bound at 4°C was not internalized by antitoxin antibody. These data suggest that toxin-sensitivity of non-confluent vs. confluent HUVEC may not be due to differential binding of toxin to these two cell types. This concept was further supported by additional data indicating that Shiga toxin could be internalized by confluent HUVEC. Incubation of confluent HUVEC with [^{125}I] Shiga toxin at 37°C resulted in a 72% increase in the amount of cell-associated toxin compared to that observed during a similar incubation performed at 4°C. Preliminary studies have shown that Shiga toxin bound to confluent HUVEC during incubation at 37°C/1h becomes refractory to neutralization by anti-Shiga toxin antibody protein. Such results have raised the possibility that protein synthesis may be inhibited by Shiga toxin in non-confluent HUVEC but not in confluent endothelial cells.

Effect of Shiga toxin on protein synthesis in HUVEC.

The rate of [^{3}H]leucine incorporation into total cellular protein was measured in non-confluent and confluent HUVEC. In the absence of Shiga toxin, the rate of protein synthesis in non-confluent cells was twice that of confluent endothelial cells. After 1h in the presence of 10nM Shiga toxin, protein synthesis was rapidly shut off in non-confluent cells. In contrast, confluent HUVEC monolayers incubated with 10nM Shiga toxin continued to synthesize protein at a rate 60% of the control rate observed in the absence of toxin. These results indicate protein synthesis in confluent HUVEC is partially refractory to Shiga toxin.

Another possible explanation for these results is that Shiga toxin preferentially inhibits uptake of [^{3}H]leucine by non-confluent HUVEC. That the latter is not responsible for our results was indicated by similar protein synthesis experiments conducted with HUVEC preincubated 4h with [^{3}H]leucine prior to addition of Shiga toxin. Again, protein synthesis was completely inhibited 2h after addition of toxin to non-confluent cells which had been equilibrated with [^{3}H]leucine. Under the same conditions of preincubation with [^{3}H]leucine, confluent HUVEC incubated with toxin exhibited a steady rate of protein synthesis equal to 60-70% that of control (minus toxin) cells.

It should be noted that partial inhibition of protein synthesis was also a characteristic of confluent HUVEC which had been incubated for a prolonged period (60h) with Shiga toxin (Fig. 5). While a portion of the reduced rates of protein synthesis in this case were due to an overall decrease in the number of viable cells, there also appears to have been a toxin dose-dependent inhibition of protein synthesis in the remaining viable confluent cells. Thus, accumulated data indicate Shiga

toxin quickly elicits a slower rate of protein synthesis in confluent HUVEC under conditions where cells remain fully viable for at least 24h and the slower rate persists in these cultures through 60h.

Shiga toxin is responsible for the cytotoxic response. It was possible that the effect observed on the endothelial cell cultures was caused by some unknown minor contaminant in the toxin preparation. To test this possibility, we examined whether specific treatments known to destroy cytotoxic activity would eliminate the effect on endothelial cells. Firstly, heat-treated toxin was without cytotoxic activity. Preincubation of the purified toxin for 15min at 90°C completely eliminated the cytotoxic response (Table 2). A similar preincubation at 37°C did not alter activity of Shiga toxin in this assay. Secondly, pretreatment of toxin with trypsin, dithiothreitol and urea reduced its cytotoxicity to zero (Table 2). This procedure has been employed previously to "activate" or increase the cell-free protein synthesis inhibitory activity of Shiga toxin (4,33). The activation procedure eliminates all biological activity of Shiga holotoxin on intact HeLa cells (15). Thirdly, antitoxin antibody neutralized the cytotoxic effects of Shiga toxin on HUVEC (Table 2). Preincubation of rabbit polyclonal anti-Shiga toxin IgG protein with purified toxin completely eliminated the cytotoxic activity when added to cultures of HUVEC. Antibody alone was without effect in the assay system.

We also examined the possibility that bacterial endotoxin may have been responsible, partially or totally, for the observed cytotoxic effects. All components of the culture system, including Shiga toxin preparations, were measured for endotoxin content using the Limulus amoebocyte lysate assay. In all cases, our results indicated that final endotoxin concentrations in the HUVEC assay system were less than 0.1 ng/ml taking into account dilution of individual components in the assays. Studies from other laboratories indicate this concentration of endotoxin has no effect on vascular endothelial cells, in vitro (43).

In summary, as mentioned above, Shiga toxin was an active inhibitor of whole cell protein synthesis in HUVEC cultures (Fig. 3,4). These data suggest that damage to human non-confluent and confluent vascular endothelial cells is due primarily to Shiga toxin inhibition of protein synthesis.

CONCLUSIONS

In summary, our results show that:

1. Shiga toxin inhibits eukaryotic protein synthesis through its primary effect on eEF-1-dependent reactions of peptide elongation.

2. Shiga toxin exhibits RNase activity when combined with an equimolar amount of RNA substrate.

3. The RNase activity of Shiga toxin is strongly inhibited by low concentrations of magnesium.

4. Shiga toxin is cytotoxic to human vascular endothelial cells, *in vitro*, in a time- and dose-dependent manner.

5. Non-confluent vascular endothelial cells are very sensitive to Shiga toxin vs. confluent endothelial cells.

6. [^{125}I]Shiga toxin binds to both non-confluent and confluent endothelial cells.

7. Shiga toxin inhibits protein synthesis in endothelial cells.

8. The direct cytotoxic activity of Shiga toxin on human vascular endothelial cells may be the cause of hemolytic uremic syndrome and hemorrhagic colitis associated with bacillary dysentery.

RECOMMENDATIONS

A complete description is now available to explain where and how Shiga toxin inhibits protein biosynthesis in eukaryotic target cells. The only unfinished business appears to be 1) a fine-tuning study of how Shiga toxin recognizes ribosomes at sites other than the site of modification ie. transient "binding" of toxin to the ribosome surface and 2) identification of the enzymatic active site of Shiga toxin.

A more ambitious study needs to be undertaken to describe the role of Shiga toxin in vascular disease syndromes which follow bacillary dysentery in humans. Our laboratory is fully involved with such an NIH-funded investigation at this time. Past funding from the Department of Defense is appreciated which has allowed us to develop this new area of medical research which will also be of value to both the Dod and the civilian population.

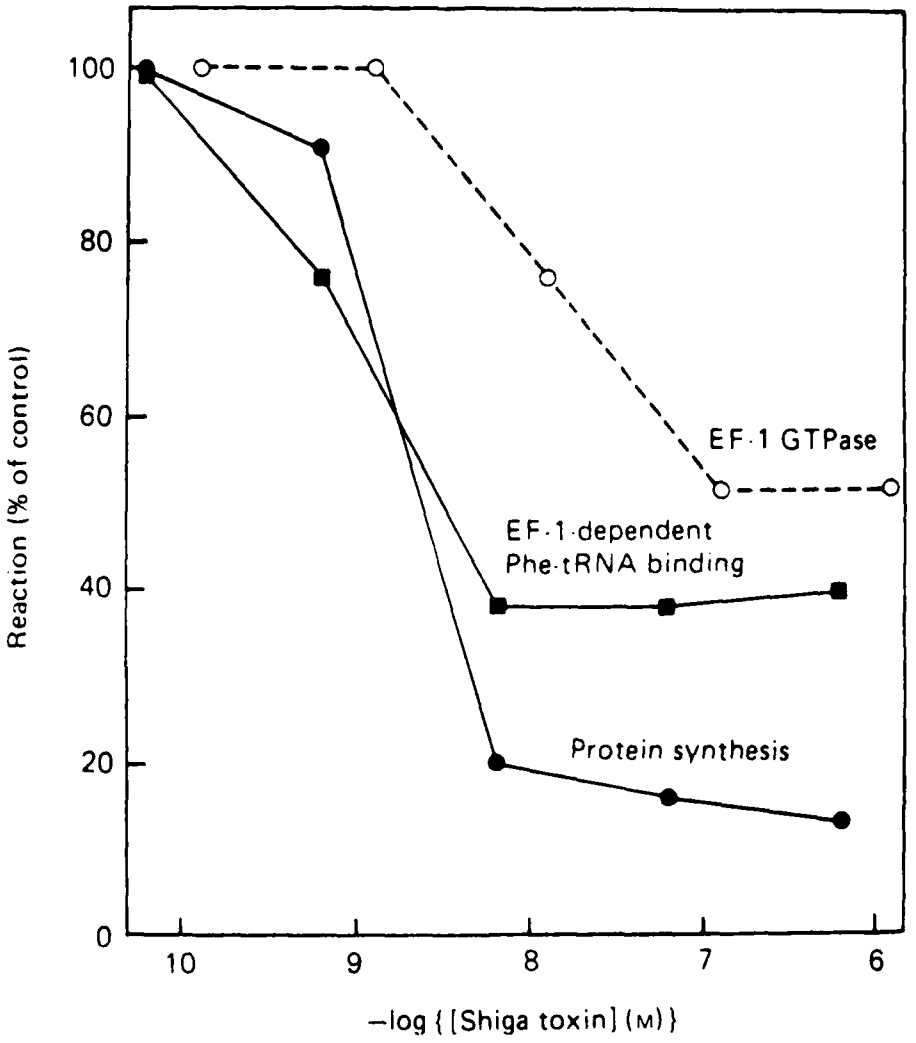

FIG. 1. The effect of Shiga toxin on total protein synthesis, eEF-1-dependent[^{3}H]Phe-tRNA binding and eEF-1 GTPase activity. Total protein synthesis (0----0) was measured by following [^{3}H]Leu incorporation into trichloroacetic acid-insoluble reticulocyte lysate protein. Codon-directed binding of [^{3}H]Phe-tRNA to 80S ribosomes ( ) was carried out in the presence of eEF-1 protein as described in Methods. eEF-1 GTPase activity (0----0) with 0.5 M KCl-washed reticulocyte ribosomes was monitored in the presence of [γ-^{32}P]GTP (see Methods). Control (100%) values for the reactions were 13,400, 3,587, and 750 cpm, respectively.

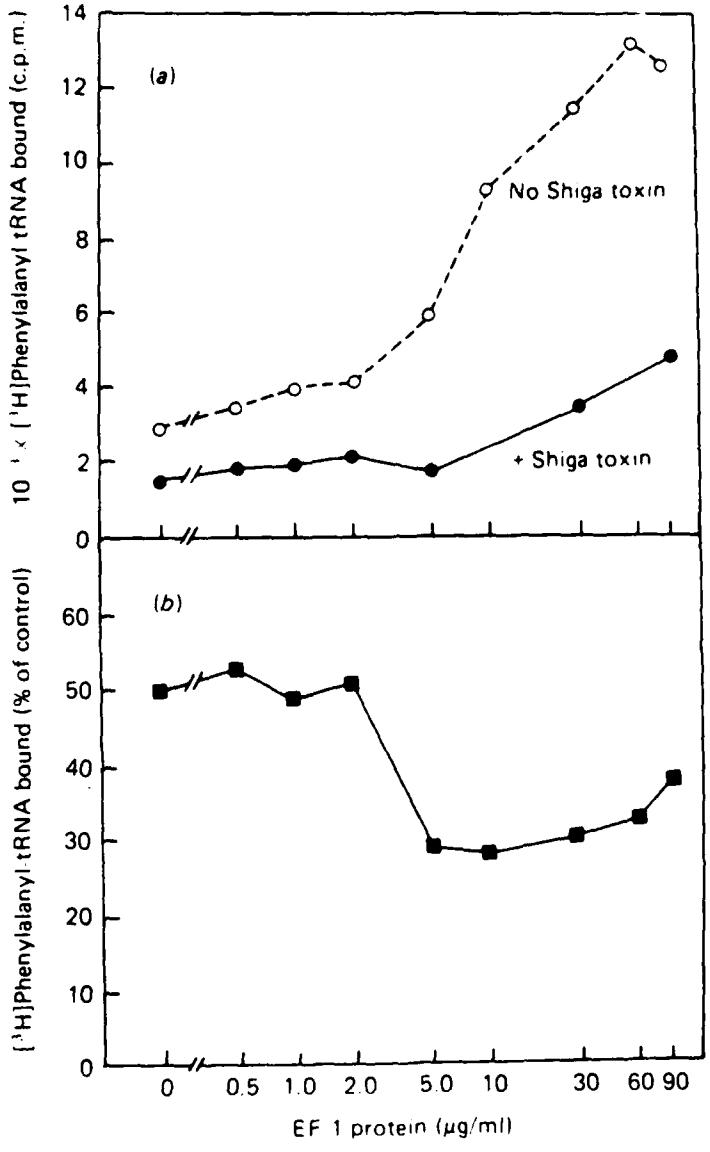

FIG. 2. The effect of eEF·1 protein concentration on Shiga toxin inhibition of enzymatic [^{3}H]Phe-tRNA binding to ribosomes. Reaction conditions were the same as those described in Fig. 1 A) Incubations carried out in the presence (0----0) or absence (0----0) of 1 uM Shiga toxin and B) data presented as percent of uninhibited control values at each EF-1 concentration ( ).

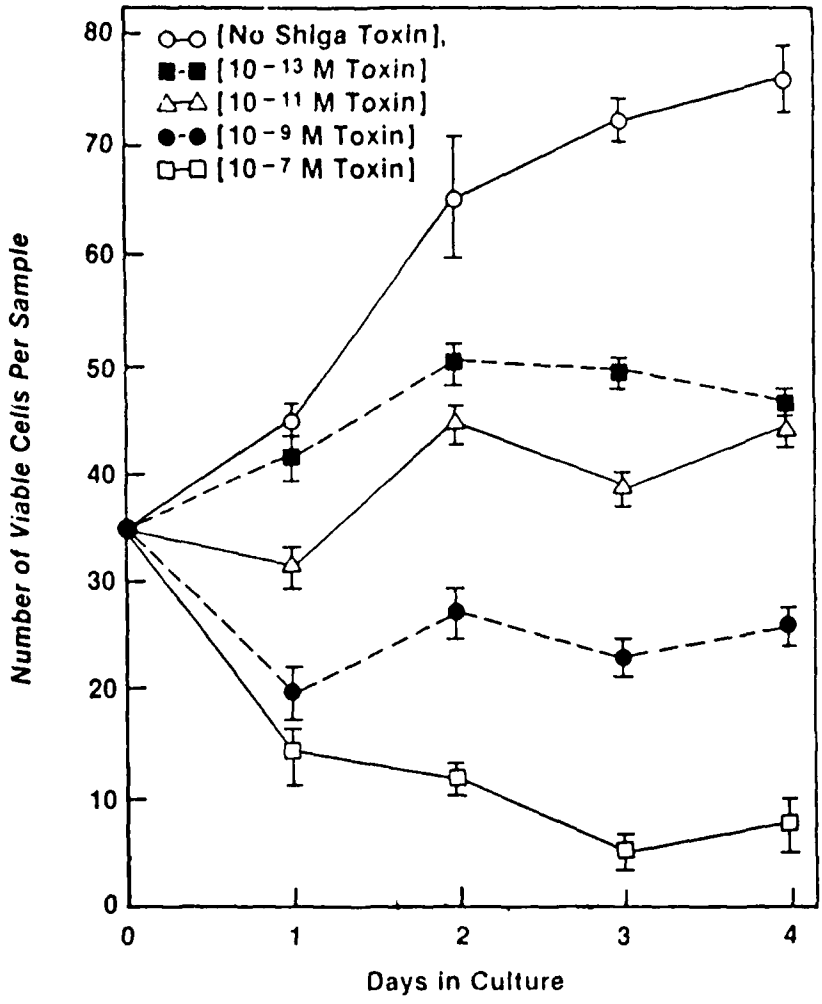

FIG. 3. Effect of Shiga toxin on non-confluent HUVEC. Cells were incubated in complete media in the presence or absence of Shiga toxin for the times indicated. The number of remaining viable substratum-attached cells in each well were then determined as described in Materials and Methods. Vertical bars represent the standard deviation of triplicate samples.

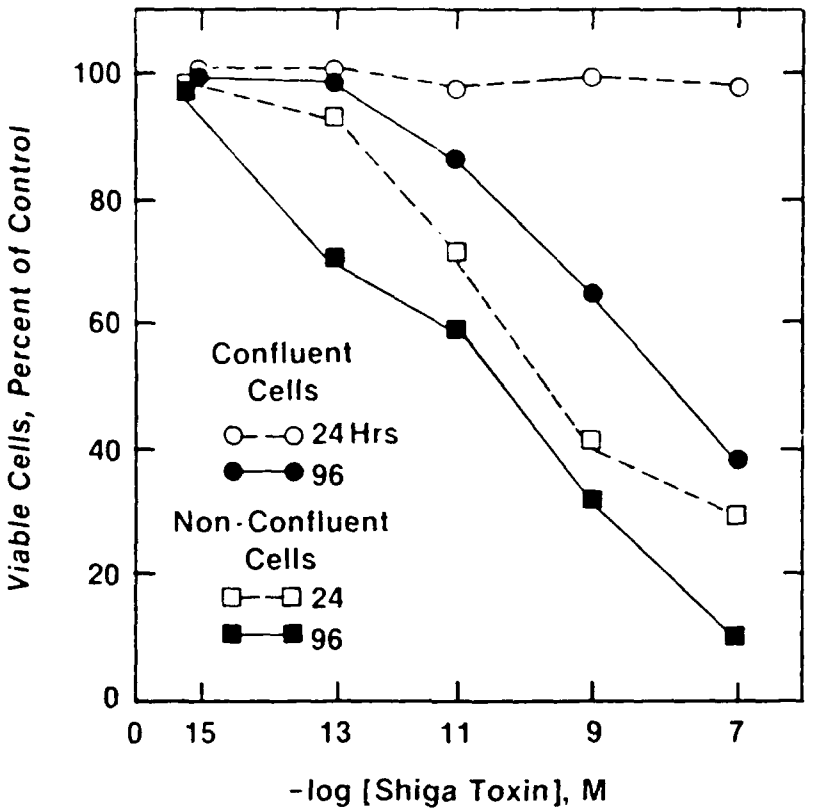

FIG. 4. A comparison of Shiga toxin effect on confluent and non-confluent HUVEC. Different concentrations of Shiga toxin were added to cell cultures at time zero and the number of viable attached cells measured at either 24 or 96h. The 100% values for 24 and 96h samples of confluent cells were 150,000 and 291,200 per well while the values for non-confluent cells were 29,000 and 76,500 cells per well.

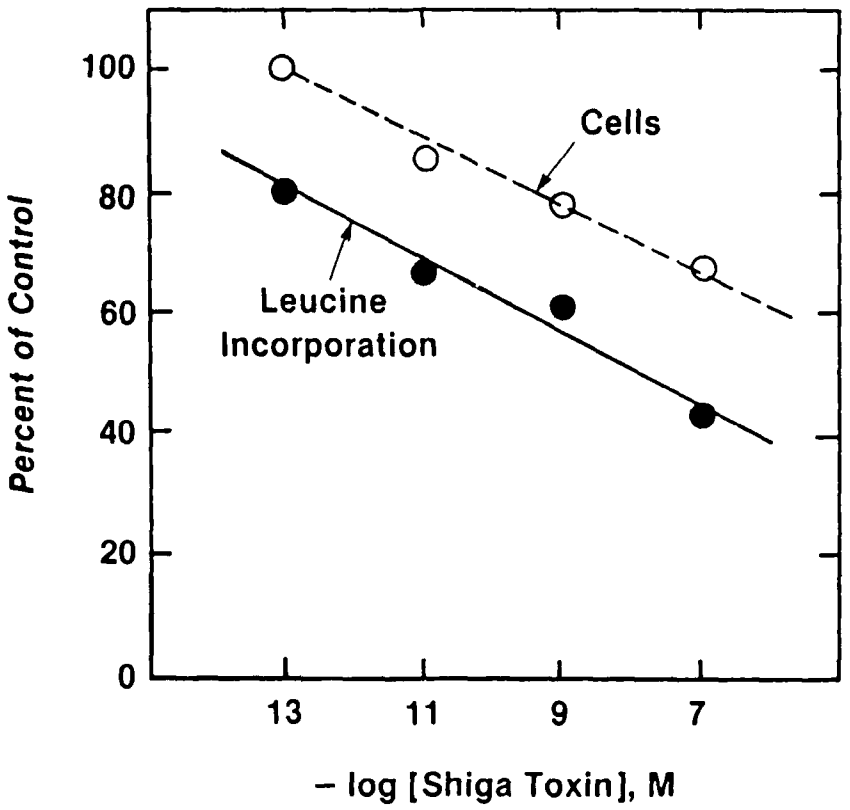

FIG. 5. Shiga toxin dose response effect on protein synthesis in confluent HUVEC. Shiga toxin was added to confluent HUVEC in 24-well culture plates at time zero. At 60h the cells in each well were pulsed with 25μCi of [^{3}H]leucine for 2h and incorporation of radioactivity into acid-insoluble protein was measured as described in Materials and Methods. The 100% values were 260,600 cells per well and 28,061 cpm per well, respectively.

TABLE 1

The effect of Shiga toxin on eEF-1 dependent Phe-tRNA binding and GTPase activities.[a]

Inhibitor		Phe-tRNA Binding		GTPase Activity	
Toxin	concn., uM	cpm	% Control	cpm	% Control
None	---	3477	100%	810	100%
Shiga toxin	0.6	1022	29%	---	---
	1.3	----	---	400	49%
Alpha sarcin	0.25	1580	45%	---	---
	1.25	---	---	0	0
Phytolaccin	0.75	---	---	542	67%
	1.1	1390	40%	---	---
Diphtheria toxin	0.3	3714	107%	795	98%

[a]Binding of [^{3}H]Phe-tRNA to 0.5 M KCl-washed reticulocyte ribosomes and eEF-1 GTPase activities were monitored as described in Fig. 1 and Methods. Toxins were added to reaction mixtures to yield the final concentrations indicated. Data are presented counts per min. and as percent incorporation compared to complete reactions performed in the absence of inhibitors.

TABLE 2

Effect of toxin pretreatments on cytotoxic activity.[a]

Pretreatment		Viable cells, percent of control
Toxin	Toxin concn	
None	--	100
Shiga toxin, non-treated	10nM	32
Shiga toxin, 90°C/15min	10nM	141
Shiga toxin, plus 1:20 diluted antitoxin	10nM	101
Shiga toxin plus trypsin, urea and dithiothreitol	10nM	108

[a]Shiga toxin was pretreated as described and added to non-confluent HUVEC at 1nM final concentration. Following incubation of toxin with cells at 37°C for 24h, HUVEC were processed as described in Materials and Methods.

LITERATURE CITED

1. Conradi, H. (1903) Ueber 18sliche durch aseptische autolyse Erhaltene Giftsoffe von Ruhrund Tryphusbazillen. Dtsch. Med. Wochenschr. 29:26.

2. LaBrec, E.H., Schneider, H., Magnani, T.J., and Formal, S.B. (1964) Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. 88:1503.

3. Formal, S.B., LaBrec, E.H., and Schneider, H. (1965) Pathogenesis of bacillary dysentery in laboratory animals. Fed. Proc. 24:29.

4. Keusch, G.T., Grady, G.F., Mata, L.J., and McIver, J. (1972) The pathogenesis of Shigella diarrhea. 1. Enterotoxin production by Shigella dysenteriae 1. J. Clin. Invest. 51:1212.

5. Vicari, G., Olitzki, A.L., and Olitzki, Z. (1960) The action of thermolabile toxin of Shigella dysenteriae on cells cultured in vitro. Br. J. Exp. Pathol. 41:179.

6. Keusch, G.T., Jacewicz, M., and Hirschman, S.Z. (1972) Quantitative microassay in cell culture for enterotoxin of Shigella dysenteriae 1. J. Infect. Dis. 125:539.

7. Gentry, M.K., and Dalrymple, J.M. (1980) Quantitative microtiter cytotoxicity assay for Shigella toxin. J. Clin. Microbiol. 12:361.

8. Eiklid, K., and Olsnes, S. (1980) Interaction of Shigella shigae cytotoxin with receptors on sensitive and insensitive cells. J. Receptor Res. 1:199.

9. Olsnes, S., Reisbig, R., and Eiklid, K. (1981) Subunit structure of Shigella cytotoxin. J. Biol. Chem. 256:8732.

10. Gill, D.M. in Bacterial Toxins and Cell Membranes (Jeljaszewicz, J. and Wadstrom, T., eds.), 1978, p. 291, Academic Press, New York.

11. Brown, J.E., Rothman, S.W., and Doctor, B.P. (1980) Inhibition of protein synthesis intact HeLa cells by Shigella dysenteriae 1 toxin. Infect. Immun. 29:98.

12. Olenick, J.G., and Wolfe, A.D. (1980) Shigella toxin inhibition of the binding and translation of polyuridylic acid by ribosomes of Escherichia coli. J. Bacteriol. 141:1246.

13. Keenan, K.P., D.D. Sharpnack, H. Collins, S.B. Formal and A.D. O'Brien. 1986. Morphologic evaluation of the effects of Shiga toxin and E. coli Shiga-like toxin on the rabbit intestine. Am. J. Pathol. 125:69-80.

14. Brown, J.E., Ussery, M.A., Leppla, S.H., and Rothman, S.W. (1981) Inhibition of protein synthesis by Shiga toxin. Activation of the toxin and inhibition of peptide elongation. FEBS Lett. 117:84.

15. Reisbig, R., Olsnes, S., and Eiklid, K. (1981) The cytotoxin activity of Shigella toxin. Evidence for catalytic inactivation of the 60S ribosomal subunit. J. Biol. Chem. 256:8739.

16. Olsnes, S., and Eiklid, K. (1980) Isolation and characterization of Shigella shigae cytotoxin. J. Biol. Chem. 255:284.

17. O'Brien, A.D., LaVeck, G.D., Griffin, D.E., and Thompson, M.R. (1980) Characterization of Shigella dysenteriae 1 (Shiga) toxin purified by anti-Shiga toxin affinity chromatography. Infect. Immun. 30:170.

18. Brown, J.E., Griffin, D.E., Rothman, S.W., and Doctor, B.P. (1982) Purification and biological characterization of Shiga toxin from S. dysenteriae 1, Infect. Immun. 36:996.

19. Olsnes, S., and Pihl, A. in The specificity and Action of Animal, Bacterial and Plant Toxins (Cutrecases, P., ed) 1976, Series B, Vol. 1, p. 128, Chapman-Hall, London.

20. Obrig, T.G., Moran, T.P. and Colinas, R.J. (1985) Ribonuclease activity associated with the 60S ribosome-inactivating proteins ricin A, phytolaccin and Shiga toxin. Biochem. Biophys. Res. Commun. 130:879-884.

21. Obrig, T.G., Moran, T.P. and Brown, J.E. (1987) The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. Biochem. J. 244:287-294.

22. Brown, J.E., Obrig, T.G., Ussery, M.A. and Moran, T.P. (1986) Shiga toxin from Shigella dysenteriae 1 inhibits protein synthesis in reticulocyte lysates by inactivation of aminoacyl-tRNA binding. Microbial Pathogenesis 1:325-334.

23. Obrig, T.G., Culp, W.J., McKeehan, W., and Hardesty, B. (1971) The mechanism by which cycloheximide and related glutarimide antibiotics inhibit protein synthesis on reticulocyte ribosomes. J. Biol. Chem. 246:174.

24. Obrig, T.G., Moran, T.P. and Brown, J.E. (1985) The catalytic toxins: probes for eukaryotic ribosome structure-function relationships. Presented at the 5th International conference on structure, function and genetics of ribosomes, Port Aransas, Texas, April, 1985.

25. Moyer, M.P., P.S. Dixon, S.W. Rothman and J.E. Brown. 1987. Cytotoxicity of Shiga toxin for primary cultures of human colonic and ileal epithelial cells. Infect. Immun. 55:1533-1535.

26. Cantey, J.R. 1985. Perspective. Shiga toxin-expanding role in the pathogenesis of infectious diseases. J. Infect. Dis. 151:766-771.

27. Keusch, G.T., A. Donohue-Rolfe and M. Jacewicz. 1985. Shigella toxin and the pathogenesis of shigellosis. p. 193-214. In D. Evered and J. Whelan (ed.), Microbial toxins and diarrhoeal disease. Ciba Foundation Symposium 112. Pitman Publishers, London.

28. Kopecko, D.J., L.S. Baron and J. Buysse. 1985. Genetic determinants of virulence in Shigella and dysenteric strains of E. coli: their involvement in pathogenesis of dysentery. Curr. Topics in Microbiol. and Immunol. 118:71-95.

29. Butler, T., M.R. Islam, A.K. Azad and P.K. Jones. 1987. Risk factors for development of the hemolytic-uremic syndrome during shigellosis: role of antimicrobial treatment. J. Pediatr. 110:894-897.

30. Gianantonio, C., M. Vitacco, F. Mendilaharzu, A. Rutty and J. Mendilaharzu. 1964. The hemolytic-uremic syndrome. J. Pediatr. 64:478-491.

31. Guillen-Alvarez, G. and E.V. Bolanos. 1974. Sindrome hemolitico uremico: reporte de 60 casos asociados a una epidemia de enterocolitis hemorragica. Revista Colombiana de Ped. Puericult. 28:414-418.

32. Koster, F., J. Levin, L. Walker, K.S.K. Tung, R.H. Gilman, M.M. Rahaman, M.A. Majid, S. Islam and R.C. Williams, Jr. 1977. Hemolytic-uremic syndrome after shigellosis. Relation to endotoxemia and circulating immune complexes. N. Engl. J. Med. 298:927-933.

33. Raghupathy, P., A. Date, J.C.M. Shastry, A. Sudarsanam and M. Jadhav. 1978. Haemolytic uraemic syndrome complicating shigella dysentery in south Indian children. Br. Med. J. 1:1518-1521.

34. Fong, J.S.C., J-P de Chadarevian and B.S. Kaplan. 1982. Hemolytic-uremic syndrome: current concepts and management. Ped. Clin. North Am. 29:835-856.

35. Bridgewater, F.A.J., R.S. Morgan, K.E.K. Rowson and G.P. Wright. 1955. The neurotoxin of Shigella shigae. Morphological and functional lesions produced in the central nervous system of rabbits. Br. J. Exp. Pathol. 36:447-453.

36. Karmali, M.A., M. Petric, C. Lim, P.C. Fleming, G.S. Arbus and H. Lior. 1985. The association between idiopathic hemolytic uremic syndrome and infection by Verotoxin-producing Escherichia coli. J. Infect. Dis. 151:775-782.

37. Koster, F.T., V. Boonpucknavig, S. Sujaho, R.H. Gilman and M.M. Rahaman. 1984. Renal histopathology in the hemolytic-uremic syndrome following shigellosis. Clin. Nephrol. 21:126-133.

38. Jaffe, E.A., R.L. Nachman, C.G. Becker and C.R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. J. Clin. Invest. 52:2745-2756.

39. Glaser, B.M., P.A. D'Amore, R.G. Michels, A. Patz and A. Fenselau. 1980. Demonstration of vasoproliferative activity from mammalian retina. J. Cell Biol. 84:298-304.

40. Lechner, J.F., A. Haugen, H. Autrup, I.A. McClendon, B.F. Trump and C.C. Harris. 1981. Clonal growth of epithelial cells from normal adult human bronchus. Cancer Res. 41:2294-2304.

41. Griffin, D.E., M.K. Gentry and J.E. Brown. 1983. Isolation and characterization of monoclonal antibodies to Shiga toxin. Infect. Immun. 41:430-433.

42. David, G.S. and R.A. Reisfeld. 1974. Protein iodination with solid state lactoperoxidase. Biochemistry 13:1014-1021.

43. Schoffel, U., J. Shiga and C. Mittemayer. 1982. The proliferation inhibiting effect of endotoxin on human endothelial cells in culture and its possible implication in states of shock. Circ. Shock 9:499-508.

DISTRIBUTION LIST

5 Copies: Director
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
ATTN: SGRD-UWZ-C
Washington, DC 20307-5100

1 Copy: Commander
US Army Medical Research and Development Command
ATTN: SGRD-RMI-S
Fort Detrick
Frederick, MD 21701-5012

2 Copies: Defense Technical Information Center (DTIC)
ATTN: DTIC-DDA
Cameron Station
Alexandria, VA 22304-6145

1 Copy: Dean
School of Medicine
Uniformed Services University
of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799

1 Copy: Commandant
Academy of Health Sciences, US Army
ATTN: AHS-CDM
Fort Sam Houston, TX 78234-6100