AD-A171 457

DTIC ACCESSION NUMBER

PHOTOGRAPH THIS SHEET

REPORT No. 1
MODE OF ACTION OF SHIGELLA TOXIN: EFFECTS ON RIBOSOME STRUCTURE AND FUNCTION
ANNUAL REPORT
15 AUG. 1983

LEVEL

INVENTORY

(1)

DOCUMENT IDENTIFICATION

DISTRIBUTION STATEMENT A
Approved for public release
Distribution Unlimited

DISTRIBUTION STATEMENT

ACCESSION FOR	
NTIS GRA&I	☑
DTIC TAB	☐
UNANNOUNCED	☐
JUSTIFICATION	
BY	
DISTRIBUTION /	
AVAILABILITY CODES	
DIST	AVAIL AND/OR SPECIAL
A-1	

DISTRIBUTION STAMP

DTIC
ELECTE
SEP 0 3 1986
S D
D

DATE ACCESSIONED

DATE RETURNED

86 9 3 061

DATE RECEIVED IN DTIC

REGISTERED OR CERTIFIED NO.

PHOTOGRAPH THIS SHEET AND RETURN TO DTIC-DDAC

DTIC FORM 70A DEC 83

DOCUMENT PROCESSING SHEET

PREVIOUS EDITION MAY BE USED UNTIL STOCK IS EXHAUSTED.

AD_____

REPORT NUMBER 1

Mode of Action of Shigella Toxin: Effects on Ribosome Structure and Function

Annual Report

Tom G. Obrig, Ph.D.

August 15, 1983

Supported by

U.S. Army Medical Research and Development Command

Fort Detrick, Frederick, Maryland 21701

Contract # DAMD 17-83-C-3035

Albany Medical College
Albany, New York 12208

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): MODE OF ACTION OF SHIGELLA TOXIN: EFFECTS ON RIBOSOME STRUCTURE AND FUNCTION	5. TYPE OF REPORT & PERIOD COVERED: Annual 11/15/82-8/1/83
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Tom G. Obrig, Ph.D.	8. CONTRACT OR GRANT NUMBER(s): DMAD17-83-C-3035
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Albany Medical College, Albany, New York 12208	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS: US: Army Medical Research Development Command, Fort Detrick, Md.	12. REPORT DATE: 8/15/83
	13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report): unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

Same as in #16

18. SUPPLEMENTARY NOTES

None

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Shigella, Shigella toxin, Shiga toxin, Ribosomes, Protein synthesis

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

AD_____

REPORT NUMBER 1

Mode of Action of Shigella Toxin: Effects on Ribosome Structure and Function

Annual Report

Tom G. Obrig, Ph.D.

August 15, 1983

Supported by

U.S. Army Medical Research and Development Command

Fort Detrick, Frederick, Maryland 21701

Contract # DAMD 17-83-C-3035

Albany Medical College
Albany, New York 12208

SUMMARY

The goals of this research contract are to examine the primary action of Shigella dysenteriae 1 toxin as an inhibitor of eukaryotic protein biosyntesis. Two major objectives of this investigation are designed to reveal Shiga toxin-induced changes in ribosome structure-function relationships. These objectives are 1) to explain, in biochemical terms, the manner by which Shiga toxin enzymatically inactivates mammalian ribosomes and 2) to define the steps of protein biosynthesis which are specifically inhibited by the toxin as a result of ribosome modification. A comparison between Shiga toxin and a similar plant-derived toxin is to be made in regard to ribosome inactivation. It is likely that information obtained from these studies will be of value in describing the role of Shiga toxin in establishment of intestinal infections by the toxin-producing Shigella species. In this contract period we have shown that Shiga toxin is not an in vitro inhibitor of initiation of reticulocyte protein synthesis which supports existing information that the toxin is a primary inhibitor of the peptide elongation process. Changes in ribosome structure as a result of toxin action was also investigated during the present contract period. It has been determined that Shiga toxin does not cause hydrolysis of ribosomal RNA to yield fragments larger than 10 nucleotides. Recent studies involving RNA sequencing indicate that the 3′ terminal region of 5.8S ribosomal RNA remains intact following toxin inactivation of ribosomes. Examination of the 5′ and 3′ termini of the other ribosomal RNA species is in progress. Finally, comparative analysis of ribosomal proteins from control and toxin-treated ribosomes using the two-dimensional gel electrophoresis technique has resulted in identical patterns. It is concluded that: 1) Shiga toxin is an inhibitor of protein synthesis elongation and is without effect on the initiation process, 2) Shiga toxin does not hydrolyze ribosomal RNA into larger fragments and 3) the toxin does not result in modification of ribosomal proteins which would cause a major change in their mass or isoelectric points.

FOREWORD

In conducting the research described in this report, the investigator 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 (DHEW Publication No. NIH-78-23, Revised, 1978).

TABLE OF CONTENTS

TITLE PAGE

SUMMARY

FOREWORD

BODY OF THE REPORT

Statement of the Problem

Technical Objectives

Background

Approach to the Problem

a. General Information

b. Initiation of Protein Synthesis

c. Elongation of Protein Synthesis

d. Ribosomal Proteins

e. Ribosomal RNAs

Results

a. Initiation of Protein Synthesis

b. Elongation of Protein Synthesis

c. Ribosomal Proteins

d. Ribosomal RNAs

Discussion of Results

a. Initiation of Protein Synthesis

b. Elongation of Protein Synthesis

c. Ribosomal Proteins

d. Ribosomal RNAs

Conclusions

Recommendations

LITERATURE CITED

DISTRIBUTION LIST

FIGURES

Scheme A	Steps in the initiation of protein synthesis
Figure 1	Initiation ternary complex formation
Figure 2	Two-dimensional electrophoresis of ribosomal proteins
Figure 3	The effect of Shiga toxin and alpha sarcin on 40S and 60S ribosomes
Figure 4	Nuclease activity of Shiga toxin and alpha sarcin on isolated rRNA of E. coli
Figure 5	Sequence of the 3'-terminus of 5.8S rRNA from toxin-treated ribosomes

TABLES

Table 1	Pentameric complex formation with purified components
Table 2	Formation of pentameric initiation complex with natural mRNA
Table 3	Methionylpuromycin formation in unfractionated reticulocyte lysate

BODY OF THE REPORT

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 $\cong 10^{-10}$ 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 by 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). 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, virtually nothing is known about the ribosomal component which is modified by Shiga toxin.

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.

Approach to the Problem

a. General Information

It seems very possible that Shiga toxin could share many of the features of the plant-derived phytolaccin toxin as an inhibitor of protein biosynthesis (24). Our research plan is designed to examine the action of Shiga toxin in the test systems we have successfully employed in the study of plant toxin mode of action. These investigations were designed, in part, because the Principal Investigator has had extensive experience in preparation and use of protein synthesis assay systems which would be useful in carrying out the Shiga toxin study (22-25).

The primary objective of this project is to describe, in biochemical terms, how Shiga toxin inhibits protein biosynthesis in mammalian cells.

To achieve this goal, two aspects of protein synthesis will be examined in reticulocyte (rabbit) cell-free protein synthesis systems. First, it will be determined what steps of protein synthesis (i.e., initiation or elongation) are affected by Shiga toxin. Second, we will determine how Shiga toxin inactivates mammalian ribosomes. More exactly, as Shiga toxin is known to specifically inactivate the large ribosomal subunit, a goal of this research plan is to determine how Shiga toxin modifies the structural integrity of the ribosome. Components of the large ribosomal subunit including its 47 proteins will be monitored for changes resulting from Shiga toxin action. The following is an abbreviated description of methodology to be used in the study of Shiga toxin mode of action as an inhibitor of protein biosynthesis. Abbreviations appearing include:

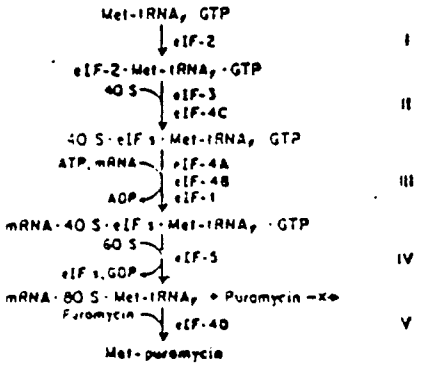
rRNA - ribosomal ribonucleic acid
rprotein - ribosomal protein
tRNA - transfer ribonucleic acid
mRNA - messenger ribonucleic acid
EF 1 - elongation factor 1 which is the aminoacyl-transfer RNA binding factor
EF 2 - elongation factor 2 which is the translocase factor
eIF - eukaryotic initiation factor
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
TCA - trichloroacetic acid

b. Initiation of Protein Synthesis

The following section is a description of refined initiation assays which are included in the research plan. It should be emphasized that we intend to examine, in detail, only those reactions affected by toxin as indicated by combined data from lysate and partially fractionated globin protein biosynthesis systems.

Initiation of protein synthesis consists of a series of reactions involving at least 8 initiation factor proteins, methionyl-$tRNA_f$, GTP, ATP, mRNA and ribosomal subunits. These reactions appear to occur in a defined sequence as shown (26). Our plan is to study the effect of toxin on formation of initiation complexes in an unfractionated reticulocyte lysate. Rationale for the assay is that exogenously added, laboratory-synthesized [^{35}S]Met·-$tRNA_f$ will take part in initiation following the "Scheme A" (Page 9). However, should toxin block one or more of these steps, the [^{35}S]Met·-$tRNA_f$ will accumulate in an intermediate complex. Each of these complexes can be detected and identified by either separation on sucrose gradients following centrifugation, or by collection on nitrocellulose filters.

Scheme A



c. Elongation of Protein Synthesis

The three steps involved in peptide elongation are 1) binding of aminoacyl-tRNA to the ribosome, 2) formation of a peptide bond between amino acids in the growing protein catalyzed by peptidyl transferase and 3) tanslocation of peptidyl-tRNA from one site to another site on the same ribosome. All of these steps will be monitored in the absence and presence of Shiga coxin as described below. Although it seems inappropriate to give a complete description of the elongation process in this report, it should be pointed out that each of these individual steps in elongation may be monitored in one or more ways by utilizing radioactivity probes in each of the different components that take place in the reactions. During our experience with these assays, we have been able to describe the site of action of several rather specific inhibitors of eukaryotic protein synthesis (22-24).

The assay systems measure: 1) binding of aminoacyl-tRNA to the ribosome, 2) translocation of aminoacyl-tRNA on ribosomes, 3) EF-2·GDP·ribosome complex formation, 3) EF-1 and EF-2 GTPase activity and 4) peptidyl transferase activity.

d. Ribosomal Proteins

All of the 48 r-proteins found in 60S ribosomal subunits are separable by two-dimensional gel electrophoresis (26-28). This procedure will be used for our examination of the 60S subunit acidic and basic r-proteins isolated from untreated and toxin-inactivated 60S subunits. Proteins extracted from 60S particles will be separated in the first dimension by their electrophoretic mobility at pH 8.6 on a cylindrical 8% acrylamide gel. The second dimension separation is a function of size of r-proteins and is carried out at pH 4.2 in an 18% acrylamide slab gel (29). Variations in this general description include acrylamide concentration, pH, voltage, time of electrophoresis, and ratio of acrylamide to bisacrylamide. These variations are considered important because of the possibility that an altered protein from toxin-treated ribosomes could assume a new co-migration pattern with any of the other r-proteins.

e. Ribosomal RNAs

Although this section was not included in the initial research plan, we plan to examine the rRNAs of toxin-treated ribosomes to fully round-out our examination of toxin action at the ribosomal level. Three rRNA species located in the 60S ribosome are 5S, 5.8S and 28S rRNAs.

Examination of these rRNAs is accomplished in two parts. First, the size of rRNAs are analyzed on polyacrylamide gels. This will allow us to detect an enzymatic hydrolysis of the rRNAs which would yield RNA fragments of 10 nucleotides or larger. Second, the individual RNA species are studied for modification at their termini by the modern technique of RNA sequencing. In the latter case, each rRNA type is radioactive labeled with [^{32}P], chemically hydrolyzed with nucleotide-specific reactions and fragments separated by gel electrophoresis. Using this technique one can determine the exact nucleotide sequence at the ends of each RN..

Results

The following results were obtained during the initial eight months of the project. Topic divisions are those appearing in the previous section, namely initiation and elongation of protein synthesis plus ribosomal proteins and RNAs.

a. Initiation of Protein Synthesis

Collaborative efforts with Dr. J. Edward Brown, Division of Biochemistry, WRAIR, have led to Shiga toxin mode of action data appearing in this section. These results were presented at the June, 1983 meeting of the American Society for Biological Chemists in San Francisco (Federation Proceedings 42:1808, 1983). The abstract is presented below. In addition, a manuscript has been submitted for publication in which are described the details of this work. Dr. Howard Noyes, WRAIR, has received a copy of this manuscript.

SHIGA TOXIN DOES NOT AFFECT INITIATION OF PROTEIN SYNTHESIS. T.G.Obrig*, T.P. Moran* and J.E. Brown* (SPON: J.L. Glenn). Albany Med. Col. Albany, NY 12208 and Walter Reed Army Inst. Res., Washington, DC, 20012.

Shiga toxin, the protein toxin of Shigella dysenteriae 1 has been shown to inhibit eukaryotic protein synthesis catalytically. We have now examined toxin action for its affect on initiation of protein synthesis. Toxin, purified to homogeneity from S. dysenteriae 1, strain 38180, was activated with TPCK-trypsin followed by phenylmethylsulfonyl fluoride, urea and dithiothreitol. A Shiga toxin concentration of 1 µg/ml which completely inhibits protein synthesis in reticulocyte lysate was utilized. In the presence of ribosomal salt wash fraction, formation of the ternary initation complex [^{35}S]Met·tRNA$_f$-eIF·2-GTP was not affected by the toxin. Further, when sucrose gradient-isolated ribosomes were added, codon-directed binding of [^{35}S]Met·tRNA$_f$ to the 40S ribosome was not inhibited by toxin. Toxin effects on protein synthesis initiation in complete reticulocyte lysates were monitored in the presence [^{35}S]Met·tRNA$_f$. Accumulation of the initiation complex [^{35}S]Met tRNA$_f$-40S ribosomal subunit-mRNA, promoted in response to NaF and β,γ -methylene GTP, was not inhibited in the presence of Shiga toxin. Finally, [^{35}S]Met:tRNA-80S ribosome-mRNA complex formed in Shiga toxin-treated lysates was fully capable of reacting with puromycin to yield [^{35}S] mechionyl-puromycin. We conclude that Shiga toxin inhibits protein synthesis without affecting the initiation process.

The process of protein synthesis initiation is depicted in Scheme "A". Individual steps as well as the overall process were examined in the presence and absence of Shiga toxin.

Scheme A

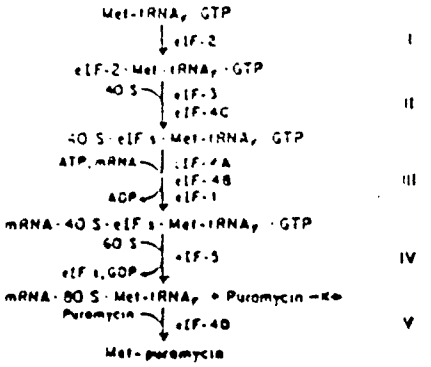
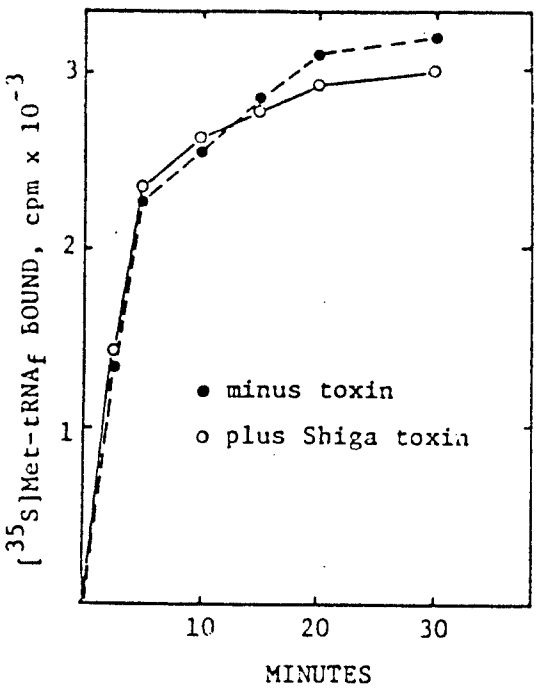


Figure 1



STEP #1 (Scheme "A"): Effect of Shiga toxin on formation of ternary initiation complex eIF-2·[^{35}S]Met-tRNA$_f$·GTP. Measurement of this step was based on the concept that ternary initiation complex was retained on nitrocellulose filters whereas the individual components passed through. The assay mixture contained in a final volume of 0.050 ml:0.02 M Tris-Cl, pH 7.4; 0.10 M KCl, 5 mM dithiothreitol; 2 mM GTP; 30,000 cpm [^{35}S]Met-tRNA$_f$ formed by charging of yeast tRNA with [^{35}S]methionine using E. coli synthetase and; 0.2 mg ribosomal salt-wash protein as a source of initiation factor eIF·2. Where indicated, Shiga toxin was added to the assay mixture at 1 ug/ml final concentration. In this and all other test systems Shiga toxin was "activated" before use by pretreatment with trypsin to "nick" the protein followed by incubation with PMSF trypsin inhibitor, dithiotreitol and urea. This process increased potency of the toxin by 100-fold in cell-free assays. The results, (Figure 1) clearly indicate Shiga toxin did not affect formation of the initiation complex formed by step #1 in Scheme "A". That is, the rate of ternary complex formation was equal in the presence or absence of Shiga toxin.

STEP #2 and 3 (Scheme "A"): Effect of Shiga toxin on formation of pentameric initiation complex; mRNA·40S ribosome·eIF-2·Met-tRNA$_f$·GTP. During initiation of protein synthesis, the ternary complex discussed above binds to 40S ribosomal subunit and is further stabilized by addition of mRNA to the growing complex (see Scheme "A"). Formation of the pentameric complex was accomplished by supplementing the previous reaction mixture (for 3° complex) with purified 40S ribosomal subunits and the artificial mRNA, poly AGU which contains the AUG initiation codon. Incorporation of

[^{35}S]Met-tRNA$_f$ into the pentameric initiation complex was monitored by its retention on nitrocellulose filters as described for the previous assay. The 3° initiation complex was very short-lived in the presence of Mg^{2+} (added to this reaction mixture as magnesium acetate) which prevented interference of 3° complex formation with measurement of the pentameric initiation complex with the filter assay procedure. Our results indicated that Shiga toxin, at 1 ug/ml, did not appreciably alter formation of the pentameric initiation complex (Table 1).

Table 1

Pentameric complex formation with purified components

Components	Filter-bound [^{35}S], cpm
minus 40S and Poly-AGU	90
minus 40S	88
minus Poly-AGU	130
minus ribosomal salt fraction	142
complete, incubation 0°C	94
complete, incubation 37°C	567
complete, 37°C, plus Shiga toxin	534

The effect of Shiga toxin on pentameric initiation complex formation was also monitored in unfractionated reticulocyte lysates. Rationale for this assay procedure follows. Pentameric initiation complex was accumulated in reticulocyte lysate in response to added NaF and β,γ-methylene GTP which inhibited the subsequent initiation step #4 (Scheme "A"), i.e., addition of 60S ribosomal subunits to the pentameric complex. If Shiga toxin activity inhibited one or more of the steps preceding step #4 in Scheme "A", one should see a reduced amount of pentameric complex formed. In this case, the sucrose gradients were employed for quantitative and qualitative analysis of [^{35}S]Met-tRNA$_f$ binding into the pentameric initiation complex. Results from this assay (Table 2) were in full agreement with those obtained with the filter assay procedure. It was concluded that Shiga toxin did not inhibit steps #1 through #3 in Scheme "A".

Table 2

Formation of pentameric initiation complex with natural messenger RNA

Assay Components	Filter-Bound [^{35}S], cpm
Complete	18,610 ± 1,070
Complete, incubated at 4°C	3,140 ± 286
Complete + 1 ug/ml Shiga toxin	14,770 ± 853
Complete + HCR*	5,360 ± 324

The HCR* protein was activated, in the absence of hemin, by preincubation (37°/1 h) of a 100,000 g supernatant of reticulocyte lysate. Results are presented as means (± S.E.M.) of four separate determinations.

Table 3

[^{35}S]Methionylpuromycin formation in unfractionated reticulocyte lysate

	[^{35}S], cpm
minus lysate	315
complete, 4°C	265
complete, 37°C	4,315
complete, 37°C, Shiga toxin	3,885
complete, 37°C, HCR*	560

*HCR is hemin control repressor, a protein kinase activated in lysates incubated at 37°C without hemin. HCR phosphorylates (inactivates) initiation factor eIF-2. HCR was added to the assay, where indicated, at zero time as a positive control.

STEP #4 and #5 (Scheme "A"): Effect of Shiga toxin on formation of the final initiation complex; 80S ribosome·mRNA·Met-tRNA$_f$. Rationale for analysis of the final initiation complex in unfractionated reticulocyte lysates was: 1) [^{35}S]Met-tRNA$_f$ incorporated into this complex became located in the ribosomal "peptidyl site", 2) by definition, aminoacyl-tRNA located in the "peptidyl site" would be reactive with exogenous puromycin, 3) the product, [^{35}S]methionylpuromycin would be rapidly released from ribosomes into the soluble phase and extractible with ethylacetate prior to measurement of radioactivity with standard liquid scintillation techniques. Results: Shiga toxin (1 ug/ml), present in the lysate during incubation with [^{35}S]Met-tRNA$_f$, was without effect on binding of [^{35}S]Met-tRNA into the final initiation complex and its reactivity with puromycin. Actual data obtained from this experiment are presented in Table 3.

b. Elongation of Protein Synthesis

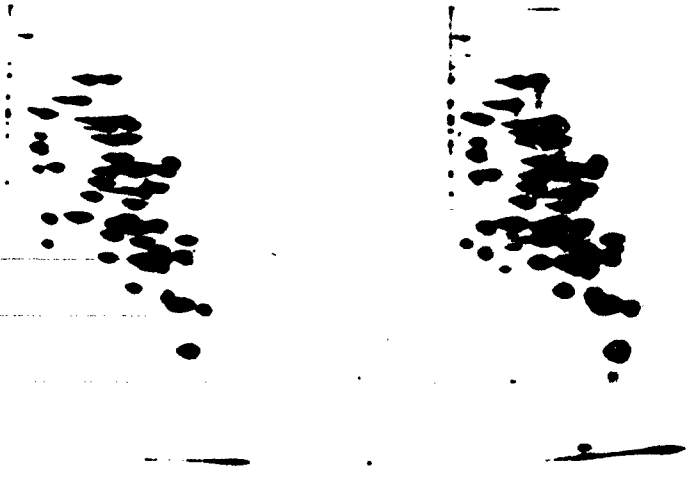
Concentration of the project goals involving the previous section on initiation of protein synthesis has precluded time allotment for this section. It is anticipated that this work will begin prior to November, 1983.

c. Ribosomal Proteins

During the period covered by this report we started analysis of ribosomal proteins for their possible alteration as a result of Shiga toxin action on 60S ribosomes. While this part of the project is yet to be completed, it appears from early data that Shiga toxin does not bring about a change in r-protein size or charge. Experimental protocol presented earlier in this report is designed to reveal changes in protein size and charge using the technique of two-dimensional (2-D) gel electrophoresis. Shown in Figure 2 are r-proteins from control and Shiga toxin-inactivated 80S ribosomes. A change in horizontal position on this gel pattern would indicate a charge modification while a vertical plane change would result from a change in protein mass. Upon close examination, we were unable to detect a significant difference between the patterns shown in Figure 2.

Figure 2

(-) Shiga toxin (+) Shiga toxin



d. Ribosomal RNAs

An abstract of our work, presented at the 1983 ASBC meeting is presented below.

ON THE MECHANISM OF ACTION OF SHIGA TOXIN IN THE INHIBITION OF EUKARYOTIC PROTEIN SYNTHESIS. T.P. Moran*, T.G. Obrig* and J.E. Brown* (SPON: J.L. Glenn). Albany Med. College, Albany, NY, 12208 and Walter Reed Army Inst. Res., Washington, DC, 20012.

We have observed that Shiga toxin appears to affect eukaryotic protein synthesis by inhibition of the aminoacyl-tRNA binding step of peptide elongation in a manner similar to that of α-sarcin. Since the mechanism of α-sarcin involves hydrolysis of 28S rRNA in 60S ribosomal subunits, the action of Shiga toxin on 28S rRNA was examined to determine if its mechanism of inhibition was similar to that of α-sarcin. Toxin purified to homogeneity from Shigella dysenteriae 1, strain 38180, was preincubated with 5 ug/ml TPCK-trypsin followed by 19 ng/ml phenylmethylsulfonyl fluoride, 2.5 mg/ml urea and 10 mM dithiothreitol. Toxin-inactivation of rabbit reticulocyte ribosomes was carried out by addition of toxin to either lysate or crude ribosomal pellet at a molar ratio of toxin to 80S ribosome of 1:10. [^{3}H]leucine incorporation into acid-insoluble material in lysate preparations was completely inhibited by this amount of toxin. Ribosomal RNA was extracted from control or toxin-treated ribosomes and analyzed by polyacrylamide-agarose gel electrophoresis. Although fragments derived from 28S rRNA were observed from α-sarcin-treated ribosomes, no hydrolysis of 28S or 18S rRNA was detected from Shiga toxin-treated ribosomes. Thus, it appears that Shiga toxin inactivates ribosomes in a manner different than α-sarcin.

Recently, we have addressed the question of how Shiga toxin inactivates the large (60S) ribosomal subunit. In particular, it has been determined whether Shiga toxin hydrolyzes the 28S rRNA contained within the 60S ribosomal subunit. Protocol for this part of the study was conducted in the following manner. "Activated" Shiga toxin was incubated with ribosomes in a molar ratio of 1:10, respectively. This amount of toxin was demonstrated, in separate experiments, to completely inhibit [^{3}H]leucine incorporation into globin protein in reticulocyte lysate. Ribosomes were isolated from the toxin-treated or control lysate by centrifugation at 100,000 x g/60 min. The 80S ribosomes were separated, by sucrose gradient centrifugation, into 40S and 60S subunits were then extracted with phenol-chloroform to obtain a rRNA preparation. Analysis of rRNA was performed by electrophoresis of 2 ug samples on polyacrylamide-agarose gels and detection of resolved RNA species with silver stain. As a positive control, rRNA from alpha-sarcin-treated lysate was also processed in an identical manner. This fungal-derived toxin has been demonstrated to specifically hydrolyze a 400 nucleotide fragment from the 3'-end of 28S rRNA. Results from these trials indicated that Shiga toxin neither changed the migration (i.e., the size) of 28S rRNA from 60S subunits in these polyacrylamide-agarose gels nor did it result in appearance of new smaller RNA species (Fig. 3F). In contrast, alpha-sarcin-treated samples contained a new easily discernible species of RNA with the approximate position of a 400 nucleotide fragment designated α-S in Figure 3E. The 5S and 5.8S rRNA species from control and toxin treated 60S ribosomes appeared similar (Fig. 3D-F). In addition, the 18S rRNA from 40S ribosomes was not modified by either toxin (Fig. 3A-C). These results show that Shiga toxin acts in a different manner than does alpha sarcin. Further support for this concept is presented in Figure 4 where alpha sarcin is shown to be a non-specific ribonuclease when incubated with isolated E. coli rRNA (Fig. 4F-H). Shiga toxin, up to 0.5 uM, did not exhibit ribonuclease activity (Fig. 4B-E).

Figure 3

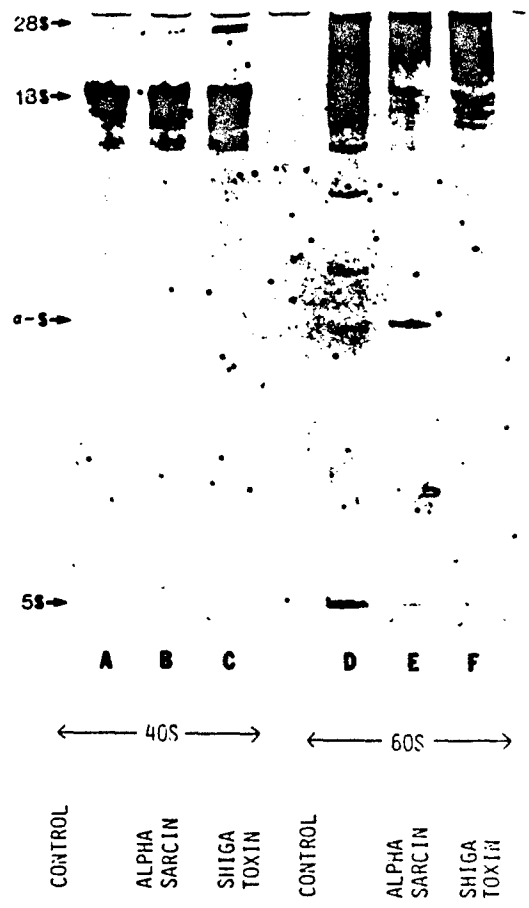
NUCLEASE ACTIVITY OF SHIGA TOXIN AND ALPHA SARCIN ON ISOLATED RIBOSOMAL RNA OF E. COLI.

INCUBATION CONDITIONS	A	B	C	D	E	F	G	H
SHIGA TOXIN, M	0	0.5	0.1	0.01	0.001	0	0	0
ALPHA SARCIN, M	0	0	0	0	0	10.0	1.0	0.1

PROCEDURE: E. coli RIBOSOMAL RNA WAS INCUBATED WITH TOXIN 5 MIN AT 37°C AND ANALYZED ON A 2.7% ACRYLAMIDE GEL

Figure 4

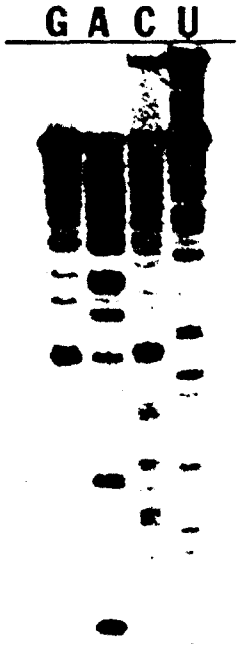
THE EFFECT OF SHIGA TOXIN AND ALPHA SARCIN ON 40S AND 60S RIBOSOMES



PROCEDURE: RIBOSOMAL SUBUNITS WERE ISOLATED FROM TOXIN-TREATED POLYSOMES (P-100) WITH ANALYSIS OF EXTRACTED rRNA ON A 2.7% ACRYLAMIDE GEL. IN A POLYPHENYLALANINE SYNTHESIS ASSAY, 60S RIBOSOMES TREATED WITH EITHER TOXIN WERE BIOLOGICALLY INACTIVE IN CONTRAST TO 40S RIBOSOMES WHICH WERE NOT AFFECTED BY THE TOXINS.

Using this technique we would be unable to detect a difference in these rRNA species should only a few nucleotides be cleaved from their termini by Shiga toxin. Thus, we have started to examine the rRNAs more closely by the Maxam-Gilbert sequencing procedure. The 5.8S rRNA has been isolated from Shiga toxin-treated 60S ribosomes, 3′ end-labeled with [^{32}P] using T4 RNA ligase, processed with base-specific chemical hydrolysis and resolved on a 12% polyacrylamide gel. An autoradiograph of this gel is presented in Figure 5. The nucleotide sequence revealed from this pattern indicates that the 3′terminus of 5.8S rRNA obtained from Shiga toxin-treated 60S ribosomes is virtually identical to that of published sequences of 5.8S rRNA from other sources. The control 5.8S rRNA has just been sequenced and agrees with this concept.

Figure 5
Sequence gel of 5.8S rRNA from toxin treated ribosomes.



Discussion of Results

a. Initiation of Protein Synthesis

It seems quite clear from the data obtained on this part of the project that Shiga toxin is not a primary inhibitor of the initiation process. Thus, Shiga toxin is without effect on each of the individual steps of initiation as depicted in Scheme "A" (p. 11). This being the case, a working hyphothesis has evolved which implicates Shiga toxin as strictly being a ribosome inactivator. Structural modification of the 60S ribosomal subunit most likely results in a single major functional change,

namely inhibition of aminoacyl-tRNA binding to ribosomes, a process which requires elongation factor 1. In simple terms, this explains why Shiga toxin inhibits elongation rather than initiation of peptides.

b. Elongation of Protein Synthesis

As described above, Shiga toxin modifies 60S ribosomes by an enzymatic process to cause a functional lesion in the EF-1-dependent elongation step. The basic question remains as to why EF-1 does not react fully with toxin-treated ribosomes. It is believed that the answer to this will come only when the structural modification in ribosomes is identified.

c. Ribosomal Proteins

In order to identify the physical modification Shiga causes in the 60S ribosome one must, at a minimum examine the 47 ribosomal proteins for changes in size or charge. This is most easily accomplished by the technique of 2-D gel electrophoresis as we have performed. Separation of proteins in the 1st dimension is a function of charge. Since most of the r-proteins are basic, they will carry positive charges at pH 8.6, and migrate towards the cathode. However, because some of these r-proteins have isoelectric points between pH 10 and 11 (more basic than histones) it is probable that small changes in charged amino acids on these proteins would go undetected by electrophoresis at pH 8.6. Thus, we have also started to examine these proteins by running the 1st dimension at either pH 9.6 or 10.6. The second dimension separation is based on size exclusion of proteins from the pores of a high percentage polyacrylamide matrix. Smaller proteins run faster throgh the gel than do larger proteins. Results from this study have not shown reproducible differences between protein patterns from control vs. Shiga toxin-treated ribosomes. However, a complete examination of the r-proteins with the varied conditions is yet to be completed.

d. Ribosomal RNAs

Of the three rRNA species present in 60S ribosomes, we have been unable to detect a change in their size as a result of Shiga toxin action on ribosomes. The limit of detection for size changes is approximately 10 nucleotides. As a positive control, we have had little difficulty resolving a 480 nucleotide fragment released from 28S rRNA by alpha sarcin toxin action on 60S ribosomes. The data presented in this report, together suggest that Shiga toxin has a mode of action different from that of alpha sarcin.

As mentioned earlier, Shiga toxin may have specific exonuclease activity whereby, 1 to 10 nucleotides are removed from either the 3′ or 5′ termini of the 5S, 5.8S or 28S rRNAs. This can only be determined by sequencing of the termini of these rRNAs. Initial sequencing data of the 3′ terminus of 5.8S rRNA indicates that this Shiga toxin does not have its action at this site. The 5.8S rRNA sequence has been strongly conserved through evolution. Our sequence data matches very closely to that of yeast and mouse hepatoma 5.8S rRNA and is identical through the first 35 nucleotides starting from the 3′ terminus. We are presently in the process of checking the other termini of rRNAs from Shiga toxin-treated 60S ribosomes.

Conclusions

The conclusions to be drawn from our experimentation to date are the following:

1. Shiga toxin does not significantly inhibit initiation of eukaryotic protein synthesis.

2. The major functional lesion caused by Shiga toxin action on ribosomes is located at the aminoacyl-tRNA binding step of the peptide elongation process.

3. We have confirmed a very recent report that Shiga toxin specifically inactivated 60S but not 40S ribosomal subunits.

4. Shiga toxin does not cause major electrophoretic or size changes in ribosomal proteins from 60S ribosomes.

5. Both Shiga toxin and alpha sarcin were shown to specifically inhibit the biological activity of 60S ribosomes in protein synthesis.

6. Incubation of ribosomes with an inhibitory concentration of either toxin yielded fragmentation of ribosomal RNA only in ribosomes treated with alpha sarcin.

7. Shiga toxin and alpha sarcin appear to interact with 60S ribosomes in a different manner although both toxins cause a similar functional lesion in aminoacyl-tRNA binding into the ribosomal A-site.

8. The 3′-terminus of 5.8S rRNA from Shiga toxin-inactivated 60S ribosomes is virtually identical to that of control 5.8S rRNAs.

Recommendations

Several modes of investigation may be pursued which will ultimately reveal, at the biochemical level, the mechanism by which Shiga toxin inactivates eukaryotic ribosomes. These directions of study are the following:

1. Analysis or ribosomal proteins using refined gel electrophoretic techniques as are presently being applied in this project.

2. To complete the sequence analysis of rRNA species of toxin treated *vs.* control ribosomes.

3. Application of the chemical crosslinking technique to this problem to help identify the topographical location of Shiga toxin binding and modification site on 60S ribosomes. That is, Shiga toxin, labeled with ^{125}I could be crosslinked to ribosomes using bifunctional chemical reagents and, following dissociation of ribosomes into their protein and RNA components, the moiety to which the toxin is attached would be identified. A line of experimentation by which to accomplish this latter approach is presented in the contract renewal protocol.

LITERATURE CITED

1. Conradi, H. (1903) Ueber 18sliche durch aseptische autolyse Erhaltene Giftsoffe von Ruhr-und 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. Eiklik, 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. Thompson, M.R., Steinberg, M.S., Gemski, P., Formal, S.B., and Doctor, B.P. (1976) Inhibition of in vitro protein synthesis by Shigella dysenteriae 1 toxin. Biochem. Biophys. Res. Commun. 71:783.

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. Benne, R., Brown-Leudi, M.L., and Hershey, J.W.B. (1979) in Methods in Enzymology, Vol. 60 (Part H), K. Moldave and L. Grossman, eds., Academic Press, New York, p. 15.

21. Obrig, T., Huston, J.S., and Cooley, L.B. (1982) Immunoaffinity purification and properties of antiviral proteins from Phytolacca americana (submitted for publication).

22. Obrig, T.G., Culp, W.J., and Hardesty, B. (1971) Inhibition of peptide initiation on reticulocyte ribosomes by edeine. Eur. J. Biochem. 21:31.

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., Irvin, J.D., and Hardesty, B. (1973) The effects of an antiviral peptide on the ribosomal reactions of the peptide elongation enzymes EF-1 and EF-2. Arch. Biochem. Biophys. 155:278.

25. Obrig, T., Shen, A., Kwoka, S., and Chudyk, M.A. (1979) Protein composition of L-cell messenger ribonucleoproteins, Biochem. Biophys. Res. Commun. 91:1062.

26. Sherton, C.C., and Wool, I.G. (1974) The extraction of proteins from eukaryotic ribosomes and ribosomal subunits. Molec. Gen. Genetics 135:97.

27. Wool, I.G. The structure and function of eukaryotic ribosomes, in G. Chambliss et al., (eds). Ribosomes: structure, function and genetics. University Park Press, Baltimore, 1979, p. 797.

28. Sherton, C.C., and Wool, I.G. (1974) Two-dimensional polyacrylamide gel electrophoresis of eukaryotic ribosomal proteins. Methods Enzymol. 30 (Part F): 506.

29. Gressner, A.M., and Wool, I.G. (1974) The phosphorylation of liver ribosomal proteins *in vivo*. J. Biol. Chem. 249:6917.

DISTRIBUTION LIST

*12 Copies: Administrator
Defense Technical Information Center
ATTN: DTIC-DDA
Cameron Station
Alexandria, VA 22314

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

1 Copy: Dean, School of Medicine
Uniformed Services University
of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20014

1? Copies: Director
Walter Reed Army Institute of Research
ATTN: SGRD-UWZ-C
Walter Reed Army Medical Center
Washington, DC 20012

4 Copies: Commander
US Army Medical Research and Development Command
ATTN: SGRD-RMS
Fort Detrick
Frederick, MD 21701