INHIBITION OF IN VITRO PROTEIN SYNTHESIS BY SHIGELLA DYSENTERIAE 1 TOXIN

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SUMMARY: A toxin produced by S. dysenteriae 1 has been purified to near homogeneity. Preliminary invesigation of its possible effect on in vitro mammalian protein synthesis shows that it has no effect on aminoacylation of tRNA. However, the transfer of amino acids from AA-tRNA to ribosomes or polysomes to effect the polypeptide chain synthesis is inhibited in the presence of microgram quantities of toxin. This is evidenced by inhibition of poly U directed polyphenylalanine synthesis using washed ribosomes as well as the synthesis of nascent polypeptide chains using polysomes and mixtures of amino acids. Unlike Diphtheria toxin NAD^+ is not required by S. dysenteriae 1 toxin for its activity in this reaction.

Shigella dysenteriae 1 is known to produce a toxin distinct from its endotoxin complex which possess cytotoxic, neurotoxic and enterotoxic properties (1-3). The role of this toxin in the pathogenesis of shigellosis and its molecular mechanism of action is yet to be elucidated. Using procedures briefly outlined here, a toxin has been purified to near homogeneity (4) and found to inhibit in vitro protein synthesis in extracts from rat liver.

MATERIALS AND METHODS: S. dysenteriae 1 strain 3818T grown in modified syncase broth for 72 hours was harvested and subjected to alkaline extraction according to the method of van Heyningen (5). The extract was subjected to 28-70% ammonium sulfate precipitation, followed by DEAE-Sephadex chromatography, gel filtration on Biogel A-0.5 M, isoelectric focusing and finally, polyacrylamide gel electrophoresis. The preparation thus obtained was protease and heat labile and did not possess nuclease-like activity as judged by lack of degradation of tRNA or [^{3}H]-labeled polyuridilic acid (poly U). A molecular weight of about 70,000 daltons was estimated for the active toxin by acrylamide gel electrophoresis. Purified toxin was stored in aliquots at -20°C. The procedure of Vicari (2) was employed to determine cytotoxicity in HeLa cell cultures. 10^{-9} µg of purified toxin demonstrated cytotoxicity. The LD_{50} for mouse neurotoxicity calculated by the method of Reed and Muench (6) was 0.1 µg. Enterotoxicity was determined using a rabbit ilial loop assay (7). Fluid secretion was induced with approximately 0.25 µg purified toxin. Details of the purification procedure, and of the chemical and biological properties of the toxin will be described elsewhere (4).

Rat liver ribosomes, essentially free of active mRNA, were prepared according to Goodwin et al., (8). Supernatant enzymes were prepared as previously described (9) and by using the method of Zomzely-Neurath et al., (10). Polysomes containing endogenous mRNA were prepared according to Pestka et al., (11). Purified [^{3}H]-phe-tRNA$_y^{phe}$ or [^{3}H]-AA-tRNA$_{c.l.}$ were prepared according to Kellog et al., (12).

Calf liver, yeast tRNA's and yeast tRNA^{phe} were obtained from Boeringer-Mannheim. [^{3}H]-poly U was obtained from Miles Laboratories. Other radio-labeled compounds were obtained from New England Nuclear Corporation. All other chemicals and reagents used were of analytical grade. Radioactivity of the samples was measured using Instabray scintillation fluid with a Packard Tri-Carb Liquid Scintillation Spectrometer.

Assay Procedure for Aminoacylation of tRNA: Incubation mixtures consisted of 100 mM Tris-HCl buffer, pH 7.6, 10 mM $MgCl_2$, 10 mM KCl, 50 mM NH_4Cl, 5 mM ATP, 0.24 µC [^{14}C]-amino acid mixture (15 amino acids), 0.05 mg yeast or calf liver tRNA and 50 µg rat liver supernatant enzymes in the presence or absence of toxin in a total volume of 1.0 ml. Incubations were carried out at 37°C for 15 min. Reactions were stopped with the addition of 2 ml cold 10% Trichloracetic acid (TCA). Samples were shaken and cooled for 5 min in an ice water bath and filtered through 0.45 µ nitrocellulose filters. Filters were washed twice with 3 ml cold 5% TCA and dissolved in Instabray for counting.

Poly U Directed Polyphenylalanine Synthesis: Two sets of reactions were carried out. In one, precharged [^{3}H]-phe-tRNA$_y^{phe}$ (specific activity 400 pmole/A_{260}) was added to the reaction mixture and in the other, 0.05 µg tRNA$_y^{phe}$ plus 3.67 µC [^{3}H]-phe (10.7 C/mM) was added. The rest of the incubation mixtures in both cases consisted of 20 mM Tris-HCl, pH 7.8, 0.17 mM GTP, 105 mM NH_4Cl, 14 mM KCl, 1.7 mM ATP, 12 mM $MgCl_2$, 6.8 µg pyruvate kinase, 8.0 mM phosphoenolpyruvate (PEP), 8.6 mM 2-mercaptoethanol, 56 µg supernatant enzymes, and ribosomes (260 µg protein) in total volume of 0.24 ml. Incubations were performed in the presence or absence of toxin for 60 min at 37°C. Reactions were initiated by adding ribosomes to the remainder of the mixture, and terminated with 2 ml of cold 10% TCA. The samples were then heated for 20 min at 90°C, cooled and filtered through nitrocellulose filters as described above.

Protein Synthesis Using Polyribosomes: The incubation mixtures in a final volume of 0.225 ml contained 50 mM Tris-HCl buffer, pH 7.8, 16 mM KCl, 13 mM $MgCl_2$; 7.5 mM 2-mercaptoethanol; 71.5 mM NH_4Cl; 11 µg pyruvate kinase; 8.5 mM PEP; 430 µg supernatant protein; 57 µg yeast tRNA; 155 µg polysomes protein; 0.02 µmoles each of unlabeled cysteine, methionine, glutamine, tryptophan, and asparagine; 1.7 mM ATP, 0.17 mM GTP; 10 µC [^{3}H]-amino acid mixture (15 amino acids) and toxin or water. Experiments utilizing precharged [^{3}H]-AA-tRNA$_{c.l.}$ contained 97 mM Tris-HCl buffer, pH 7.8, 55 mM NH_4Cl; 15 mM KCl, 12 mM $MgCl_2$; 7 mM 2-mercaptoethanol; 1.6 mM ATP; 0.16 mM GTP; 11 µg pyruvate kinase, 8.2 mM PEP, 215 µg supernatant enzymes; 155 µg polysome protein, and toxin or water in 0.225 ml. Incubation of the complete polysome system was 1 hr at 37°C; the precharged tRNA containing reaction was incubated for 20 min at the same temperature. Both reactions were stopped with cold TCA as described above.

RESULTS AND DISCUSSION: The effect of shiga toxin on aminoacylation of calf liver tRNA and yeast tRNA was studied using a rat liver supernatant enzyme preparation. Increasing the toxin concentration to 60 µg/assay

TABLE I

INHIBITION of [^{3}H]-AMINO ACID TRANSFER FROM AMINOACYL tRNA INTO POLYPEPTIDES BY S. DYSENTERIAE 1 TOXIN

Toxin Concentration (μg)	[^{3}H]-phe Polymerized (cpm)	Inhibition %	[^{3}H]-Nascent Polypeptide Synthesized (cpm)	Inhibition %
0	27,080 ± 550	0	7,350 ± 40	0
2.2			6,490 ± 150	12
4.4	14,460 ± 580	47	5,870 ± 200	21
7.7			5,710 ± 150	22

cpm represents an average of two samples. Details of the experimental procedures are described under Materials and Methods. 66,880 cpm yeast [^{3}H]-phe-tRNA and 39,460 cpm calf liver [^{3}H]-AA-tRNA were added in the appropriate reaction samples.

failed to produce an inhibitory effect on this reaction. Changing the tRNA or enzyme concentration in the reaction mixture did not alter this observation. Hence, in the mammalian system we employed there is no inhibitory effect by shiga toxin on in vitro aminoacylation of tRNA.

When transfer of [^{3}H]-phe from [^{3}H]-phe tRNA^{phe} to NH_4Cl washed ribosomes in a poly U directed system was studied, the results presented in Table I were obtained. Also shown are the results of the effect of toxin on transfer of [^{3}H]-amino acids from [^{3}H]-AA-tRNA to nascent polypeptide chains using polysomes and [^{3}H]-AA-tRNA calf liver. The results demonstrate this toxin affects the transfer reaction.

In order to further establish that the toxin inhibits the transfer of amino acids from tRNA to ribosomes, the following experiments were carried out. First the effect of various concentrations of toxin on poly U-directed polyphenylalanine synthesis was studied using purified yeast tRNA^{phe} and NH_4Cl washed ribosomes. As can be seen in Table II

TABLE II

INHIBITION OF POLYPHENYLALANINE SYNTHESIS BY PURIFIED *S. DYSENTERIAE* 1 TOXIN

Toxin Concentration (μg)	[^{3}H]-phe Polymerized (cpm)	Inhibition %
0	147,880 ± 18,500	0
2.2	59,850 ± 12,200	60
4.4	37,091 ± 9,800	75
11.0	29,022 ± 7,400	80
55.0	16,123 ± 3,500	89

Details of the experiment are described under Materials and Methods. The cpm represents an average of three samples.

the inhibition of polyphenylalanine synthesis by *S. dysenteriae* 1 toxin is dependent upon concentration of toxin in the incubation mixture. Secondly, the effect of toxin on the nascent polypeptide chain synthesis using polysomes (containing mRNA) and amino acid mixture was studied (Table III).

Our findings thus show that shiga toxin effects both polyphenylalanine synthesis as well as nascent polypeptide chain synthesis in a mammalian cell-free protein synthetic system. The transfer of amino acids from tRNA to nascent polypeptide chain is inhibited.

It has been shown by Collier (13) that diphtheria toxin fragment A inactivates the free form of elongation factor -2 (EF-2) by catalyzing attachment of the ADP-ribose moiety of intracellular NAD^+ to an amino acid residue on EF-2. The modified factor is inactive in promoting translocation on ribosomes. The effect of cholera (14), diphtheria (15), and shiga toxin in the presence and absence of NAD^+ on nascent polypeptide chain

TABLE III

INHIBITION OF NASCENT POLYPEPTIDE CHAIN SYNTHESIS BY PURIFIED S. DYSENTERIAE 1 TOXIN

Toxin Concentration (μg)	Nascent Peptide Chain Synthesized (cpm)	Inhibition %
0.0	33,230 ± 110	0
0.9	26,750 ± 100	19
1.8	21,600 ± 160	35
2.2	19,590 ± 1,010	41
4.4	17,950 ± 1,100	46
7.7	13,320 ± 1,400	60

Details of the experimental procedures are described under Materials and Methods. Figures represent an average of three samples.

TABLE IV

COMPARISON OF EFFECT OF CHOLERA, SHIGA AND DIPHTHERIA TOXINS ON IN VITRO PROTEIN SYNTHESIS

Toxin	Nascent Polypeptide Synthesized - NAD^+ (cpm)	Nascent Polypeptide Synthesized + NAD^+ (cpm)
None	30,785 ± 1,000	26,770 ± 300
Cholera	27,732 ± 100	
Shiga	11,432 ± 1,100	10,900 ± 350
Diphtheria	25,730 ± 450	13,308 ± 450

The details of the experiment are described under Materials and Methods. The cpm represents an average of two samples. 6.6 μg toxin was used for each assay; the concentration of NAD^+ was 10 μg/assay.

synthesis using polysomes and amino acid mixture is shown in Table IV. These results reveal that shiga toxin, unlike diphtheria toxin does not require NAD^+ to inhibit cell-free protein synthesis.

These studies have been based on toxin purified from a highly virulent strain of S. dysenteriae 1 isolated from a recent epidemic of shigellosis in Guatamala. Toxin from another strain, 60 R, has also been purified in this laboratory. Preliminary experiments indicate that toxin from 60R also inhibits cell-free protein synthesis in a similar manner. The exact mode of action is unknown at this time. However, it is evident from the data presented in Table IV that the mode of action of shiga toxin is different from the known mode of action of diphtheria toxin. Experiments are underway to elucidate the exact mechanism of action of toxin from S. dysenteriae 1.

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