SYNTHESIS OF DIPHTHERIA TOXIN IN E. COLI CELL-FREE LYSATE

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Summary. An E. coli cell-free lysate was used to translate C. diphtheriae RNA from nontoxinogenic $C_7(-)$, C_7 infected with β tox^+ corynebacteriophage, and C. diphtheriae strain PW8. De novo synthesis of toxin was detected by immune precipitation with antitoxin, ADP-ribosylation of mammalian elongation factor 2 and rabbit skin test. The results indicated that toxin is produced in the E. coli protein synthesizing system primed with RNA from cells infected with tox^+ bacteriophage and is absent in systems primed with RNA from $C_7(-)$ cells.

Diphtheria toxin, a single polypeptide of MW 62,000, is synthesized by members of C. diphtheriae infected by tox^+ corynebacteriophages (1). Production and release of toxin by cells infected with temperate or lytic tox^+ bacteriophages can occur independent of phage production (2). Recent isolation of phage mutants which produce altered non-toxic molecules which cross-react with antitoxin (CRM mutants) provides strong evidence that diphtheria toxin is specified by the bacteriophage DNA (3), although its particular role in the life cycle of corynebacteriophages remains to be elucidated.

In order to understand the control of toxin synthesis at the molecular level it is desirable to develop a cell-free system in which de novo synthesis of toxin can be studied. In this paper we report that C. diphtheriae RNA is translated in E. coli cell-free lysate, but that toxin is produced only when the in vitro system is primed with RNA from tox^+ bacteriophage-infected cells and is absent in systems primed with RNA from nontoxinogenic $C_7(-)$ cells.

Materials and Methods

A. Growth of bacteria: C. diphtheriae $C_7(-)$ and C_7 infected with a hypervirulent β tox^+ bacteriophage (C_7 β tox^+) were grown in deferrated PGT medium (4). Cells were harvested at 45 min post-infection, washed once with buffer (10 mM Tris-HCl pH 7.8, 10 mM magnesium acetate, 60 mM NH_4Cl and 6 mM 2-mer-

captoethanol), rapidly frozen on dry ice and stored at -60 C. C. diphtheriae Park-Williams-8 (PW8) lysogenized with a tox^+ corynebacteriophage was grown in deferrated CY medium (5), harvested in mid-log phase, and treated as above.

B. Isolation of RNA from C. diphtheriae: 2-4 g frozen $C_7(-)$, C_7 β tox^+ and PW8 cells were ground with alumina. The RNA was extracted by the SDS-phenol method according to Franklin (6), incubated with 10 μg/ml DNase at 37 C for 15 min, re-extracted with phenol and precipitated with 2 volume of ethanol.

C. E. coli cell-free protein synthesizing system: Preparation of E. coli Q_{13} S-30 extract and other components of the cell-free protein synthesizing system as well as determination of ^{14}C-lysine incorporation into proteins were as described by Modolell (7). Reaction mixtures in 500 μl were incubated for 10 or 15 min with each RNA preparation. Peptides were released with EDTA (0.1 M), ribosomes removed by centrifugation and the supernatants were examined for presence of diphtheria toxin and toxin fragments. In experiments where ADP-ribosylation assay and rabbit skin tests were to be performed, the reaction mixtures were incubated with non-labeled amino acids and the supernatants were collected as above.

D. Immune precipitation: Horse diphtheria antitoxin (a kind gift of Dr. R. Thompson, Wellcome Research Laboratories) was tested for presence of antibodies against $C_7(-)$ and E. coli Q_{13} cell extracts by double immunodiffusion in agar gel, and found to contain precipitating antibodies against both extracts. Therefore, this antitoxin was absorbed four times with lyophylized extracts of $C_7(-)$ cells and E. coli Q_{13} to remove antibodies against the two extracts. Rabbit diphtheria antitoxin was prepared by immunization with toxoid (8) made from electrophoretically pure diphtheria toxin (courtesy of Dr. R. J. Collier, Univ. Calif. Los Angeles). Supernatants derived from the in vitro systems were dialyzed against borate-saline buffer pH 8.4. The protein concentration was measured by Lowry's method (9), and the total acid-precipitable radioactivity was determined for each supernatant. A volume of 500 μl of each supernatant containing approximately 750 μg protein was precipitated with antitoxin in

the presence of unlabeled carrier toxin for 1 hr at 37 C and 48 hr at 4 C. Immune precipitates were washed three times with 5 ml borate-saline buffer, collected on Millipore filters and counted in a liquid scintillation counter. Non-specifically precipitable radioactivity was determined using normal rabbit serum.

E. ADP-ribosylation of mammalian elongation factor 2 (EF2) enzyme: The assay was similar to that described by Collier and Kandel (10). The supernatants of the reaction mixtures were dialyzed against 50 mM Tris-HCl pH 8.2, 0.1 mM EDTA for 48 hr. A volume of 100 µl of each supernatant was assayed in presence of 100 µl EF2 enzyme prepared from rabbit reticulocytes (11) and 12 p mole ^{14}C-adenine NAD$^+$ in a total volume of 250 µl.

F. Rabbit skin test: Rabbit skin test was performed by the method of Matsuda and Barksdale (12). Test supernatants were concentrated tenfold, and 3 mg protein in 0.2 ml was injected intradermally. An additional 3 mg protein from each concentrated supernatant was mixed with rabbit antitoxin and injected in the opposite site 6 hr later. Test sites were read 96 hr after inoculation.

Results and discussion: The RNA isolated from C_7(-), C_7 β tox$^+$ and PW8 cells stimulated incorporation of ^{14}C-lysine in the E. coli cell-free lysate 6 to 30 times over the endogenous incorporation. The degree of stimulation obtained depended on the RNA preparation rather than the cell source. In preliminary experiments, the optimum concentration of magnesium ion for maximum incorporation of label with each preparation of RNA was determined to be 12 mM. Since magnesium ion concentration slightly lower than optimum for amino acid incorporation has been shown to improve the fidelity of translation (13), concentration of 10 mM magnesium ion was used throughout the experiments.

Immune precipitation with specific antitoxins (see Methods) was used to identify toxin or toxin fragments in supernatants derived from the in vitro protein synthesizing reaction mixtures. Table 1 shows that between 1.5 and 3%

Table 1. Immune precipitation of supernatants from cell-free E. coli lysate primed with RNA from tox^+ and tox^- cells. E. coli lysate was incubated for 10 min with added RNA in presence of ^{14}C-lysine. Polypeptides were released with EDTA, dialyzed against borate-saline buffer pH 8.4 and 500 µl samples containing 750 µg protein were precipitated with horse or rabbit antitoxins using unlabeled toxin carrier.

	CPM/mg protein		
Source of RNA	Acid insoluble ^{14}C-lysine	Horse* antitoxin precipitable	Rabbit* antitoxin precipitable
PW8 tox^+	29,560	425	260
C_7 β tox^+	4,240	115	60
C_7(-) tox^-	4,125	0	0
Endogenous	660	0	0

*The results were corrected for non-specifically precipitable polypeptides by subtracting the CPM/mg protein precipitated by normal rabbit serum from the CPM/mg protein precipitated by the antitoxins.

of the total acid insoluble radioactivity was specifically precipitated with both horse and rabbit antitoxins when RNA from cells infected with tox^+ bacteriophage was used to prime in vitro protein synthesis. Although the amino acid incorporation depended on the particular RNA preparation (Lightfoot unpublished observations), the percent of total counts precipitable with antitoxin depended on the source of RNA. Therefore, while the C_7(-) RNA used in these experiments directed protein synthesis as efficiently as the C_7 β tox^+ RNA, the products of C_7(-) RNA or those of endogenous incorporation were equally precipitable both by normal rabbit serum and by antitoxin. These results indicated that toxin or cross-reacting proteins were only synthesized when RNA from tox^+ bacteriophage-infected cells primed the cell-free protein synthesizing system.

While immune precipitation detected de novo synthesis of toxin in vitro,

it could not distinguish between biologically active and inactive products. Fragment A of diphtheria toxin has a unique enzymatic activity. It covalently links the adenosine diphosphate ribose moiety of NAD^+ to the mammalian elongation factor 2 (EF2), thereby inhibiting protein synthesis in the cell (14). Since minute quantities of Fragment A can be detected by its ADP-ribosylation activity, this assay was employed to determine if enzymatically active Fragment A was synthesized in vitro. Unlabeled NAD^+ in the derived supernatants was removed by dialysis (see Methods) and each supernatant was tested for ADP-ribosylation activity using ^{14}C-adenine NAD^+. Table 2 shows that the supernatant from cell-free system primed with $C_7(-)$ RNA did not have ADP-ribosylation activity higher than the EF2 background, whereas both supernatants from cell-free systems primed with C_7 β tox^+ and PW8 RNA possessed Fragment A enzymatic activity. The RNA preparations themselves showed no activity in the ADP-ribosylation assay. Therefore, the results indicated that enzymatically active Fragment A had been synthesized only in the system primed with RNA from tox^+ bacteriophage-infected cells.

Although Fragment A of diphtheria toxin inhibits mammalian cell-free protein synthesis, the whole toxin molecule is required to intoxicate animal cells (15). Rabbit skin is a very sensitive tissue and only 2×10^8 toxin molecules (1 MRD) injected intradermally leads to necrosis which can be specifically neutralized with antitoxin. Rabbit skin tests were performed by intradermal injection of 3 mg of concentrated in vitro supernatants in a total volume of 0.2 ml. Diphtheria toxin (5 MRD) was also injected as a positive control. Tests were read 96 hr later. A positive lesion consisted of a central necrotic zone surrounded by an area of erythema. These lesions were readily distinguishable from the localized erythema produced by the E. coli S-30. Necrosis was evident at the injection sites of toxin and supernatants from cell-free systems primed with C_7 β tox^+ RNA or PW8 RNA, but not $C_7(-)$ RNA. Furthermore, there was no necrosis at the injection site of toxin or supernatants neutralized with rabbit antitoxin.

Table 2. ADP-ribosylation activity of supernatants of cell-free system primed with RNA from tox^+ and tox^- cells. E. coli lysate was incubated for 15 min with added RNA in presence of unlabeled amino acids. The polypeptides were dialyzed against Tris-HCl EDTA buffer and 100 µl of each supernatant (150 µg) was assayed for ADP-ribosylating activity using ^{14}C-NAD. A minimum of ten separate experiments were carried out with each RNA species.

Source of RNA	CPM-acid-insoluble radioactivity	
C. diphtheriae PW8	100 ± 15	$p < 0.05$
C. diphtheriae β tox^+	129 ± 12	$p < 0.05$
C. diphtheriae $C_7(-)$	43 ± 17	$p > 0.05$
Elongation Factor 2	42 ± 11	

The detection of Fragment A activity by the ADP-ribosylation of EF2 enzyme in the absence of trypsinization of the supernatants as well as the skin necrosis reaction indicated that the supernatants contained free Fragment A as well as whole toxin. Free Fragment A could have resulted from premature release of polypeptide following EDTA treatment and/or cleavage of intact toxin by the proteolytic enzymes present in the S-30.

In summary, the E. coli cell-free protein synthesizing system translated RNA from C. diphtheriae. Furthermore, the results indicated that de novo synthesis of a specific polypeptide namely diphtheria toxin occurred when RNA from toxinogenic cells was used to prime the system. RNA from non-toxinogenic C. diphtheriae was able to prime the cell-free protein synthesizing system but did not synthesize diphtheria toxin. In this respect $C_7(-)$ RNA acted as a control for the specificity of the assay systems employed to identify the toxin.

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