Studies on the Mode of Action of Diphtheria Toxin
VI. Inhibition of Protein Synthesis Induced by Local Infection with Toxinogenic *Corynebacterium diphtheriae*[1] (36953)

C. B. SAELINGER,[2] J. G. IMHOFF, AND P. F. BONVENTRE
(Introduced by H. C. Lichstein)

Department of Microbiology, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45219

It has been well documented that the production of an extracellular protein toxin constitutes the prime virulence mechanism of *Corynebacterium diphtheriae,* and that this toxin is responsible for the pathological lesions and the mortality associated with diphtheria infections. The toxin has been characterized extensively and is known to inhibit protein synthesis in eukaryotic cells by enzymatically splitting nicotinamide adenine dinucleotide into nicotinamide and adenosine diphosphoribose. The latter moiety binds with the eucaryotic translocase, transferase II, rendering it inactive (1). This toxin-catalyzed reaction has been shown to occur in established tissue culture cell lines (2), in cell-free systems derived from sensitive and resistant mammalian cells (2, 3) and in sensitive laboratory animals (4, 5). Previous experiments have utilized bacteria-free crude or purified toxin preparations, and none has employed the viable toxinogenic diphtheria bacillus as the source of toxin. It has been an implicit assumption that the action of purified toxin and of toxin produced by the bacterium in host tissues is the same. Early investigators speculated that factors other than toxin produced by the bacillus may modify the severity of the disease (6), and although the exact nature and role of these factors were not understood, they were thought to facilitate the dissemination of the toxin (*i.e.,* other bacterial products, in addition to toxin, could influence the course of diphtheritic infection).

It is the purpose of the present series of experiments to compare the action of toxin produced and released *in vivo* by viable diphtheria bacilli with the action of a crystalline toxin preparation used in our earlier studies (4, 7, 8).

Materials and Methods. C. diphtheriae *culture.* The SM-1 strain of *C. diphtheriae* was maintained as a stock culture on blood agar slants and stored at 4° for the present experiments a shake-culture was grown overnight in Wadsworth Medium at 37°, washed three times in phosphate-buffered saline (pH 6.9) to remove extracellular toxin and then resuspended to an optical density of 0.15. Sonication and toxin assay of the bacterial cell extract showed that between 1–2 Minimal Lethal Doses (MLD) preformed toxin was present in the 0.5 ml inoculum used in these experiments.

Guinea pigs. Hartley strain albino, male guinea pigs weighing between 250 and 350 g were used in all experiments. Animals were purchased from the Paul Hamm Rabbitry, Greenwood, Indiana.

Materials. Neuraminidase and deoxyribonuclease were obtained from Calbiochem, Los Angeles, California. Both were prepared for injection in isotonic, pyrogen-free saline.

General experimental procedure. Guinea pigs were fasted for a period of 24 hr prior to the start of each experiment. Guinea pigs were injected intramuscularly with 0.5 ml of the suspension of washed *C. diphtheriae.* This concentration of bacteria resulted in a fatal infection within 20–24 hr, a time to death similar to that found after im injection of 10 MLD purified toxin. Symptoms were similar in animals infected with viable diph-

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[2] Present address: Dept. of Microbiology, Health Sciences Center, Louisville, Kentucky 40201.

TABLE I. Protein Synthesis in Muscle Tissues of Guinea Pigs after Intramuscular Challenge with *C. diphtheriae* or Purified Diphtheria Toxin.

Tissue	Reduction in normal protein synthesis (%)[a]			
	C. diphtheriae			Toxin
	Exp. 1	Exp. 2	Exp. 3	(10 MLD)
Heart	61	38	73	42
Skeletal muscle	—	—	50	51
Diaphragm	68	34	38	35

[a] Composite data from a total of 24 guinea pigs. Data is expressed as the percent of inhibition of incorporation of tritiated leucine into tissue protein in experimental animals as compared to control animals.

$$\frac{\text{cpm/mg (experimental)}}{\text{cpm/mg (normal)}} \times 100 = \%\ \text{inhibition.}$$

theria bacilli and in those challenged with purified toxin. Microscopic examination and bacteriologic culture of tissue taken from the site of injection with *C. diphtheriae* indicated that the bacteria remained localized and did not proliferate to any extent in the tissues of the guinea pig. Inflammation at the site of infection was moderate. An exudate primarily of polymorphonuclear leukocytes 24 hr after injection was demonstrated by hematoxylin and eosin staining of sections prepared from infected muscle tissue. A third, untreated, group of guinea pigs served as normal controls.

Protein synthesis was evaluated by measuring the extent of incorporation of tritiated leucine into tissue proteins *in vivo*. Approximately 18 hr after challenge with toxinogenic bacteria, the animals were injected with 1.0 mc ^{3}H-leucine intraperitoneally (L-leucine 4,5 ^{3}H; New England Nuclear Corp., Boston, specific activity, 30–50 Ci/mmole). At this time the animals showed visible signs of illness, but had not reached a moribund state. After a precise two or three hour exchange period, the animals were anesthetized and subjected to cardiac perfusion with warm Ringer's solution containing 3% sucrose to remove blood from tissues and organs. Tissues then were excised and frozen until analyzed. To determine the amount of tritiated leucine incorporated into tissue protein during the *in vivo* exchange period, the tissues were homogenized in saline and precipitated with cold 5% trichloracetic acid (TCA). The precipitates were washed two times with cold TCA, extracted in successive steps with 70%, 95%, and absolute alcohols, then with a 1:1 mixture of absolute alcohol and ether, and finally three times with ether. The dried residue was digested in 0.1 *N* NaOH in preparation for liquid scintillation counting and protein was assayed by the method of Lowry *et al.* as modified by Oyama and Eagle (9).

Guinea pigs were challenged with viable *C. diphtheriae* in three separate experiments using a total of 18 guinea pigs. The data from each experiment compares results from two groups of animals, control and experimental. It should be pointed out that there is some variability in incorporation of leucine within each individual experiment and thus differences of less than 15% between comparable tissues in the two groups of animals was not considered experimentally significant.

In one additional experiment, involving a total of six animals, one group of animals was inoculated im with 25 MLD diphtheria toxin in a hind leg and with a mixture of 1.5 mg DN'ase and 100 units neuraminidase in the opposite member. A second group received only toxin. All animals were sacrificed 20 hr later, following a 2-hr exchange period with tritiated leucine to allow measurement of protein synthesis, as described above.

Results. The sites of biochemical activity

TABLE II. Protein Synthesis in Non-Muscle Tissues of Guinea Pigs after Intramuscular Challenge with *C. diphtheriae* or Purified Toxin.

Tissue	Reduction in normal protein synthesis (%)[a]			
	C. diphtheriae			Toxin
	Exp. 1	Exp. 2	Exp. 3	(10 MLD)
Kidney	31	7[b]	33	0
Liver	52	40	75	0
Adrenal gland	50	52	19	0
Spleen	0	0	0	12[b]
Small intestine	0	20	9[b]	—
Brain	0	0	0	0
Lung	0	0	0	0

[a] Footnotes same as in Table I.

[b] Not statistically significant.

of diphtheria toxin produced *in vivo* by a toxinogenic strain of *C. diphtheriae* were compared with those of a purified toxin preparation. Toxin released *in vivo* from viable *C. diphtheriae* was found to reduce protein synthesis in several tissues of infected animals. In three separate experiments, guinea pigs were given an intramuscular injection of washed, viable *C. diphtheriae* bacilli and 18 hr later, the level of protein synthesis in the infected animals was evaluated by determining the amount of incorporation of tritiated leucine into tissue proteins *in vivo*. Data in Tables I and II compares the effect on protein synthesis of toxin synthesized and excreted by toxinogenic bacilli in muscle tissue with that of 10 MLD crystalline toxin administered by the same route. The results are expressed as percent inhibition of tritiated leucine incorporation into tissue proteins in infected animals as compared with normal control animals. It should be noted that the average time to death of animals challenged with the bacterial suspension or with 10 MLD was approximately the same (*ca.* 24 hr), and that both groups of animals received leucine 4–5 hr before death normally would have occurred. Protein synthesis is markedly reduced in muscle tissues of animals given either toxinogenic bacilli or preformed toxin (Table I). This inhibition of protein synthesis is a consistent finding in the heart, skeletal muscle, and diaphragm from both groups of animals. No significant difference in the level of inhibition at these sites in animals inoculated with bacteria and those receiving purified toxin was noted.

The pattern of inhibition of leucine incorporation in non-muscle tissues, however was not identical following injection of toxinogenic bacilli or 10 MLD purified diphtheria toxin intramuscularly (Table II). Inhibition appeared to be more widespread in animals receiving bacteria than in those challenged with a purified preparation of toxin. Several non-muscle tissues showed decreased levels of protein synthesis as a result of infection with *C. diphtheriae*. A reduction of at least 30% in the level of protein synthesis was observed in kidney, liver and adrenal gland from guinea pigs receiving the viable bacilli. On the other hand, no significant reduction in the incorporation of leucine occurred in the same tissues following challenge with 10 MLD crystalline toxin. Protein synthesis remained normal in spleen, small intestine, lung or brain tissues from animals in either group. Toxin released from the bacilli *in vivo* appears to act in a more generalized manner, while the action of purified toxin, under similar conditions, is restricted to muscle tissues.

This difference in response must be reconciled with the fact that animals in both groups showed similar symptoms and died at approximately the same time, as well as the observation that extensive bacterial multiplication at the site of bacterial inoculation did not occur. Infection represents a complex situation in which several factors, in addition to the toxin itself, could conceivably be contributing to the overall pathogenesis of the disease. *C. diphtheriae* produces several enzymes, *e.g.*, neuraminidase, deoxyribonuclease, and phospholipase, which in the natural or experimental infection might potentiate the biochemical action of the toxin. Therefore an additional experiment was performed to determine the effect of two of these enzymes on the pattern of inhibition of protein synthesis in toxin-treated animals (Table III). Guinea pigs were injected im in one leg with 25 MLD purified diphtheria toxin, and in the opposite member with a mixture of 1.5 mg DN'ase and 100 units neuraminidase; a second group of animals received only toxin. Eighteen hours later animals received 0.5 mCi tritiated leucine ip and were sacrificed after 2 hr. Initial studies involving the enzymes alone had shown that they were not toxic for the animals at the concentrations employed. Data in Table III compares the level of protein synthesis in guinea pigs given toxin alone or in combination with neuraminidase and DN'ase. The results are expressed as counts per min per mg tissue protein. The presence of enzymes did not enlarge or alter the range of tissues inhibited by diphtheria toxin. The level of protein synthesis was similar in the tissues of animals receiving either enzymes and toxin or toxin alone. In both cases the circumscribed inhibition of synthesis in heart and other muscle tissues was noted.

Discussion. In the guinea pig model used

TABLE III. Effect of Neuraminidase and Deoxyribonuclease on Protein Synthesis in Tissues of Guinea Pigs Challenged with Diphtheria Toxin.[a]

Tissue	cpm × 10^{-3}/mg Protein	
	Toxin Alone	Toxin and Enzymes
Heart	0.4	0.7
Skeletal muscle	0.2	0.2
Diaphragm	1.6	2.2
Kidney	2.1	3.5
Spleen	3.8	5.0
Pancreas	8.8	12.0
Intestine	15.7	16.2
Lung	3.7	3.9
Liver	3.2	3.9
Brain	2.1	2.5

[a] Composite data from a total of 6 guinea pigs.

for this study, diphtheria toxin produced *in vivo* by viable *C. diphtheriae* inhibits protein synthesis in a more widespread fashion than does intramuscular injection of a small dose of purified toxin. The difference in the pattern of inhibition in the two groups of animals has no readily apparent explanation. Symptoms and time to death in both cases were similar; therefore, presumably the stage of illness during which *in vivo* protein synthesis was assayed was the same in both situations. It has been noted previously that parenteral injection of high levels of diphtheria toxin (greater than 100 MLD) results in a generalized inhibition of protein synthesis (4, 10), and thus one might consider synthesis of toxin by the bacteria in the tissues as a possible explanation for the results. It is difficult, however, to reconcile production of such high levels of toxin *in vivo* with the absence of extensive bacterial multiplication at the site of infection. The fact that less than two MLD was present in the original inoculum would preclude the bacterial suspension *per se* as the source of significant quantities of preformed toxin.

A second possibility may be considered. Toxin produced *in vivo* may be disseminated in a different manner than is purified toxin. Inhibition of protein synthesis after iv injection of purified toxin into guinea pigs is more widespread than after im challenge (10). Similarly the distribution, detoxification and excretion of toxin following infection may be different from that following injection of the soluble protein toxin alone. Although no evidence is presented for an enhancement of purified toxin activity by deoxyribonuclease and neuraminidase, the experimental method represents an artificial situation and may not adequately mimic conditions of the diphtheritic infection. Thus the possibility that auxiliary virulence factors other than toxin play a role in the total pathophysiology of the intoxication cannot be totally discounted.

Finally, a third possibility may be considered. Diphtheria toxin is synthesized as a single polypeptide chain with a molecular weight of 62,000 daltons. Treatment of the intact toxin molecule with proteases converts it into an active or "nicked" form of toxin. The activated (nicked) toxin consists of two fragments, A and B, held together by internal disulfide linkages. Fragment A is enzymatically active and inhibits the mammalian translocase, thus stopping protein synthesis. Fragment B is non-enzymatic but apparently is required for attachment and entry of toxin into the cell. Neither fragment A nor B alone is toxic for animal cells (11, 12). Purified toxin preparations have been shown to contain varying ratios of intact-to-nicked toxin (12). Therefore, a possible explanation for the different patterns of inhibition following injection of toxin and toxinogenic diphtheria bacilli is that toxin produced *in vivo* had a higher percentage of active toxin molecules than was present in the crystalline preparations employed in our experiments. Several reasons for such a difference can be offered. First, there may be local release of bacterial proteases which specifically activate the toxin. Secondly, toxin may be activated by host enzymes, *e.g.*, cathepsin (13), or other lysosomal proteases present in the inflammatory neutrophils mobilized to the site of bacterial injection. This increase in the amount of active toxin could conceivably result in a generalized inhibition of protein synthesis encompassing many tissues. Finally, since neither fragment alone is toxic to intact cells, reduction of nicked toxin to fragments A and B probably occurs on or in the sensitive cells themselves. Cell types within the diphtheria-sensitive animal may vary in ability to activate intact toxin and thus in the absence of bacteria-derived activating enzymes, the lim-

ited tissue spectrum of activity demonstrated by purified toxin may represent a target effect on those cells most capable of activating toxin themselves.

In conclusion, diphtheria toxin, synthesized and released *in vivo* by *C. diphtheriae* impairs protein synthesis in several tissues, both muscle and non-muscle in nature.

Summary. Protein synthesis in the tissues of guinea pigs after intramuscular challenge with viable, toxinogenic diphtheria bacilli was evaluated by measuring incorporation of ^{3}H-leucine into cellular proteins. Significant inhibition of protein synthesis occurred in muscle tissues (heart, diaphragm, and skeletal muscle) and several non-muscle tissues (kidneys, liver, and adrenal gland). Protein synthesis in the spleen, small intestine, lung and brain was unimpaired. Inhibition of protein synthesis subsequent to intramuscular injection of 10 MLD purified diphtheria toxin was restricted to the three muscle tissues while non-muscle tissues were unaffected. Purified neuraminidase and deoxyribonuclease injected simultaneously with toxin failed to increase the range of tissues inhibited. Possible reasons for the difference in the patterns of inhibition obtained with *C. diphtheriae* bacilli and purified toxin are considered.

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