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Studies on the Mode of Action of Diphtheria Toxin

V. Protein Metabolism in a Guinea Pig Model Simulating Chronic Diphtheritic Toxemia

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An experimental model of "chronic" diphtheria intoxication in the guinea pig was developed. Adult guinea pigs, subjected to a regimen of multiple sublethal doses of purified diphtheria toxin (total of 1.4 minimum lethal doses divided equally in four daily doses), developed a toxemia which terminated in death between 6 and 8 days. During an advanced stage of illness, de novo protein synthesis was assessed by in vivo incorporation of tritiated leucine into tissue proteins. With the exception of pancreas and skeletal muscle, protein synthesis in healthy, control and toxin-treated guinea pigs was comparable. The absence of notable impairment of protein synthesis in this experimental situation is discussed in terms of the mode of action of the diphtheria toxin and the pathophysiology of the disease.

The molecular biology of diphtheria toxin action has been established beyond doubt, primarily as a result of a series of investigations by Collier, Pappenheimer, and their colleagues (8–13, 23), spanning the last decade. The protein toxin is characterized as a single polypeptide chain with a molecular weight of 62,000; it contains two disulfide bridges which covalently link two toxin fragments generated by proteolysis. Fragment A and B are both required for intoxication of tissue culture cells or animals. Whole toxin has been identified as a unique enzyme with nicotinamide adenine dinucleotide (NAD) as substrate. The active site is located on fragment A, since all enzyme activity is associated with that toxin fragment and none is associated with fragment B. The reaction results in the cessation of peptide bond formation by the transfer of the adenosine diphosphate ribosyl moiety of NAD to eukaryotic cell translocase (EF-2). As a result of this catalytic binding of EF-2, the translocase is inactivated and protein synthesis is abruptly halted. This reaction has been demonstrated in diphtheria-sensitive tissue culture cells (13), in cell-free protein-synthesizing mixtures derived from sensitive and resistant tissue culture cells (8, 13, 15), in tissue extracts from guinea pigs and rats (5, 15), and in intact animals (1, 5). The assumption, therefore, is that the series of biochemical events described above results in disruption of normal physiological function, onset of a pathological state, and ultimately in death. Previous experiments utilizing the guinea pig as an experimental animal, and assessing protein metabolism (1, 5, 15), can be considered to be of an acute nature. The time of death of 300- to 400-g albino guinea pigs subsequent to challenge with diphtheria toxin is dose dependent but usually occurs between 12 and 72 h at minimum lethal doses (LD_{min}) between 2,000 at one extreme and 5 at the lower extreme (Bonventre, unpublished data; reference 1). Time of death becomes erratic at doses approaching 1 LD_{min} and at times may be protracted beyond 96 to 120 h. Our previous experiments and those of Baseman et al. (1), in which the information regarding protein synthesis in vivo was generated, utilized toxin doses in which the time of death of guinea pigs was less than 36 h. Therefore, one might say that the assessment of protein synthesis inhibition as a factor in the genesis of diphtheritic toxemia has been obtained exclusively in an animal model not simulating the time course usually observed in natural human infections. The clinical course in the most rapidly fatal diphtheria infections runs about 10 days and usually is protracted to 2 or more weeks before cardiovascular complications and electrocardiographic changes ensue (7). Therefore, it was considered of some interest to assess protein synthesis in guinea pigs suffering from a more chronic form of diphtheria intoxication, since this more closely simulates the human form of the disease than do the acute models on which all of our currently available knowledge is based. As the data will show, guinea pigs receiving a regimen of toxin in multiple sublethal quantities eventually die of a toxin-induced illness but show minimal evidence of protein synthesis impairment even at late stages of the toxemia.

MATERIALS AND METHODS

Toxin. Diphtheria toxin was obtained originally from C. G. Pope and M. Stevens of the Wellcome Research Lab., Beckenham, England. When reconstituted, the crystalline toxin contained 324 limit of flocculation (L_f) U/mg; 1 L_f U was equivalent to approximately 60 guinea pig LD_{min}. The toxin was standardized with a flocculating antitoxin of equine origin obtained from L. Levine of the Massachusetts Health Department, Boston, Mass., and by titration in HEp-2 monolayers. Toxin was solubilized in phosphate buffer (pH 6.9; 0.01 M) containing 0.1% bovine serum albumin and stored at −70 C until used.

Guinea pigs. Hartley strain, male guinea pigs weighing between 400 and 500 g were used. Animals were supplied by Camm Research Institute, Wayne, N.J. The guinea pigs were conditioned in isolator cages in an environmentally controlled room for at least a week prior to initiation of experiments.

Regimen of toxin injection. Preliminary titrations to establish a reliable and reproducible guinea pig model of chronic diphtheria intoxication showed that total toxin doses of between 1 and 3 LD_{min} administered in daily, sublethal amounts (i.e., between 0.2 and 0.4 LD_{min}) usually caused death of guinea pigs between 5 and 17 days after the start of the regimen (24). A predictable regimen chosen for the experiments reported here was the injection of 0.4 LD_{min} for 4 successive days by the intraperitoneal route. Animals challenged in this way usually died between the 6th and 7th day (i.e., 2 to 3 days after the total of 1.6 LD_{min} had been administered). Occasionally animals died on the 5th or the 8th days.

Monitoring of animals during intoxication. During the regimen described, guinea pigs were examined daily for weight loss, temperature changes, heart rate, and electrocardiographic changes. Blood chemistries were also obtained on the day the experiment was performed; analysis of blood sera or plasma was done in a SMA 12/60 autoanalyzer (Technicol Corp., Tarrytown, N.Y.).

Experimental protocols. Preconditioned guinea pigs were divided into two experimental subgroups: (i) diphtheria toxin-treated animals subjected to the regimen described above, and (ii) normal controls injected with phosphate buffer daily in lieu of the toxin. Between 24 to 36 h after the last injection, manipulations to ascertain in vivo protein synthesis in the two groups were started. At this time, the toxin-treated animals demonstrated visible signs of illness. According to the time of death data obtained previously, the natural course of the toxemia would have terminated in death approximately 24 h later. A sufficient number of animals were subjected to the toxin regimen to ensure that those selected for measurement of protein synthesis would show visible signs of the toxemia; although all animals subjected to the toxin regimen succumbed, not all of them demonstrated hypothermia and electrocardiographic changes which were considered hallmarks. Each experiment was carried out with three to four animals in each subgroup. Guinea pigs in both groups were injected intraperitoneally with 0.5 mCi of a radiolabeled protein precursor in sterile aqueous solution (^{3}H-L-leucine, 4,5,T; sp act 30–50 Ci/mmol, New England Nuclear Corp., Boston, Mass.). After a precise 2-h exchange period, the animals were anesthetized and perfused through the left heart with Ringer solution containing 3% sucrose; this was done primarily to remove blood from organs and viscera, since earlier experiments showed that failure to do so resulted in a significant amount of nonspecific radioactivity being extracted with the tissue samples. After perfusion, tissues were excised rapidly, frozen with dry ice, and stored at −70 C until processed. Proteins from the tissues were precipitated with trichloroacetic acid, extracted, processed, solubilized, and prepared for liquid scintillation counting as described in detail elsewhere (2). The method of Lowry et al. (19) was used to measure protein. Radioactivity and protein were determined in duplicate samples from each tissue extract, and protein synthesis during the 2-hr exchange period was expressed as counts per minute per microgram of protein. This protocol allowed a valid comparison between in vivo protein synthesis in the tissues of normal guinea pigs and of guinea pigs late in the course of the chronic diphtheritic toxemia.

RESULTS

Signs and symptoms. Several vital parameters were measured during the course of diphtheria intoxication of guinea pigs. Animals subjected to the dosage regimen described in Materials and Methods exhibited an average weight loss of 10 to 15% in 5 days, became hypothermic (temperature reduction of >2.0 C), demonstrated elevated serum potassium levels (values ranged from 5.2 to 17 meq/liter, with an average of 9.0; average value in normal guinea pigs is 4.0 meq/liter), and electrocardiogram tracings became abnormal in more than 70% of the animals. Abnormalities included bradycardia, inverted T waves, and irregular QRS complexes (J. M. Weister, Ph.D. thesis, Univ. of Cincinnati, Ohio, 1971). Isolated working heart preparations from these "chronic" diphtheritic animals reflected direct myocardial damage by impaired contractability, atrial-ventricular conduction defects, and loss of coronary flow autoregulation (24).

In vivo protein synthesis. Two separate experiments in which three toxin-treated and three normal control animals for the first experiment, and four toxin-treated and three normal animals for the second were evaluated for in vivo protein synthesis. Table 1 shows the composite data for the two experiments; the mean values and standard deviations for the individual tissues in both

TABLE 1. *Effect of "chronic" diphtheria intoxication on protein metabolism of guinea pigs*[a]

Tissue	^{3}H-leucine incorporation (counts per min × 10^{-3} per mg of protein)		Inhibition[b] (%)
	Toxin treated	Normal controls	
Heart 1[c]	2.03 ± 0.18	2.05 ± 0.09	N[d]
2[e]	1.95 ± 0.12	2.20 ± 0.03	NS
Skeletal muscle 1	0.63 ± 0.15	0.75 ± 0.07	16
2	0.44 ± 0.13	0.82 ± 0.11	46
Diaphragm 1	3.59 ± 0.35	3.18 ± 0.45	N
2			
Liver 1	8.82 ± 0.81	6.28 ± 1.04	N
2	8.08 ± 1.25	5.58 ± 0.31	N
Lung 1	5.60 ± 0.61	3.39 ± 0.07	N
2	5.27 ± 0.35	3.78 ± 0.29	N
Spleen 1	4.50 ± 0.23	5.50 ± 0.50	18
2	5.92 ± 0.98	6.03 ± 0.70	N
Small intestine 1	8.17 ± 1.53	8.49 ± 1.21	NS
2	8.90 ± 1.70	10.01 ± 1.20	NS
Brain 1	1.48 ± 0.17	1.50 ± 0.19	N
2	1.86 ± 0.30	1.76 ± 0.22	N
Adrenals 1	4.76 ± 0.34	4.79 ± 0.52	N
2	6.01 ± 1.31	5.56 ± 0.84	N
Pancreas 1	13.76 ± 0.47	27.72 ± 5.16	52
2	13.50 ± 1.95	23.53 ± 2.23	43
Kidney 1	4.37 ± 0.60	3.47 ± 0.28	N
2	5.97 ± 0.37	4.82 ± 0.41	N
Sciatic nerve[f] 1	1.02	1.00	
2	1.46 ± 0.30	1.38 ± 0.25	N

[a] Protein synthesis in vivo was assessed by measuring ^{3}H-leucine incorporated during a 2-h period in the two groups of animals. Results are expressed as the means and standard deviations for each tissue; radioactivity and protein determinations were done in duplicate or triplicate for each sample.

[b] Percent inhibition equals (counts per min toxin mean values/counts per min control mean values) × 100.

[c] Experiment 1. Data were obtained by using three normal and three toxin-treated guinea pigs.

[d] N, None; NS, not statistically significant. Values varied as much as 10 to 15% for tissues within each group of animals; thus any difference between normal and toxin-treated tissue samples of 15% or less was not considered significant.

[e] Experiment 2. Data were obtained by using three controls and four toxin-treated guinea pigs.

[f] No standard deviations since these were pooled samples.

experiments are provided, so that variation within each subgroup can be readily appreciated. The overall pattern of protein synthesis in the guinea pigs during the terminal stages of the "chronic" toxemia was essentially normal. Of all the tissues examined, only pancreas and skeletal muscle incorporated significantly less ^{3}H-leucine than did comparable tissues from normal animals. This is in sharp contrast to results obtained with guinea pigs given doses of toxin resulting in acute deaths. In those cases, significant inhibition of protein synthesis occurred and has been assumed to represent an important biochemical factor in the pathophysiology of diphtheria. The impairment of protein synthesis in pancreas and skeletal muscle may not represent a direct effect of toxin via the NAD-mediated translocase reaction by which toxin acts. Pancreatic enzymes are synthesized largely in response to dietary intake, and the "chronic" animals studied consumed little or no food in the 2 days prior to assessment of protein synthesis. Thus, the differences observed in the uptake of leucine into pancreatic proteins by normal and toxin-treated animals may be due to nutritional factors rather than toxin. The defect in protein synthesis in skeletal muscle may also represent an indirect effect of the toxemia. Weight loss and increased urinary nitrogen excretion are commonplace in a variety of infectious and toxemic states; often the source of nitrogen in these cases is skeletal muscle (20) and thus muscle wasting ensues. Therefore, a defect in protein metabolism in skeletal muscle may be due to factors other than a direct effect of diphtheria toxin at that site. The marked reduction in protein synthesis in the myocardium seen in the

"acute" guinea pigs (2, 6) given toxin in low doses by the intramuscular route and the generalized inhibition noted when toxin is administered intramuscularly in large doses (5) or intravenously (1, 4) does not occur in the guinea pig model simulating "chronic" diphtheritic toxemia.

DISCUSSION

Several years ago we addressed ourselves to the task of assessing cessation of protein synthesis by diphtheria toxin as a factor in the genesis of experimental diphtheritic toxemia. Experiments were designed to answer several important questions: (i) the extent to which one could extrapolate the molecular biology of toxin action to the intact laboratory animals (and presumably to humans); (ii) the pattern of protein synthesis inhibition induced by toxin in animals; and (iii) the cell types, tissues, and organs most prominently affected. The results of these experiments have been documented (P. F. Bonventre and C. B. Saelinger, Abstr. Amer. Soc. Microbiol., p. 105, 1972; references 2–6), but a summary of the principal findings is presented here to provide a reasonable perspective for the data presented in this communication. The extent to which protein synthesis is reduced in the tissues of diphtheria-intoxicated guinea pigs appears to be dependent upon both the dose and route of toxin injection. Intramuscular injection of 10 to 20 guinea pig LD_{min} does not result in a widespread inhibition of protein synthesis during the latter stages of the intoxication as measured by incorporation of ^{3}H-leucine into tissue proteins, but rather appears to be restricted to cardiac and other muscle tissues; occasionally nonmuscle tissues are inhibited to some extent, but this is inconsistent. When large doses of toxin are administered (>200 LD_{min}) by the intramuscular route, the inhibition of protein synthesis in guinea pig tissues is generalized and is demonstrable in nonmuscle as well as muscle tissues. Toxin injected directly into the blood stream either at the low- or high-dosage levels results in a generalized inhibition of protein synthesis during the overtly toxemic stage of illness (1, 4). Animals inoculated with viable, toxinogenic *Corynebacterium diphtheriae* bacilli also demonstrate an impairment of in vivo protein synthesis (P. F. Bonventre and C. B. Saelinger, Abstr. Amer. Soc. Microbiol., p. 105, 1972). Therefore, coupled with the in vitro studies cited previously, all of these observations suggested that the enzymatic reaction of toxin which results in cessation of protein synthesis is of prime significance in the pathophysiology of diphtheria. As has already been pointed out, natural diphtheria is not as rapid a process as is the toxemia in guinea pigs induced by 5 or more LD_{min} administered parenterally. Although the chronic toxemia we describe in the current study cannot be equated with the human disease, the sublethal doses given in four daily doses and the progression of the illness induced in guinea pigs by this means at least mimics the natural disease in humans more closely than does the acute guinea pig experimental model used in the past. By using the chronic guinea pig model, we have made the rather surprising observation that in vivo protein synthesis is not severely impaired during the latter stages of the toxemia. It is not likely that protein synthesis was impaired during earlier stages of the toxemia. Guinea pigs subjected to two daily injections of 0.4 LD_{min} of toxin (rather than four) were normal with respect to all vital signs (temperature, electrocardiogram, blood chemistries, and body weight). In addition, no tissue pathology was evident at that time. Therefore, the possibility that cell death due to toxin activity had already occurred and that we were measuring protein synthesis only of the remaining normal cells at day five can be dismissed. Conversely, it might be suggested that, had protein synthesis been measured later, inhibition might have been apparent. This argument is difficult to accept, however, since the animals had reached the late stages of the toxemia and soon would have been in the agonal stage of illness. Another criticism which might be raised is that our assay procedures are not suitable for assessing protein synthesis and that consequently the data are artifactual. Although such a criticism would be impossible to disprove, it is unlikely that artifact can account for the data. In vivo incorporation of radiolabeled amino acids into trichloroacetic acid-insoluble material is a widely used and accepted procedure for measuring de novo macromolecular synthesis (14, 16–18, 21, 22). In addition, we used identical procedures for assessment of protein synthesis in guinea pigs injected with known inhibitors such as cycloheximide, and generalized inhibition of protein synthesis in all tissues examined was readily demonstrable (6).

If the data are valid, therefore, one is forced to ask several provocative questions concerning the pathogenesis of diphtheria intoxication. First, one must ask whether the acute or the chronic experimental models of the disease more closely resemble the course of human disease. Secondly, we may have to reopen the polemic of whether or not inhibition of protein synthesis per se is the most important factor in the genesis of diphtheria, since these experiments suggest that it may not be. If it is the critical biochemical lesion induced, however, perhaps looking for inhibition at the level of organ or tissue has been inappropriate and our attention should be turned to specialized

proteins such as hormones, enzymes, or unique proteins made only in a region of a differentiated tissue. The task of identifying such a hypothetical protein(s) inhibited by toxin in the intact organism would be a much more difficult task than merely measuring overall protein synthesis. In the final analysis, however, it may be necessary to undertake that task if a complete understanding of what is currently the best of all understood infectious diseases is to be achieved.

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