STUDIES ON THE EFFECT OF DIPHTHERIA TOXIN ON PROTEIN SYNTHESIS IN MICE

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DIPHTHERIA toxin seems to be the sole factor responsible for the pathological lesions and mortality associated with human infections caused by *Corynebacterium diphtheriae*. The protein toxin has been studied extensively and much is now known concerning its chemistry, immunology and biochemical action on living cells (Pope and Stevens, 1958; Collier, 1967; Honjo *et al.*, 1968; Collier and Kandel, 1971; Gill and Pappenheimer, 1971; Uchida, Pappenheimer and Harper, 1972). These investigations and others have established that toxin, in the presence of the mammalian translocase, transferase II (EF2), acts as a unique enzyme that cleaves nicotinamide adenine dinucleotide (NAD) to yield nicotinamide and adenosine diphosphoribose (ADP). The transferase is subsequently ADP-ribosylated and inactivated and this ultimately leads to a cessation of protein synthesis. Toxin has been shown to inhibit protein synthesis by this reaction in (i) established tissue culture cells (Strauss and Hendee, 1959), (ii) cell-free protein-synthesising systems derived from tissue cultures (Collier and Pappenheimer, 1964), (iii) laboratory animals (Baseman *et al.*, 1970; Bowman and Bonventre, 1970), and (iv) cell-free systems derived from animal tissues (Bowman and Bonventre). Nevertheless it is apparent that the events leading to death in man or animals from natural or experimental diphtheria are exceedingly complex, and much remains to be learned before the pathophysiology of this disease is completely understood.

Species susceptibility or resistance to diphtheria toxin varies considerably. Man, dogs and guinea-pigs are sensitive species, whereas rats and mice are relatively resistant. Approximately 0.06 µg protein toxin is lethal for 250-g guinea-pigs, and an inadvertent tragedy with Japanese children who received an injection of non-detoxified "toxoid" revealed that man is five times more sensitive to the lethal effect of the toxin than is the guinea-pig (Barksdale, Garmise and Horibata, 1960). On a weight basis, rats and mice are several orders of magnitude more resistant, since they can withstand 10³–10⁴ guinea-pig lethal doses (Andrewes *et al.*, 1923). The same relative differences in sensitivity to toxin have been observed in primary and established tissue cultures derived from susceptible and resistant animal species (Gablíks and Solotorovsky, 1962). An important aspect of innate resistance is that cell integrity is required, since cell-free protein-synthesising systems derived from sensitive and resistant cell cultures are inhibited equally by direct addition of toxin (Johnson, Kuchler and Solotorovsky, 1968; Bowman and Bonventre). This shows that human and rat cells do not differ in their toxin-sensitive enzyme systems but suggests rather that membrane-associated cellular functions differ in the two species. It has been suggested

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that the absence of specific receptors (Pappenheimer and Brown, 1968) or failure to pinocytose toxin (Moehring and Moehring, 1968), or both, might account for resistance, but it now appears that these possibilities are unlikely (Amorini, Ivins and Bonventre, 1972), and thus the essential difference between cells that are susceptible or resistant to diphtheria toxin is still unknown.

We previously demonstrated that a marked reduction in protein synthesis, particularly in muscle tissues of guinea-pigs, occurs after the intramuscular injection of 20 MLD of toxin (Bonventre and Imhoff, 1966; Bowman and Bonventre); a consistent inhibition of synthesis as measured by ^3H -leucine incorporation *in vivo* was observed in cardiac and skeletal muscle whilst protein synthesis in non-muscle tissues remained relatively normal. On the other hand, when larger doses of toxin (1000 MLD) were administered to guinea-pigs intramuscularly, the inhibition of protein synthesis was more generalised and included other tissues as well as muscle (Bowman and Bonventre).

To date, nothing has been published about the effects of diphtheria toxin on protein synthesis in resistant animal species. Because toxin inhibits synthesis in extracts derived from the tissues of both sensitive and resistant animal species we considered it of interest to ascertain the extent to which protein synthesis is inhibited in the tissues of mice given lethal amounts of diphtheria toxin intramuscularly. Such information might provide an insight into the nature of resistance to toxin and the mechanism of toxin action in the intact animal.

MATERIALS AND METHODS

Toxin and antitoxin. Crystalline diphtheria toxin was obtained from Drs Pope and Stevens (Wellcome Research Laboratory, Beckenham, England). When reconstituted, the toxin contained 324 Lf units per mg and approximately 60 guinea-pig MLD per Lf. A batch of toxin was also purified from culture filtrates of the SM-1 strain of *C. diphtheriae* grown in a modified Mueller-Miller medium (Mueller and Miller, 1941); the filtrate was purified by ammonium sulphate precipitation followed by chromatography on Sephadex and TEAE-cellulose. The final preparation contained 6000 MLD per ml and approximately 25 MLD per Lf unit and was stored at -70°C until used. A flocculating antitoxin obtained from Dr L. Levine of the Massachusetts Department of Health was used to standardise toxin.

Animals. Random-bred Swiss Albino mice weighing between 25–28 g were obtained from the Laboratory Supply Co., Indianapolis, Indiana.

In-vivo protein synthesis. The synthesis of proteins in mouse tissues was estimated by the incorporation of tritiated leucine into trichloroacetic acid (TCA)-insoluble fractions during a defined exchange period *in vivo*. Mice received 0.5 millicurie of ^3H -L-leucine (4, 5 ^3H ; specific activity 30–50 Ci per mmole, New England Nuclear, Boston, Mass.) intraperitoneally and were killed precisely 2 hr after injection of the isotope. Tissues were excised rapidly, quick-frozen, and stored at -70°C until processed. Processing of tissues and measurements of ^3H -leucine incorporation into tissue proteins were done as described in detail previously (Bonventre and Imhoff, 1966). In one experiment (see text) the animals were killed by cardiac perfusion with Ringer's solution containing 3 per cent. sucrose to remove blood from the tissues and organs to be processed. TCA precipitates were dissolved in 1.0-N NaOH and protein was determined by the method of Lowry *et al.* (1951).

General experimental protocols. Mice were divided into two groups for each of the four experiments: (a) those treated with diphtheria toxin, (b) untreated controls. An experiment used either three or four animals in each sub-group. The toxin-treated mice were given 2000 guinea-pig MLD intramuscularly. The average time to death with this dose was approximately 30 hr. Consequently, the tritiated leucine was injected 24 hr after toxin challenge and 2 hr later, the mice were killed and the tissues taken for processing. At that time, the toxin-treated mice were visibly ill but not moribund. In view of the average 30-hr time-course from injection to death, evaluation of in-vivo protein synthesis between the

24th and 26th hr is at a comparatively late stage of the intoxication. The untreated control mice also received radioactively labelled leucine intraperitoneally 2 hr before they were killed.

Protein synthesis was calculated as a function of ^3H -leucine incorporation and is expressed as counts per minute per mg protein. A comparison of protein synthesis in the tissues of normal and toxin-treated mice was then made for each experiment.

RESULTS

Four separate experiments were done in which a total of 17 toxin-treated and 13 normal control mice were utilised. The experiments were designed

TABLE I
*Protein synthesis in tissues of mice given lethal doses of diphtheria toxin**

Tissue	^3H -leucine incorporation† (cpm $\times 10^{-3}$ per mg protein) in		Inhibition‡
	control mice	toxin-treated mice	
Heart	9.6	10.1	none
Diaphragm	13.0	12.6	none
Skeletal muscle	4.1	2.3	44 per cent.
Liver	26.5	26.3	none
Lung	13.4	16.1	none
Brain	7.5	11.6	none
Spleen	83.7	74.7	11 per cent.§
Pancreas	108.8	107.3	none
Kidney	23.7	15.9	37 per cent.
Small intestine	61.2	114.0	none

* The data were obtained from three separate experiments in which a total of 11 toxin-treated and nine normal control mice was employed. Animals were not perfused before processing the tissues for radioactivity and protein determination.

† Each tissue protein sample was counted in duplicate. Average radioactivity values of the three experiments are shown. cpm = Counts per minute.

‡ $\frac{\text{cpm per mg toxin}}{\text{cpm per mg control}} \times 100$.

§ Not statistically significant.

so that in-vivo protein synthesis could be evaluated in mice given lethal quantities of toxin and during the irreversible stage of the toxæmia. The rationale was that inhibition of protein synthesis as a result of the biochemical action of toxin would be most apparent, if present, during the few hours preceding death.

The first three experiments were done as described. None of the animals was perfused with fluid through the circulation to remove blood although as much excess blood as possible was removed by thorough rinsing of the excised tissue slices in Ringer's solution. The composite data of the experiments are given in table I. All of the tissues from the toxin-treated mice, with the exception of skeletal muscle and kidney, incorporated ^3H -leucine *in vivo* to the same extent or to a greater extent than the comparable tissues from normal animals. In view of the fact that the values of leucine incorporation at times varied by as much as 15 per cent. for comparable tissues in each sub-group, we

considered that contaminating blood containing labelled TCA-insoluble material might be responsible in part for this variation. Therefore, in the fourth experiment, the animals were perfused through the heart so that all blood was removed from the viscera and organs. Results of this experiment are shown in table II. The data are presented with standard deviations, and it is clear that variation was much reduced. The pattern of protein synthesis observed, however, was not changed. Again, with the exception of kidney and skeletal muscle, leucine incorporation was equal to or greater than normal.

TABLE II
*Protein synthesis in tissues of mice given lethal doses of diphtheria toxin and perfused before processing**

Tissue	³ H-leucine incorporation† (cpm × 10 ⁻³ per mg protein) in	
	control mice	toxin-treated mice
Heart	10.4 ± 1.3	11.4 ± 1.6
Diaphragm	14.8 ± 0.3	15.1 ± 3.0
Skeletal muscle	3.8 ± 0.3	2.0 ± 0.2
Liver	56.7 ± 6.4	41.1 ± 2.8
Lung	25.3 ± 2.2	24.7 ± 2.2
Brain	12.3 ± 0.4	14.7 ± 1.0
Spleen	113.5 ± 19.5	127.2 ± 22.9
Pancreas	145.5 ± 25.7	155.2 ± 25.3
Kidney	41.1 ± 4.1	20.7 ± 3.9
Small intestine	111.1 ± 18.0	145.7 ± 36.2

* Data were obtained from a single experiment in which six toxin-treated and four control mice were used. The mice in this experiment were anaesthetised and perfused with phosphate-buffered saline through the heart to remove blood from organs and tissues before processing.

† Results expressed as means ± standard deviations.

These results with the diphtheria-resistant mouse are strikingly different from those previously obtained with the sensitive guinea-pig. The cardiac specificity observed with small intramuscular doses in guinea-pigs (Bonventre and Imhoff, 1966), and the generalised depression of synthesis observed with intravenous or high intramuscular toxin doses (Bonventre and Saelinger, 1972) did not occur in mice. The amount of inhibition observed in the kidney and skeletal muscle tissues of toxin-treated mice was appreciable but probably is not sufficient to cause death. Therefore, one is led to conclude that gross inhibition of protein synthesis by toxin, detectable by the examination of tissues and organs, is probably not responsible directly or indirectly for the ultimate death of the mice.

DISCUSSION

Mice, which are approximately 10⁴ times more resistant to diphtheria toxin than guinea-pigs, succumb to a form of diphtheritic toxæmia if sufficiently large quantities of toxin are administered parenterally. We report here the

pattern and extent of in-vivo protein synthesis in mice during the terminal stages of illness induced by lethal quantities of toxin.

The biochemical action of toxin as an inhibitor of protein synthesis is well established, but the precise contribution of this biochemical lesion in the genesis of the disease is unknown. It has been established that protein synthesis in the intact guinea-pig is reduced but not in a completely uniform manner. Synthesis in heart and other muscle tissues appears to be inhibited markedly in a consistent fashion whereas synthesis in non-muscle tissues remains relatively normal following injection of 15 MLD toxin intramuscularly (Bonventre and Imhoff, 1966). These observations suggested that toxin does not affect all tissues equally and that within a sensitive animal species some tissues may act more like target organs than others. Myocarditis, electrocardiographic abnormalities and cardiac failure are recognised complications of severe human diphtheria (Naiditch and Bower, 1954; Ledbetter, Cannon and Costa, 1964; Christie, 1969) and thus the guinea-pig data provide a biochemical explanation of the clinical and pathological signs associated with the disease in man. Indeed, this possibility is reinforced by a recent study showing that cardiac function as measured in an isolated working-heart preparation is significantly impaired by diphtheritic toxæmia (Weister, Bonventre and Grupp, 1973). Inhibition of protein synthesis in non-muscle tissues of guinea-pigs becomes significant when large doses of toxin are administered intramuscularly or if the toxin is given intravenously (Baseman *et al.*, 1970; Bonventre and Saelinger, 1972). Thus, it appears that factors such as toxin dosage and route of administration, as well as species differences *per se* are significant in influencing the course of the experimental disease. Whether or not these factors are important in the natural pathophysiology of diphtheria infection remains to be established.

The fact that mice do not respond to diphtheria toxin in the same manner as guinea-pigs is not completely surprising. The data presented here, however, are interesting from several standpoints. First, they show that the consistent and pronounced inhibition of protein synthesis found in the cardiac tissues of guinea-pigs does not occur in mice challenged with lethal doses of toxin. In addition, protein synthesis was significantly less than normal only in mouse kidney and skeletal muscle, whereas in several tissues, notably small intestine and pancreas, an elevated level of synthesis was often demonstrable. Stimulation of protein synthesis in intestinal tissue of mice given sub-lethal doses (100 guinea-pig MLD) of toxin has also been observed (Bonventre and Imhoff, 1966). The absence of observable depression of protein synthesis in heart tissues suggests that diphtheria-resistant mice, unlike guinea-pigs and man, are refractory to direct cardiac damage by the toxin. This is also substantiated by studies with cell cultures derived from newborn-rat heart tissues (Bonventre and Imhoff, 1967). Reasons for the increase in protein synthesis in some mouse tissues are not clear. The fact that skeletal-muscle protein synthesis appears to be inhibited, while heart muscle is unaffected, is also puzzling. Observations on systemic bacterial and viral infections in experimental animals and man may, however, provide a basis for speculation. Generalised infection usually results in an increased rate of urinary nitrogen excretion and a depletion

of body protein. Evidence suggests that in this situation skeletal muscle supplies a major portion of the excreted nitrogen (Beisel, 1966). Thus, in the case of mice given lethal amounts of diphtheria toxin, the effects on muscle tissue may be indirect and not a result of the NAD-mediated ADP-ribosylation of transferase II. On the other hand, an increase in the rate of protein synthesis in several non-muscle tissues has been noted during the course of bacterial or viral infections (Lust, 1966).

Perhaps the most interesting insight provided by the data is that the complete pattern of protein synthesis remains essentially normal in spite of the fact that the mice are destined to die within several hours. The inhibition of synthesis observed in the kidney was a consistent phenomenon and may be significant. It is known, however, that mice excrete toxin much more rapidly than do sensitive guinea-pigs, and thus the local inhibition may be due to a sustained, high concentration of toxin reaching the kidney tissues; this remains conjectural until experimental evidence substantiates it. The maintenance of normal synthesis of protein at a terminal stage of the toxæmia in the mouse provides a striking contrast to the effects observed with the diphtheria-sensitive guinea-pig. In that species, and presumably the human and other sensitive species, inhibition of protein synthesis resulting from the action of toxin probably plays a significant role in the pathophysiology and mortality of the disease. Our data suggest that gross impairment of protein synthesis *per se* may not be a critical determinant of the ultimate outcome.

Because both sensitive and resistant cells possess a toxin-sensitive translocase, it might have been expected that, once the membrane barrier of the mouse cells was breached by sufficiently high concentrations of toxin, the pattern of inhibition of protein synthesis would have been similar to that seen in the guinea-pig challenged with much smaller amounts of toxin. This clearly did not occur, and it seems possible that the difference in the responses of the two animal species is not merely dependent on the dose of toxin necessary to overcome a permeability or other membrane-associated barrier, but rather that the pathophysiology of diphtheritic toxæmia may be significantly different in the two species. If inhibition of protein synthesis is a significant factor leading to death of resistant mice given overwhelming amounts of diphtheria toxin, it is obvious that the particular proteins inhibited are not of a kind measurable by our assays. Perhaps we have been looking for an impairment of protein synthesis at an inappropriate level; for example, a critical enzyme system essential for maintenance of life may be specifically blocked. Another alternative is that diphtheria toxin may possess more than one biochemical action, and that, in the resistant mouse at least, inhibition of protein synthesis may not be of critical importance in the genesis of the disease and may not be the direct cause of death. These possibilities warrant further study.

SUMMARY

Mice are several orders of magnitude more resistant to diphtheria toxin than are guinea-pigs and man. Resistance is, however, relative rather than

absolute, because 2000 guinea-pig lethal doses cause death of mice within 36 hr. Mice subjected to this quantity of toxin subsequently received an injection of tritiated leucine during the latter stages of the toxæmia in order to assess de novo protein synthesis *in vivo*. All tissues from toxin-treated mice, with the exception of skeletal muscle and kidney, incorporated ^3H -leucine to the same extent as did comparable tissues of normal animals, or to a greater extent. In view of the fact that the biochemical action of diphtheria toxin on more susceptible animals is known to result in a cessation of protein synthesis, the implications of the unexpected results are discussed in terms of species resistance to toxin and the pathophysiology of diphtheritic toxæmia.

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