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The diphtheria bacillus and its toxin: a model system

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INTRODUCTION

Friedrich Loeffler's classical paper entitled 'Untersuchungen über die Bedeutung der Mikroorganismen für die Entstehung der Diphtherie beim Menschen, bei der Taube und der Kalbe' (Studies on the significance of bacteria in causing diphtheria in man, pigeons and calves), was published in 1884. In this paper and those which followed during the next few years, Loeffler described the diphtheria bacillus and its isolation in pure culture, and proved its relationship to the disease diphtheria. While the credit for the discovery of diphtheria toxin must go to Roux & Yersin (1888), Loeffler clearly predicted its existence in his original paper. Because, at autopsy, living 'virulent' diphtheria bacilli could only be recovered from experimental animals at the site of injection, Loeffler postulated that the bacteria must have released into the bloodstream a 'chemical poison' which caused the characteristic *sterile* haemorrhagic lesions in remote organs. He even noted early in 1888 that 'the bacterial poison resembles in its action the poison [now known as abrin] obtained from jaquiriti seeds, which causes inflammation and the production of false membranes when placed on the mucous membranes either of men or animals' (cited in Loeffler, 1908). Today we know the reason for this astute observation. Both abrin and diphtheria toxin block protein synthesis in sensitive eucaryotic cells (Collier, 1975; Olsnes & Pihl, 1976; Pappenheimer, 1977), albeit by different mechanisms. Almost a quarter of a century after the discovery of the diphtheria bacillus, the British Medical Research Council published a 718-page volume on diphtheria. In the first chapter Loeffler (1908) reviewed the history of the disease and reminisced at some length about his own early work. By 1908 when this book appeared, a good deal was already known about the epidemiology of diphtheria, the bacteriology of the diphtheria bacillus, its mode of spread by healthy adults and the protective effect of antitoxin. However, other than its proteinaceous nature almost nothing was known of the chemistry of diphtheria toxin or its mode of action. The interpretation of many of Loeffler's astute early observations and questions that puzzled him remained obscure and unanswerable until the discovery of lysogenic conversion to toxinogenicity by Freeman (1951) and the realization that the *tox* structural gene was carried by a bacteriophage Uchida, Gill & Pappenheimer, 1971).

Lysogeny and Toxinogenicity

In his early observations on *Corynebacterium diphtheriae* isolated from the nasopharynges of man and animals and their relation to the aetiology, diagnosis and spread of diphtheria, Loeffler stressed time and again the importance of distinguishing between 'virulent and non-virulent true diphtheria bacilli, on the

one hand, and the pseudobacilli (that we now call diphtheroids) on the other'. I suspect that he would have been delighted to learn that the only difference between his 'virulent and non-virulent true diphtheria bacilli' was that the former contained, integrated into its chromosome, a small prophage, one of whose genes carried structural information for the synthesis of diphtheria toxin. Moreover, it is now known that spread of this *tox* gene among a human population may take place not only by the toxinogenic bacteria themselves but also by the tox^+ corynephage which they carry. Individuals that harbour non-toxinogenic *C. diphtheriae* among their normal throat flora may become carriers of a toxinogenic strain by lysogenic conversion *in situ*, even if they themselves have already been immunized with diphtheria toxoid (Pappenheimer & Murphy, 1983).

Production and Chemical Properties of Diphtheria Toxin

In their original demonstration of a lethal toxin, Roux & Yersin injected sterile filtrates from a 14-day-old culture into guinea-pigs, which died several days later. Loeffler attributed his own failure to find the toxin to the fact that he only used relatively young, growing cultures. It is now realized that toxin is only released in significant amounts by cultures whose growth rate has become limited by exhaustion of available iron in the medium. In their original experiments, Roux & Yersin injected the enormous dose of 35 ml of culture filtrate into guinea-pigs in order to cause death within five or six days. Today, culture filtrates containing 10000–20000 guinea-pig lethal doses per ml can be produced without difficulty. Thus, by using a low iron concentration and limiting the growth rate in a chemostat with glucose to only 0·051 divisions/h, Rhigelato & van Hemert (1969) succeeded in producing 0·3 g secreted toxin/g bacterial protein continuously for weeks. Because of the high yields now obtainable, isolation of the toxin as a crystalline protein in a high state of purity (lethal dose $\leqslant 0{\cdot}1$ μg/kg sensitive animal) is relatively easy. In fact, it is now possible to obtain large crystals of the toxic protein suitable for X-ray crystallographic analysis (see Fig. 1) (Collier *et al.* 1982; McKeever & Sarma, 1982). It is not unlikely that before the end of 1984 the three-dimensional structure of the diphtheria toxin molecule will be revealed in detail.

We already know a good deal about the diphtheria toxin molecule. The complete sequence of the nucleotides that code for its 535 amino acids has been determined (Greenfield *et al.* 1983; Ratti, Rappuoli & Giannini, 1983). The molecule, shown diagrammatically in Fig. 2, contains two cystine residues near which are clustered 10 of the 17 arginine residues. The first disulphide bridge (*cys* 186–201) subtends an exposed loop of 14 amino acids containing three *arg* residues. The loop is easily 'nicked' by serine proteases and the –S–S– linkage is readily broken by reduction. Two dissimilar fragments, A and B, are thus formed which, as discussed below, differ markedly from one another both in structure and function. Even after carboxymethylation of the two SH groups, A and B remain tightly held together by weak interactions without significant loss of toxicity. Nevertheless, this treatment must cause a marked conformational change, since in contrast to native toxin the altered molecule is now enzymically active and catalyses the translocation of the ADP-ribose moiety of NAD to the imidazole ring of 'diphthamide', a unique post-translationally modified histidine residue in polypeptidyl elongation factor 2

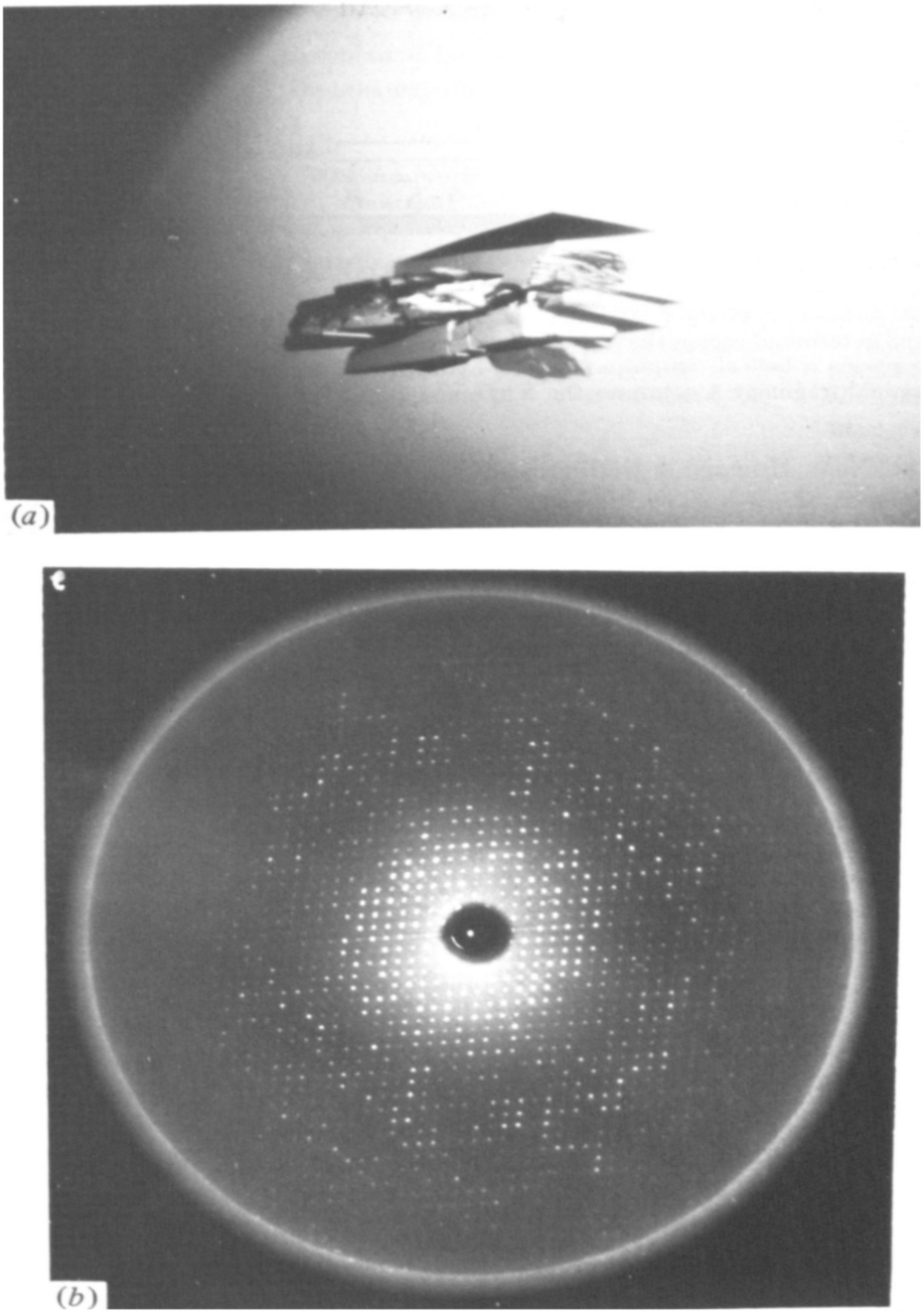

Fig. 1. (*a*) Crystal of diphtheria toxin complexed with the endogenous nucleotide ApUp (adenyl-(3′,5′)-uridine-3′monophosphate). (*b*) X-ray precession photograph of ApUp-complexed crystal of diphtheria toxin. Outer edge of the photograph corresponds to a resolution of 2·5 Å (Collier *et al.* 1982, with permission).

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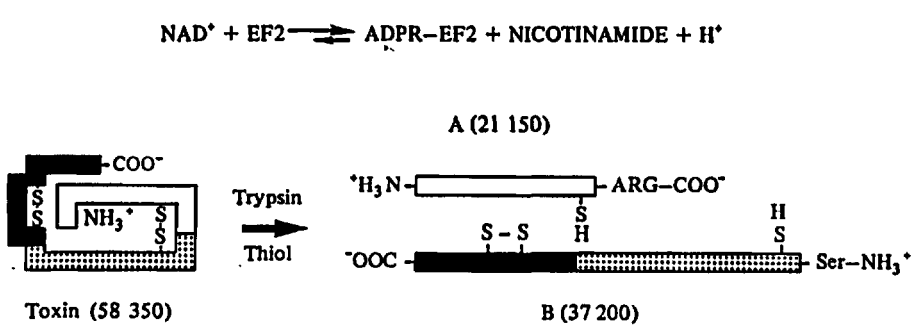

Fig. 2. Activation of the diphtheria toxin molecule. Solid bar represents positively charged C-terminal region that recognizes receptors on sensitive cell surface. Stippled bar contains α-helical, amphipathic and hydrophobic regions of the molecule. The N-terminal fragment A catalyses the NAD-mediated ADP-ribosylation reaction.

(EF2) (van Ness, Howard & Bodley, 1980), an essential enzyme that is present in the cytoplasm of all eucaryotic cells. Inhibition of protein synthesis by ADP-ribosylation of EF2 is the primary event at the molecular level, leading to the necrotic lesions seen in diphtheria intoxication.

Once separated from one another, neither fragment A nor fragment B is toxic even at high concentration. Although the ADP-ribosylating activity is located on fragment A (Collier, 1975), fragment B is required for recognition of receptors on the surface of sensitive cells and for translocation of the A fragment across the plasma membrane to reach the cytoplasm. The elegant experiments of Yamaizumi *et al.* (1978) have shown that if a single molecule of diphtherial fragment A is introduced into the cytoplasm of a mammalian cell, it is lethal within a matter of hours even if the cell is from a toxin-resistant species. For highly sensitive cell lines in culture, a concentration of *ca.* 10^{-13} M intact toxin is lethal within 24 h. Yet even 10000000 times this concentration of *free* fragment A has no effect on cell growth (Pappenheimer, 1982).

Recent studies have revealed that the diphtheria toxin molecule contains three functional domains, namely: (1) an amino-terminal, negatively charged 21000 dalton fragment A which is responsible for enzymic activity; (2) a *ca.* 15000 dalton carboxy terminal region which recognizes specific receptors on the surface of sensitive cells; and (3) an intermediate hydrophobic region in which most of the α-helical stretches are located. Fragments containing this hydrophobic region form ion-conducting channels in phospholipid membranes that are gated by an applied voltage or by a pH gradient (Kagan, Finkelstein & Colomboni, 1981; Donovan *et al.* 1981; Eidels, Proia & Hart, 1983). Such channels are large enough to permit passage of fragment A in its extended denatured form. Similar domains have now been demonstrated in a number of other protein toxins including, amongst others, pseudomonas toxin A (Lory & Collier, 1980), the plant toxins abrin and ricin (Olsnes & Pihl, 1982), *Shigella dysenteriae* toxin, *Vibrio cholera* and *Escherichia coli* LT toxins (Gill, 1978), pertussis toxin and tetanus toxin (Boquet & Duflot, 1982). In each of these toxins, which are listed in Table 1, there is an N-terminal enzymically active A fragment or A chain, a C-terminal receptor recognition domain and an intermediate hydrophobic region. The table shows that these toxic

Table 1. *Biological activity of certain protein toxins*

Toxin	Substrate	Cytoplasmic target	Product	Pathologic effect
Diphtheria Pseudomonas exotoxin A	NAD	Diphthamide-EF2	ADPR–diphthamide-EF2	Inhibition of protein synthesis
Abrin Ricin	—	60S eucaryotic ribosomal component	Inactivation of 60S ribosome	Inhibition of protein synthesis
V. cholera *E. coli* LT	NAD	GTP-binding stimulatory subunit of adenylate cyclase N_s	ADPR-derivative of α_s subunit of N_s	Activation of adenylate cyclase
Pertussis	NAD	Inhibitory subunit of adenylate cyclase N_i	ADPR-derivative of α_i subunit of N_i	Blockade of inhibition of adenylate cyclase
Tetanus	—	—	—	Blocks release of neurotransmitters

proteins also share biochemical and physiological properties that are similar or analogous to those of diphtheria toxin. Thus pseudomonas exotoxin A, though it is serologically unrelated to diphtheria toxin and recognizes different receptors on target cells (Vasil & Iglewski, 1978), contains an Mr 26000 A fragment that inhibits eucaryotic protein synthesis by ADP-ribosylation of the diphthamide residue on EF2. The A chains of abrin, ricin and shigella toxins also inhibit eucaryotic protein synthesis but by a different mechanism, as yet not fully understood, that causes inactivation of the 60 S ribosomal subunit (Olsnes & Pihl, 1982; Jacewicz & Keusch, 1983). The A subunits of the two serologically related toxins of *V. cholera* and *E. coli* use NAD as a substrate to activate eucaryotic adenylate cyclase by ADP ribosylation of its GTP-binding stimulatory subunit N_s (Gill *et al.* 1981). Pertussis toxin also uses NAD as a substrate to bring about intracellular accumulation of cyclic AMP (Katada & Ui, 1982). In this case, however, it is the GTP-binding inhibitory subunit N_i that is ADP ribosylated and is no longer able to exert its regulatory function by inhibition of adenylate cyclase activity (Codina *et al.* 1983). The molecular mechanism by which tetanus toxin A fragment acts pre-synaptically to block acetylcholine release in the peripheral nervous system and central nervous system transmitters is not known.

It is of interest that the target molecules which are inactivated by every one of the bacterial toxins listed in Table 1 are only found in the cytosol or inner membrane surface of eucaryotic cells. There is no evidence that any of the toxin molecules are involved in the metabolism of the bacteria that produce them. Their only role seems to be to confer virulence on the bacteria so that they may proliferate within an animal host. However, although the ability to produce a toxin may confer a temporary selective advantage upon a bacterial species, it is not essential for its survival in an animal host. Thus while mass immunization of man with diphtheria toxin has resulted in eliminating the *tox* gene, the prevalence of *C. diphtheriae* in the human nasopharynx has not diminished (Saragea, Maximescu & Meitert, 1979). Similarly, *E. coli* strains lacking the LT toxin structural gene thrive happily in the intestinal tract of man and animals.

What is the origin of genes coding for production of bacterial toxic enzymes

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which attack substrates that are only found in eucaryotes and which appear to have limited if any survival value for the bacteria which produce them? It is now known that many bacterial *tox* genes are carried by transmissible genetic elements or small replicons. For example, the diphtherial *tox* operon is inserted into the bacterial genome by a temperate corynephage. The structural genes for *E. coli* LT toxin (Gyles, So & Falkow, 1974) and for tetanus toxin (Finn *et al.* 1984) are present on plasmids. Even though the cholera *tox* gene is carried on the *V. cholerae* chromosome, a recent study by Mekalanos (1983) suggests that the cholera toxin operon is carried on a 'transposon-like element'. Since these genes appear to be carried by mobile transferable elements and their products act only upon target molecules located inside eucaryotic membranes, might they have had a eucaryotic ancestry?

Is it possible that the diphtheria bacillus acquired its *tox* gene in relatively recent times? In opening his chapter on the history of diphtheria, Loeffler (1908) wrote:

> On the one hand, the occurrence of epidemics, especially amongst children, and the characteristic clinical manifestations, render diphtheria an easily recognisable disease even to lay men, and we are consequently forced to the conclusion that the old Greeks, Hippocrates and his pupils had no knowledge of it. We find in the writings of these physicians the most exact and minute accounts of various diseases of the throat, pharynx and air passages, but not one of these accords with the picture of diphtheria, and if it had occurred, such excellent observers would not have failed to recognize it.

Recently, the sequence of the 1942 nucleotide base pairs comprising the entire diphtheria *tox* operon, including its promoter, leader sequence and structural gene, has been independently determined in two laboratories (Greenfield *et al.* 1983; Ratti, Rappuoli & Giannini, 1983). The two groups used different strains of *C. diphtheriae*, one of them isolated 90 years ago in New York, the other 50 years later in California. Despite the fact that the bacterial strains differ strikingly in morphology, cultural requirements and metabolic properties, the sequences of all 1942 base pairs found were identical! This fact, and the fact that all known strains from whatever source appear to synthesize immunochemically indistinguishable toxin molecules, would be consistent with the idea that diphtheria as a human disease may have originated within the last two or three millennia.

Toxins as Therapeutic Agents?

It will be recalled that even before Roux & Yersin demonstrated a heat-labile toxin in culture filtrates of the diphtheria bacillus, Loeffler had been impressed by the similarity between the necrotic lesions caused by the 'chemical poison' produced by his 'virulent' bacteria and the heat-labile toxic substance (abrin) extracted from jaquiriti seeds. In fact when Roux & Yersin's paper appeared he was attempting to extract the diphtherial 'poison' from bacteria using a method developed for extraction and purification of abrin from seeds by a group of Danish workers. The use of jaquiriti seed extracts had been introduced into European medicine in 1882 by de Wecker (cited in Olsnes & Pihl, 1976) and was being used in the treatment of chronic eye diseases such as trachoma. Because of the severe necrotic effects of such extracts, their use was soon abandoned. Now, 100 years later, the use of toxins in therapy is once again being considered. Many laboratories are currently attempting to construct molecules containing toxin A-chains or A

fragments linked to polypeptide sequences that will specifically recognize malignant cell lines, enter and destroy them. Some promising preliminary results have been obtained in animals (Uhr & Vitetta, 1983; Bacha, Murphy & Reichlin, 1983) and it may be that the latest chapter in the diphtheria toxin story will involve its use in specific therapy.

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