Diphtheria

Recent studies have clarified the molecular mechanisms involved in its pathogenesis.

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Among infectious diseases of bacterial etiology, diphtheria is the only one of major importance, the near disappearance of which can be attributed to active immunization applied on a mass scale. By 1953, Sir Macfarlane Burnet (*1*) was able to state: "Other diseases are more important causes of death, and some have been just as carefully and extensively studied as diphtheria, but no other common disease has been so successfully studied." Despite the spectacular success resulting from mass immunization, however, much remained to be learned about the biology of this bacterial disease. In this article we review recent progress toward understanding the primary events involved in pathogenesis of diphtheria at the cellular and molecular levels.

The success in prevention of diphtheria is easy to explain. As early as 1884, Loeffler (*2*) noted that while the causative organism, *Corynebacterium diphtheriae*, can ordinarily be found only in a superficial, membranous lesion localized in the throat, fatal cases invariably show sterile, hemorrhagic, and necrotic damage in many organs of the body. Roux and Yersin (*3*) showed that these lesions were caused by a potent, extracellular, heat-labile toxin produced by the bacteria and transported to remote tissues by the blood. Antibodies directed against the toxin protect against the disease itself (*4*). Fortunately, treatment with dilute formalin detoxifies the toxin without affecting its serological specificity or immunogenicity (*5*). Upon injection into man, Formol diphtheria toxoid stimulates specific antitoxin formation and thereby confers protection.

Even a toxigenic strain of *C. diphtheriae* must possess limited powers of "invasiveness" before it can become established on the mucous membrane of the human throat or nasopharynx to initiate a local lesion. Apart from the poorly understood factors concerned with invasiveness, however, the problem of pathogenesis is simply one of understanding the nature and mode of action of the toxic protein.

Studies on diphtheria toxin have been aided by its availability in relatively large amounts and by the accuracy and sensitivity of methods for its bioassay, originally worked out by Paul Ehrlich (*6*).

Biosynthesis and Properties of Toxin

With certain exceptional strains of *C. diphtheriae*, such as PW8, under optimal conditions, toxin may represent more than 5 percent of the total bacterial protein synthesized and over 75 percent of all the protein secreted. Yields of 500 mg per liter of culture may be obtained (*7*) and purification of the toxin is not difficult (*8*). Two important factors concerning toxin production should be noted. (i) Only strains of *C. diphtheriae* lysogenic for, or infected with, bacteriophage carrying the *tox* gene are capable of toxin biosynthesis (*9*). Thus the sensitive C7(−)$^{tox-}$ diphtherial strain may be converted to a lysogenic and toxigenic strain by treatment with the temperate phage βtox+ to yield C7(β)$^{tox+}$. Although strains such as C7(−)$^{tox-}$ are capable of causing mild transient fever and sore throat in human beings, only toxin-producing lysogens can cause true diphtheria (*10*). (ii) Even when the *tox* gene has been introduced into the bacterial host, toxin is not produced in appreciable amounts by growing diphtheria bacilli until the inorganic iron of the culture medium is largely depleted and the bacterial iron content has decreased to a critical amount (*11*). It is still uncertain how the effect of iron is mediated, but recent evidence suggests that all diphtherial cells, whether grown in media containing high or low concentrations of Fe and whether lysogenic or not, may contain a factor that can specifically reduce the expression of the *tox* gene upon the addition of iron (*12*). In any event, while the structural information for toxin biosynthesis is now known to be carried by the phage genome (*13*), its expression is regulated, at least in part, by the bacterial host.

The minimum lethal dose (MLD) of toxin, which for the most active purified preparations is less than 40 nanograms, was originally defined by Ehrlich as the least amount of toxin that, when injected subcutaneously, would kill a 250-gram guinea pig within 4 or 5 days (*6*). Only a few animals are required to determine the MLD of a given preparation to within 20 to 25 percent. Injected into rabbit skin, a few picograms of toxin will produce a visible reaction. The toxicity of certain preparations often declines upon storage without parallel loss in serological activity.

Different animal species vary widely in susceptibility (*14*). Human beings, rabbits, and guinea pigs are highly sensitive whereas rats and mice are relatively resistant. Although most invertebrates appear to be completely resistant, toxin does arrest morphogenesis of certain species of insects (*15*).

Diphtheria toxin can readily be isolated as a purified protein of molecular weight 62,000 to 63,000. The purified toxin may form dimers and aggregates of higher molecular weight without appreciable loss of toxicity (*16*). The conditions for such aggregation are not well understood, but it is obvious that weak interactions are involved since only monomers exist in 0.1 percent sodium dodecyl sulfate. The toxin coagulates rapidly when heated to 55°C at *p*H 6. The amino acid composition of the toxic protein has been determined in several laboratories (*17*) and studies of its amino acid sequence are now in progress (*18, 19*). No special prosthetic group or sugar residues have been found in purified preparations.

The toxin molecule is released from the bacterial cell as a single polypeptide chain (*20*). It has two nonoverlapping cystine bridges, one of which lies about

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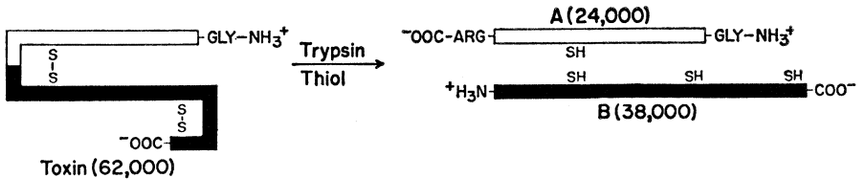

Fig. 1. The diphtheria toxin molecule, before and after reduction followed by mild hydrolysis with trypsin.

40 percent along the chain from the amino-terminal glycine and spans a stretch of about ten amino acids (*18*). The sequence, which contains three arginine residues, presumably represents an exposed loop in the intact molecule, since it is abnormally sensitive to proteolytic attack. Short treatment with proteases of trypsin-like specificity yields two large peptides; an amino-terminal fragment A (24,000 daltons) and a carboxyl-terminal fragment B (38,000 daltons) (*20*) (Fig. 1). When these are still connected by the disulfide bridge, we call the product "nicked toxin." It has been convenient to treat fragments A and B as if they were homogeneous species, even though each is usually obtained as a mixture of closely related molecules. For example, in the case of trypsin, the peptide bond cleaved may be located after any one of the three arginine residues (*19*). Even after reduction and alkylation of the sulfhydryl groups, there is little loss of toxicity; the A and B chains still remain firmly held together by weak interactions at a concentration of $10^{-9}M$ or less. It is probable that much of the A chain is masked in the intact toxin since antibodies against purified A fail to precipitate monomeric toxin and antibodies against intact toxin precipitate purified A little if at all (*21*).

Fragments A and B are most satisfactorily separated by electrophoresis or gel filtration in denaturing solvents such as 6*M* urea or 0.1 percent sodium dodecyl sulfate. Fragment B cannot then be recovered in a native state, for it spontaneously aggregates. Because of its extreme instability, it is often impossible to perform biological experiments on B alone. However, fragment A is easily renatured in a fully enzymically active form. The A fragment is surprisingly stable and can withstand brief exposure to 100°C, quite severe extremes of *p*H, and is relatively resistant to further proteolytic degradation.

If the intact toxin molecule is initially hydrolyzed within the B region, NH_2-terminal fragments containing A are formed. Like fragment A, any of these larger fragments are extremely water soluble and resistant to denaturing conditions. The region near the COOH-terminal, on the other hand, appears to contain many hydrophobic residues so that fragment B and related peptides are easy to denature and difficult or impossible to maintain in solution in the native state. Nearly all toxin preparations used in research or for commercial production of toxoid have already suffered limited proteolytic damage from the action of a serine protease present in the crude culture filtrate and partially purified together with the toxin. In favorable cases, the damage is slight and the preparations consist mainly of intact and nicked toxin.

Effect of Toxin on Protein Synthesis

The breakthrough that eventually led to an understanding of the mode of action of diphtheria toxin was made by Strauss and Hendee (*22*) who showed that the incorporation of amino acids into protein by growing HeLa cells ceased within 2 or 3 hours in the presence of a low concentration of toxin. There was no inhibition in the presence of an equivalent amount of specific antitoxin. The effect on protein synthesis appeared to result from a primary action of the toxin, since other metabolic activities remained normal for several hours after amino acid incorporation had ceased. There was no leakage of potassium or of inorganic phosphate as would be expected if toxin caused damage directly to the cell membrane (*23*).

In a system in vitro derived from mammalian cells, toxin inhibits polypeptide chain elongation, provided that nicotinamide adenine dinucleotide (NAD) is present (*24*), by inactivating the elongation factor, EF-2 (*25*). This factor is required for translocation of polypeptidyl-transfer RNA from the so-called acceptor site to the donor site on the eukaryotic ribosome, a step that is coupled to the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) (*26*). Toxin preparations catalyze EF-2 inactivation according to the following reaction (*27, 28*):

$$NAD^+ + \text{EF-2} \rightleftharpoons \underset{\text{inactive}}{\text{ADPR-EF-2}} + \text{nicotinamide} + H^+$$

where ADPR is adenosine diphosphate ribose (*29*). The *p*H optima are approximately 8.5 and 5.2 for the forward and reverse reactions, respectively. The equilibrium of this reaction at physiological *p*H lies far to the right ($K \sim 10{,}000$). Nevertheless, its reversibility can be demonstrated by subsequently removing the NAD and adding an excess of nicotinamide, at which time there is a renewed burst of amino acid incorporation (*30*). Likewise, the ADPR-EF-2 in extracts from intoxicated cells can be reactivated provided some toxin is added to catalyze the reverse reaction (*28*). In a similar way, the protein synthesizing system has been reactivated in extracts of heart muscle taken from severely intoxicated guinea pigs (*31*). Analyses of organs from intoxicated guinea pigs and rabbits have shown a reduced EF-2 content in tissues damaged by the toxin (*32*). There can thus no longer be any reasonable doubt that inactivation of EF-2 by ADP-ribosylation is the primary biochemical lesion caused by toxin.

No eukaryotic protein other than EF-2 has been found capable of accepting ADPR from NAD in the presence of diphtheria toxin preparations. Thus when crude NAD-free tissue extracts are incubated for a few minutes with ^{32}P-labeled or (^{14}C-adenine)-labeled NAD in the presence of toxin, the label that is precipitated in trichloroacetic acid gives a quantitative measure of its EF-2 content (*33*) provided conditions are chosen that minimize interfering reactions (*34*).

Relatively few animal or plant species are susceptible to the toxic action of diphtheria toxin. Nevertheless, in NAD-containing cell-free extracts of all eukaryotic species tested, toxin preparations catalyze the transfer of ADP-ribose to inactivate EF-2. Those tested include extracts from yeast, wheat, arbacia embryos, arthropods, insects, reptiles, amphibia, birds, and mammals (*35*). Toxin has no effect on polypeptide chain elongation systems derived from prokaryotes or from mitochondria (*36*) in which polypeptide chain translocation is catalyzed not by EF-2, but by an unrelated protein,

EF-G (*26*). It is clear that the ADPR group must be bound to a conserved functional site on the EF-2 molecule that is different from the functional site on the corresponding prokaryotic translocase.

A number of substances inhibit the ADP-ribosylation reaction. These include adenine, adenine nucleotides, reduced NAD (NADH), and nicotinamide (*37*). The last is not only a substrate for the reverse reaction, but competitively inhibits the forward reaction. The most interesting inhibition is that brought about by ribosomes, for, under certain circumstances, this reflects a physiologically important process. During normal protein synthesis EF-2 cycles on and off ribosomes (*38*). Cells generally contain a slightly greater number of EF-2 molecules than of ribosomes and, at any given moment, the majority of these molecules are bound to ribosomes (*33*). This bound material is not a substrate for toxin and is not inactivated in vivo or in vitro until it dissociates (*8, 39, 40*). In vitro, the following chain of events has been deduced (*41*). First, EF-2 binds to GTP to form EF-2 · GTP. This interacts with a ribosome, with hydrolysis of the GTP, to form EF-2 · GDP · ribosome which, after translocation, dissociates to liberate EF-2 · GDP. Although EF-2, EF-2 · GTP, and EF-2 · GDP are substrates of toxin, EF-2 · GDP · ribosome is not. ADPR-EF-2 appears to interact normally with GTP (*41, 42*) and according to Chuang and Weissbach (*41*) with ribosomes also.

The situation in vitro is often complicated by additional binding of EF-2 to ribosomes without the assistance of GTP, presumably at nonfunctional sites. Such nonfunctional binding, which at low salt concentrations may exceed the functional binding, also results in the protection of EF-2 from toxin. Nonfunctionally bound EF-2 can, in fact, be liberated from the ribosomes by GTP or GDP and thereby become susceptible to inactivation by toxin (*39*). Similarly, in the absence of guanine nucleotides, EF-2 may bind to prokaryotic ribosomes, isolated ribosomal subunits, ribosomal RNA, and ribonucleotide homopolymers, and even DNA (*43*). When bound in this way, the EF-2 is protected from toxin. That these effects are artifacts of the system in vitro seems more likely than that they reflect biologically important processes.

Although the primary effect of toxin on living cells is inactivation of EF-2 by the ADP-ribosylation reaction, there is ordinarily no correlation between the enzymic activity of toxin preparations and their toxicity for susceptible animals. Collier and Cole (*44*) were the first to note that toxin could be "activated" by reduction of its disulfide bonds and that after reduction a substantial fraction of its enzymic activity could be found associated with a polypeptide fraction of molecular weight considerably lower than intact toxin. We now know that the NAD : EF-2 ADPR-transferase active site is located on fragment A, but is masked, since intact toxin is enzymically inactive (*45*). Activation must be associated with a major conformational change, since *both* disulfide reduction and cleavage of a peptide bond in the loop formed by the disulfide bridge joining the two fragments is required.

No enzymic activity of any kind is known for the B moiety. Its function appears to be concerned with binding toxin to specific sites on the sensitive membrane and facilitating in some way the transport of fragment A to the cytoplasm.

Table 1. Some properties of diphtheria toxin and related proteins.

Protein	Molecular weight	Toxicity (MLD/μg)	Enzymic activity*	Blocking activity†
Toxin	62,000	25–30	100	
Toxoid	62,000	Nontoxic	None	None
CRM_{30}	30,000	Nontoxic	100	Not done
CRM_{45}	45,000	0	100	None
CRM_{176}	62,000	0.05–0.1	8–10	
CRM_{197}	62,000	0	None	100
CRM_{228}	62,000	0	None	10–15
Hybrid A_{45}-B_{228}	62,000	4–5	100	15
Fragment A	24,000	0	100	None

* NAD : EF-2 ADPR-transferase activity per mole after activation by trypsin and reduction relative to fragment A taken as 100. † Ability to inhibit competitively the action of toxin on HeLa cells. The blocking activity of CRM_{197} is taken as 100.

Properties of Altered Tox Gene Products

The *tox* gene, although present in the phage genome, is expressed independently of other phage genes (*46*). Expression of *tox* is not essential for phage growth and the gene may be modified without even affecting the kinetics of phage replication (*47*). When replicating phage is treated with a mutagen such as nitrosoguanidine, a relatively high proportion of the survivors (up to 2 to 3 percent) carry modifications in their *tox* gene which, when expressed by the diphtherial host, result in production of nontoxic proteins of reduced toxicity that cross-react with diphtheria antitoxin. These altered proteins, or CRM's, have been particularly useful in studies on the interaction of diphtheria toxin with the sensitive cell membrane (*48*).

In Table 1, we summarize the properties of five purified CRM's of altered toxicity and compare them with those of intact toxin, Formol toxoid, and isolated fragment A. The mutant proteins fall into two classes. (i) Those CRM's that are presumed to result from missense mutations leading to one or more amino acid substitutions in fragment A. The CRM's of class (i) may show no enzymic activity at all (CRM_{197}, CRM_{228}) or greatly reduced activity (CRM_{176}). These three proteins appear to be immunologically indistinguishable from toxin. (ii) There are CRM's resulting from mutations leading to an altered fragment B in which the process involving entry of fragment A into the sensitive cell is impaired. These CRM's may or may not contain a normal fragment A. For example, CRM_{30} and CRM_{45} are apparently prematurely terminated proteins (30,000 and 45,000 daltons, respectively) each containing a single disulfide bond. Upon reduction of the trypsin-nicked proteins, 24,000-dalton fragments may be isolated that cannot be distinguished from fragment A derived from intact toxin. In CRM_{30} and CRM_{45}, the active sites must be at least partly exposed, since both show considerable NAD : EF-2 ADPR-transferase activity even without nicking or reduction. Finally, CRM_{228} appears to represent the product of a double mutation in the *tox* gene affecting the activity of both fragments. Not only is A_{228} enzymically inactive, but B_{228} may in addition have an amino acid substitution which results in increased sensitivity to tryptic

digestion and an impaired ability to interact with the sensitive cell membrane.

When nicked and reduced CRM's from each of the above classes are mixed and allowed to reoxidize, hybrid molecules may be formed (*49*). For example, when the two nontoxic proteins, CRM_{45} and CRM_{197}, are nicked with trypsin in the presence of a thiol, mixed, and the disulfides allowed to reform during dialysis, among the products a fully toxic hybrid, A_{45}-B_{197}, is formed that cannot be distinguished from nicked toxin. The hybrid A_{45}-B_{176} also appears to be identical with the nicked wild type molecule. Both CRM_{197} and CRM_{176}, therefore, must contain B fragments that, functionally at least, are normal. A_{45}-B_{228}, however, is only about 15 percent as toxic as native toxin.

The ease with which toxic hybrids such as A_{45}-B_{197} may be formed has provided a simple method for introducing a label such as iodine-125 into either fragment A or fragment B. Such labeled reconstituted molecules should prove useful in further studies on the interaction of toxin with cells.

Interaction of Toxin with the Cell Membrane

There are few well-documented examples in which it has been demonstrated that intact protein molecules cross cell membranes and reach the cytosol while still functional. Protein, in soluble or in particulate form, may be taken up through a pinocytotic or phagocytic process by a large variety of cell types. But in most instances, proteolytic degradation takes place within the endocytotic vesicles and only free amino acids or small peptides actually traverse the membrane. In the case of toxin, it is clear that a few molecules of toxic protein, or at least of its A fragment, must reach the cytoplasm in an active form in order to inactivate EF-2. We have attempted to study the entry process by measuring amino acid incorporation by cultured HeLa cells after they have been exposed to toxin or cross-reacting proteins (*48, 50, 51*). Curve A, Fig. 2, shows that the rate of [^{14}C]leucine incorporation by normal HeLa cells in culture at 37°C doubles in about 22 hours. When diphtheria toxin is added, the rate of incorporation increases normally for a short period and then declines according to a first order process. As the concentration of toxin is increased (curves B and C), the rate of fall increases and the lag period shortens until, as the toxin concentration nears $10^{-6}M$ (curve D), the rate of inactivation of the system approaches a maximum and the lag period a minimum. Inactivation proceeds at half-maximal rate when the toxin concentration is about $10^{-8}M$.

These results may be interpreted as follows. The HeLa cell membrane contains a limited number of entry sites per cell that specifically interact with groupings on the B fragment. These sites are not present on the cell membranes of resistant species such as mouse L cells. We do not know how many entry sites there are per HeLa cell, but studies with ^{125}I-labeled toxin suggest an upper limit of 10,000 to 20,000. It is still not known whether nicking and reduction of intact toxin takes place at or in the membrane or whether the entire molecule reaches the cytoplasm before activation and rapid breakdown of its labile B fragment takes place. In any event, in the presence of sufficient toxin to saturate the entry sites, fragment A rapidly reaches a maximum steady-state level in the cytosol. However, an effect on protein synthesis is not seen immediately, but only after EF-2 becomes the rate-limiting factor in protein synthesis. There is, therefore, a lag before the rate of protein synthesis starts to fall. At lower toxin concentrations, when only a fraction of the entry sites are occupied at any given moment, the steady-state level of fragment A is lower and the lag before EF-2 becomes limiting is prolonged. It has been calculated from studies in vitro that, at the intracellular NAD concentration of HeLa cells (about 0.5 mM) a steady-state concentration of only a single molecule of fragment A per cell may be enough to inactivate all the EF-2 within a day.

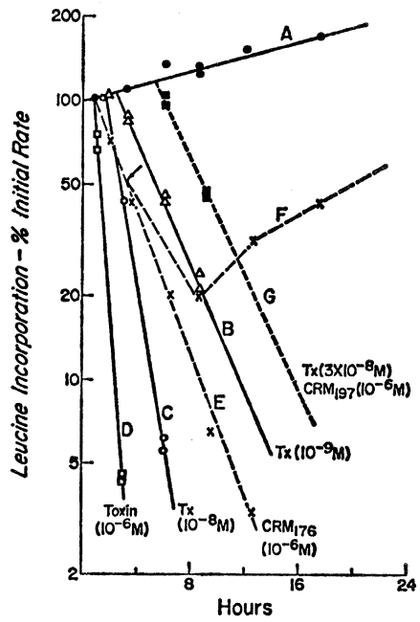

Fig. 2. Effect of diphtheria toxin and related proteins on rate of protein synthesis. The HeLa cells were collected during exponential growth and resuspended in Eagle's medium containing 2 percent calf serum and distributed in spinner flasks containing toxin or related proteins, or both. At intervals, 2-milliliter duplicate samples were removed to roller tubes, each containing 0.4 microcurie of [^{14}C]-leucine. After rotation at 5 rev/min for 1 hour at 37°C, the cells were collected on Millipore filters and counted. Curve A, control; curve B, $10^{-9}M$ toxin; curve C, $10^{-8}M$ toxin; curve D, $10^{-6}M$ toxin; curves E and F, $10^{-6}M$ CRM_{176}; in F, cells were removed at 3 hours (arrow) and resuspended in fresh medium containing $10^{-6}M$ CRM_{197} to inhibit entry of traces of CRM_{176} still present; curve G, $3 \times 10^{-8}M$ toxin plus $10^{-6}M$ CRM_{197}.

We have shown that in CRM_{176}, fragment B is normal, but A_{176} has only 8 to 10 percent the specific enzymic activity of wild type A. Obviously, a higher cytoplasmic concentration of A_{176} is required in order to produce the same rate of inactivation of EF-2. Figure 2, curve E, shows that at $10^{-6}M$ CRM_{176}, a concentration that saturates the HeLa cell entry sites, the rate of inactivation was only equivalent to that expected for less than $5 \times 10^{-9}M$ toxin. Moreover, if the CRM_{176}-treated cells were removed within 3 hours and resuspended in fresh toxin-free medium containing antitoxin, they were not killed even though (curve F) their rate of protein synthesis had fallen to 20 to 25 percent the initial rate. Although the rate of protein synthesis continued to be low, most of the cells remained viable, since 24 hours later their numbers had doubled. The studies with CRM_{176} suggest that A_{176} is continuously taken up by the cells where it is slowly degraded upon reaching the cytoplasm. By analogy, it seems reasonable to suppose that the same is true for the fragment A of toxin.

Nontoxic mutant proteins, such as CRM_{197}, that contain a normal B fragment, can compete with toxin for entry into the sensitive cell. In studies on the inhibition of protein synthesis in HeLa cells by mixtures of toxin and CRM_{197} in which entry sites are kept saturated by maintaining their total concentration at $10^{-6}M$, the slopes of

the inactivation curves are those expected for free competition. Curve G of Fig. 2 shows that in the presence of $10^{-6}M$ CRM_{197}, $3 \times 10^{-8}M$ toxin exhibits the prolonged lag period and inhibition curve that would be given by about $3 \times 10^{-10}M$ toxin alone. Interference with the entry process is clearly what is involved, because CRM_{197} in vitro, even in 100 : 1 excess, has no effect on the enzymic activity of fragment A from toxin. From careful quantitative studies of the competition for entry, Ittelson and Gill (*50*) calculated an apparent binding constant of 10^8 liters per mole for CRM_{197} to sites on the HeLa membrane. At $10^{-8}M$, toxin alone inactivates protein synthesis in HeLa cells at about half the maximal rate, supporting the notion that 10^8 liters per mole may also be the binding constant between toxin itself and the HeLa membrane sites. Significant competition can only be expected when there is enough CRM_{197} to occupy the vast majority of entry sites in a population of cells. For this reason, demonstration of protection of whole animals by CRM_{197} against the lethal action of toxin has not been feasible. Nevertheless, it has been possible to demonstrate the blocking of local reactions in vivo by CRM_{197}. Skin reactions in rabbits to picogram amounts of toxin are almost completely inhibited when the toxin is injected together with a 10^5-fold excess of CRM_{197} (*50, 51*).

Is Specific Entry a General Phenomenon?

Some recent studies show an interesting parallelism between diphtheria toxin and two highly toxic seed proteins, ricin from the castor bean (*Ricinus communis*) and abrin from *Abrus precatorius*. Although these two proteins are found in the seeds of taxonomically distant plants, they resemble one another structurally and in their mode of action. Both have molecular weights of about 65,000 and upon treatment with thiols yield two fragments, A and B, neither of which is toxic by itself (*52*). The fragment A from each protein blocks polypeptide chain elongation in extracts from eukaryotic cells by a mechanism that is different from diphtheria toxin. Fragment B from abrin or ricin binds to sensitive cell membranes and are required for entry of A. An especially interesting observation is that both abrin and ricin B fragments bind saccharides containing a terminal nonreducing galactose. Moreover, the toxicity of ricin for mouse lymphocytes (*53*) and for HeLa cells (*54*) can be inhibited by lactose or by galactose. It seems likely, therefore, that ricin and abrin interact with a specific membrane site containing a terminal galactose.

The fact that three toxic proteins of widely different origin are now known to be composed of two dissimilar fragments, one of which in each case is required for attachment to the sensitive cell membrane and for penetration of the other fragment, which has a lethal enzymic activity, suggests that the mechanism may be a general one and that other toxic proteins may reach the cytoplasm by analogous processes.

It is clear that interaction of diphtheria toxin with the cell membrane and entry of its A fragment into the cytoplasm is a highly specific process—in the natural disease in man, in laboratory animals injected with toxin, and in cell cultures in vitro to which toxin is added. If an occasional molecule of toxin were able to cross the cell membrane by some other nonspecific mechanism, it would not be detected. It has long been known that certain mammalian species, notably rats and mice, are extremely refractory to the action of diphtheria toxin as are cell lines derived from these species. Thus 10,000 to 100,000 times as much toxin is required to inhibit protein synthesis in cultured mouse cells as in cultured cells of human origin (*55*). At high toxin concentrations, it is possible that a few molecules of fragment A escape destruction in endocytotic vesicles and reach the cytoplasm by a nonspecific mechanism. Mouse L cells can be rendered more "permeable" and their sensitivity to toxin somewhat increased in the presence of nonspecific stimulators of pinocytosis such as polyornithine (*56*) and diethylaminoethyl cellulose (DEAE) dextran (*57*). These basic polymers increase sensitivity when they are present in concentrations that are close to their toxic concentrations; the increased sensitivity may therefore be related to membrane damage. In large amounts, even isolated fragment A becomes toxic for L cells (*57*). By what is presumed to be a nonspecific mechanism, it has proved possible to kill mice with large doses of CRM_{45} (> 100 micrograms per 20-gram mouse) (*58*). These observations probably have little bearing on the natural disease process.

Concluding Remarks

Although fatal infections in animals with toxigenic strains of *C. diphtheriae* can be produced experimentally, the occurrence in nature of diphtheria as a disease seems to be restricted to man. How did the *tox* gene originate and how was its perpetuation in the phage genome ensured? The gene is not essential for any known phage function and may be modified or eliminated without any effect that has been noticed on β-phage replication. Nor does lysogenic conversion of a diphtherial strain to the toxigenic condition confer any obvious advantage to the bacteria under laboratory conditions. How then has the *tox* gene, whose product is an enzyme specific for a eukaryotic substrate, managed to become established and survive in a prokaryotic host? As we have argued elsewhere (*13, 35*), the ability to produce toxin does have survival value for both β-phage and for its bacterial host in a human population that has not been subjected to artificial immunization. Clinical cases provide a source of large numbers of toxigenic organisms which can initiate spread through droplet infection to other susceptible individuals directly or via healthy immune carriers or a series of healthy carriers. In a largely immune population, the advantage of toxigenicity is almost eliminated and bacterial spread from person to person will progressively diminish. Indeed the disappearance of toxigenic diphtheria bacilli as a common member of the normal bacterial flora of the human throat and nasopharynx has been a striking consequence of the immunization program.

It seems likely that observations on the pathogenesis of diphtheria can be generalized to help explain the mechanism of the infectious process in other bacterial diseases in which extracellular toxins play an important role. Thus, the production of scarlet fever toxin is linked to lysogeny in certain strains of hemolytic streptococci (*59*) and the same has been reported for certain lysogenic toxin-producing staphylococci (*60*). It has recently been shown that nontoxigenic *Clostridium botulinum*, type C, may be converted to toxigenesis by lysogenization with a particular phage (*61*). Like diphtheria toxin, cholera, scarlet fever, tetanus, and many other bacterial toxins involved in bacterial infections act on systems that are present in higher animals and that have no obvious counterpart among prokaryotes.

Did the genes for these toxins evolve by chance from other prokaryotic genes, specifying proteins that were of value to bacteria or their viruses, which then with slight modification lost their previous function and specified toxins instead? This seems to us very unlikely. The alternative, which by default remains the more attractive, is that many of the toxins produced by pathogenic bacteria are the products of descendents of eukaryotic genes that were randomly incorporated into phage genomes during chance association with certain eukaryotic cells.

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Parameters of Technological Growth

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In recent studies of the limits to population growth in a finite world, the assumptions concerning the future availability of new technologies are crucial. The predictive scenarios developed by Meadows *et al.* (*1*) and Forrester (*2*) assumed only a few discrete contributions of technology to ease the world stresses produced by growth. Meadows states, "The basic behavior mode of the world system is exponential growth of population and capital, followed by collapse . . . . When we introduce technological developments that successfully lift some restraint to growth or avoid some collapse, the system simply grows to another limit, temporarily surpasses it, and falls back." On the other hand, Boyd (*3*) has shown (Fig. 1) that a "technological optimist" approach of a continuous flow of technological change completely alters the conclusions from Forrester's world model simulation, and avoids the overshoot, collapse, and human tragedy implications of Meadows *et al.* (*1*) and Forrester (*2*).

It is, of course, an obvious outcome of elementary mathematics that an exponential will overtake any fixed quantity or some fixed multiple of it. In

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