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MOLECULAR PHARMACOLOGY OF BOTULINUM TOXIN AND TETANUS TOXIN

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INTRODUCTION

Botulinum toxin is a term that has been used to describe eight different substances designated types A, B, C_1, C_2, D, E, F, and G. For many years it was assumed that these eight substances acted at the neuromuscular junction to block acetylcholine release. It is now known that this assumption is not entirely correct. Seven of the substances do act at cholinergic junctions (types A, B, C_1, D, E, F, and G), and they are properly referred to as botulinum neurotoxins. The eighth substance (type C_2) is unique in its structure and pharmacological actions. It is called the botulinum binary toxin.

Tetanus toxin is a term that has been used to describe a single substance that acts mainly in the central nervous system to block inhibitory transmission. There are many similarities between tetanus toxin and botulinum neurotoxin, including a common origin, a closely related structure, and perhaps the same subcellular mechanism of action. The similarities between tetanus toxin and the botulinum binary toxin are less clear.

The purpose of the present review is to discuss the proposed mechanism(s) of action of the three groups of clostridial toxins. Recent findings suggest that research is moving quickly toward a full determination of the cellular and subcellular actions of these substances. This is an encouraging turn of events, because the clostridial toxins are generally regarded as the most poisonous substances known to mankind.

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MECHANISM OF ACTION

In 1980, the author proposed a three-step model to account for the neuromuscular blocking actions of botulinum neurotoxin (1). This model has been expanded in recent years (2), and it can be summarized in the following way. The neurotoxin binds to receptors on the external surface of the plasma membrane. The binding step is essential for development of paralysis, but it does not produce any adverse effect on nerve cell function.

The binding step is followed by an internalization step, which is itself a sequence of events. The toxin enters the cell by the process of receptor-mediated endocytosis. This allows the toxin to cross the plasma membrane but leaves it within an endocytic vesicle. The toxin enters the cytoplasm by a mechanism that has not been fully clarified but that may involve formation of channels.

The final step produces nerve cell dysfunction. The toxin acts in the cytoplasm or at the internal face of the plasma membrane to alter a molecule involved in exocytosis. The precise nature of this poisoning event has not been established. However, to the extent that botulinum neurotoxin mimics the intracellular actions of other potent protein toxins, one can reasonably propose a catalytic mechanism. Botulinum neurotoxin may block transmitter release by enzymatically modifying a subtrate involved in excitation-secretion coupling.

The model for toxin action can be closely linked to the structure of the toxin molecule. Botulinum neurotoxin is produced by an anaerobic organism, *Clostridium botulinum* (3). The molecule is synthesized as a single chain polypeptide having a molecular weight of $M_r \sim 150{,}000$ (Figure 1). This molecule undergoes proteolytic cleavage to yield a dichain polypeptide composed of a heavy chain ($M_r \sim 100{,}000$) and a light chain ($M_r \sim 50{,}000$). The single chain polypeptide is only weakly active, but the dichain molecule is fully active (4).

When the molecule is exposed to additional proteolytic cleavage with papain, the heavy chain is split into two components roughly equivalent in molecular weight. The free portion of the heavy chain is the carboxyterminus, and the bound portion covalently linked to the light chain is the aminoterminus. Reduction of the disulfide bond or proteolytic cleavage of the heavy chain produces loss of toxicity (4).

The structure of the molecule can be related to the proposed model for toxin action. It is envisioned that the carboxyterminus of the heavy chain possesses a binding domain, the aminoterminus of the heavy chain possesses a channel-forming domain, and the light chain possesses a poisoning domain. None of the domains alone is neurotoxic; only the holotoxin can block transmitter release.

Tetanus toxin is also produced by an anaerobic organism, *Clostridium tetani* (5–7). The synthesis and proteolytic processing of tetanus toxin is virtually identical to that of botulinum neurotoxin (8). Thus, the molecule is synthesized

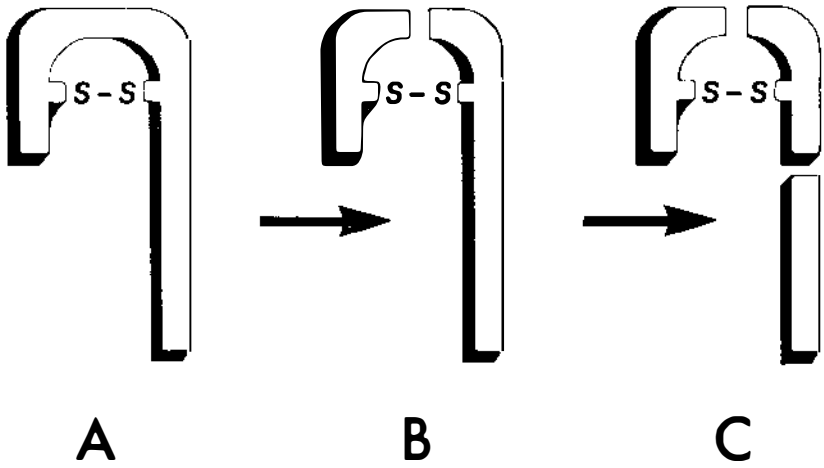

Figure 1 Botulinum neurotoxin is synthesized as a relatively inactive, single chain polypeptide that has a molecular weight of approximately 150,000 daltons (*A*). The single chain molecule possesses at least one intrachain disulfide bond. When exposed to trypsin or trypsin-like enzymes, the neurotoxin is nicked to yield an active dichain molecule with an interchain disulfide bond (*B*). The two chains are referred to as light (ca 50,000 daltons) and heavy (ca 100,000 daltons). When exposed to additional proteolytic cleavage with papain, the heavy chain is cleaved again (*C*). The result is two nontoxic polypeptides. The free portion of the heavy chain is the carboxyterminus, and it has a molecular weight of approximately 50,000 daltons. The bound portion of the heavy chain is the aminoterminus, and it remains covalently linked to the light chain.

The synthesis and proteolytic processing of tetanus toxin is identical to that of botulinum neurotoxin. The synthesis of the botulinum binary toxin is different. The binary toxin is synthesized as two polypeptide chains that have no disulfide bonds. There is a heavy chain (ca 100,000) and a light chain (ca 50,000), which mimics the status of botulinum neurotoxin and tetanus toxin. In addition, there is proteolytic processing. When the heavy chain is exposed to trypsin, it is converted from an inactive to an active form.

as an inactive, single chain polypeptide ($M_r \sim 150{,}000$) that is converted to an active, dichain molecule ($M_r \sim 100{,}000$ and 50,000). Tetanus toxin is known for its effects on inhibitory interneurons, but it too can block neuromuscular transmission (9–12). In fact, the general features of its actions are very similar to those of botulinum neurotoxin (13–15). The data suggest that the molecule proceeds through a sequence of steps, including an extracellular binding step, a membrane penetration step, and an intracellular poisoning step (14). The data also suggest that the tetanus toxin molecule has three functional domains. As with botulinum neurotoxin, the carboxyterminus of the heavy chain mediates binding (16, 17), the aminoterminus of the heavy chain mediates internalization (18, 19), and the light chain is assumed to be responsible for poisoning nerve cell function.

The botulinum binary toxin is produced by *Clostridium botulinum* (3). There are some organisms that simultaneously produce both botulinum neurotoxin

and the binary toxin. The structure of the latter is somewhat different from that of the botulinum and tetanus neurotoxins. It is synthesized as two separate polypeptide chains ($M_r \sim 100{,}000$ and 50,000) that have no interchain covalent bonds (20, 21). Neither chain alone is very active, but the combination of chains is extremely toxic (20–22). Hence, it has been called a binary toxin (22).

The details of the mechanism of action of the binary toxin are in some respects less well understood and in other respects better understood than the details of neurotoxin action. The heavy chain of the binary toxin plays a key role in cell surface binding (22, 23); the light chain is an enzyme whose catalytic effects have been partially characterized (S. Leppla, personal communication; 24). The sequence of events that brings the toxin from an extracellular binding site to a supposed intracellular poisoning site has not been described.

When the general features of botulinum neurotoxin action were first proposed, the model was met by two sharply contrasting responses. Investigators in neuromuscular pharmacology tended to view it with skepticism, whereas investigators in microbiology and cell biology readily adopted it, in some cases viewing it as "intuitively obvious." There is an easily identifiable explanation for these dissimilar responses. The proposed model for botulinum neurotoxin action is seemingly complex and certainly different from models used to explain the mechanism of action of other neuromuscular blocking agents. However, the proposed action of botulinum neurotoxin shares many features with the known actions of potent protein toxins commonly studied by microbiologists and cell biologists (25). Examples of the latter include diphtheria toxin, which is microbial in origin, and abrin and ricin, which are plant lectins.

The trend during the past five years has been for an increasing number of pharmacologists to accept the model for botulinum neurotoxin action. This trend has been hastened by two discoveries: (*a*) the finding that the model is generalizable and appears to apply to tetanus toxin (14), and (*b*) the finding that the botulinum binary toxin possesses enzymatic activity similar to that of other potent protein toxins (S. Leppla, personal communication; 24). The current state of affairs is that most investigators now agree that the proposed model has merit and is worthy of experimental testing.

EVIDENCE TO SUPPORT THE MODEL

Binding Step

In 1949, Burgen and his associates published a manuscript that is regarded as one of the most important contributions to the botulinum neurotoxin literature (26). Their report introduced the isolated neuromuscular junction (phrenic nerve-hemidiaphragm) as a preparation on which to analyze toxin action. The phrenic nerve-hemidiaphragm preparation continues to be the mainstay of research on botulinum neurotoxin. In the recent past, Habermann and his

associates have shown that tetanus toxin also blocks transmission at the phrenic nerve-hemidiaphragm (13). This was a particularly noteworthy finding, because it showed that a single tissue could be used to compare the actions of the two most potent neurotoxins.

Among the data reported in the paper by Burgen et al was the discovery that botulinum neurotoxin "fixed" to tissues very rapidly. Neuromuscular preparations that were exposed to toxin for short periods of time and then washed extensively continued to become paralyzed. Although the data were not discussed as such, the discovery of rapid fixation was the first indication that toxin action could be divided into at least two phases: an initial binding step and a later paralytic step. The fact that binding is rapid and that it is a separate event from paralysis has been confirmed by virtually every worker in the field.

Ideally, these suggestive findings on binding should be complemented by four specific types of research: (*a*) histological studies to demonstrate binding at the cholinergic nerve terminal, (*b*) radioligand studies to show selective and saturable binding at nerve endings, (*c*) peptide chemistry studies to show that fractionation of the toxin molecule will yield a nontoxic component with binding activity, and (*d*) isolation studies to extract an authentic receptor from nerve tissue. Varying degrees of success have been achieved in these four areas.

Hirokawa & Kitamura have shown binding of botulinum neurotoxin to the mouse phrenic nerve-hemidiaphragm (27). Using iodine-labeled toxin and light microscopic resolution, they obtained autoradiograms in which binding of neurotoxin was coincident with areas of acetylcholinesterase staining, which along with other evidence suggested localization at endplates. Dolly et al similarly showed binding of iodine-labeled toxin at the neuromuscular junction, but the work was more refined (28). They used a highly purified preparation of toxin, they exposed tissues to a much smaller amount of toxin (2.5×10^3 mouse LD_{50} versus 1.3×10^6 LD_{50}), and they provided evidence for selectivity and saturability of binding. Subsequent studies by the same group have localized the binding of iodine-labeled neurotoxin at the electron microscopic level (29, 30). Binding sites were found on nerve terminals of motoneurons, where there were approximately 150 to 500 binding sites per micrometer of membrane.

No one has reported radioligand binding studies to characterize the interaction between botulinum neurotoxin and its receptor in peripheral nerves. Because of the presumably small number or receptors in peripheral tissue, such work may require a toxin preparation that is labeled to an unusually high specific activity. However, ligand binding studies have been reported for synaptosome preparations from brain. Early studies provided data that were difficult to accept, owing to the questionable purity of the ligand and to the relatively high concentrations added to membrane preparations, but later work has overcome these difficulties. The most recent studies indicate that the

binding of botulinum neurotoxin to brain synaptosomes involves two classes of receptors. A high affinity site has an apparent K_d of 1 to 6×10^{-10} M, and a low affinity site has an apparent K_d of 5×10^{-8} M (31, 32).

There is evidence to suggest that the heavy chain of the neurotoxin molecule is responsible for binding. When added to neuromuscular preparations, the heavy chain does not paralyze transmission but it does protect tissues from the neuromuscular blocking actions of the intact molecule (L. L. Simpson and B. R. DasGupta, unpublished data). The proposed explanation for this finding is that the isolated heavy chain binds to and occludes receptors, thus preventing the intact molecule from binding. Evidence obtained from histological studies on the neuromuscular junction confirms that the heavy chain mediates binding (29, 30). Experiments on synaptosomes (28, 31–36) have shown that intact toxin binds to plasma membranes; the heavy chain competes for binding sites with the intact toxin; and monoclonal antibody directed against epitopes in the heavy chain prevents binding of the intact molecule. To date, no one has reported use of the labeled heavy chain as a ligand to characterize binding sites.

The receptor for botulinum neurotoxin has not been identified. Simpson & Rapport have shown that botulinum neurotoxin interacts with gangliosides, and this remains the only demonstration of a meaningful interactions between the toxin and a naturally occurring constituent of nerve tissue (37). The work has been reproduced by Kitamura et al, who reported that ganglioside G_{T1b} is most effective at inactivating the toxin (38). The ability of this species of ganglioside to produce inactivation is not equivalent for all serotypes. Of the six that have been tested, types A, B, E, and F were markedly inactivated, but types C and D were only mildly inactivated (39).

Various interpretations can be assigned to the finding that botulinum neurotoxin interacts with gangliosides; they include the following: (*a*) Gangliosides are merely acceptors and have no real role in the neuroparalytic process; (*b*) gangliosides are true receptors that mediate the first step in the neuroparalytic process; (*c*) a sialic acid-containing molecule other than ganglioside, such as a sialoglycoprotein, is the true receptor, and gangliosides partially mimic the behavior of the true receptor; or (*d*) gangliosides or sialoglycoproteins are involved in something other than the binding step, such as clustering and/or internalization.

Relatively little work is being done to further characterize the interaction between botulinum neurotoxin and gangliosides. Indeed, there is no research program that is entirely devoted to isolating and identifying the toxin receptor. In part, this absence of effort is related to the proposed mechanism of toxin action. It is widely acknowledged that poisoning is due to an intracellular action rather than to membrane binding; therefore, research interest has shifted to the cell interior. However, this shift in focus does not detract from the data that show that binding is the first step in the overall paralytic process, that binding

does not have observable effects on cell function, and that binding is mediated by the heavy chain of the toxin molecule.

The literature on the binding of tetanus toxin has evolved quite differently from that on botulinum neurotoxin. Because the former substance is known for its effects on the central nervous system, it has received comparatively little attention as a neuromuscular blocking agent. There are no histological studies that demonstrate binding of tetanus toxin to nerve terminals of motor fibers, and there are few studies that characterize the pharmacological aspects of this binding. Schmitt et al have shown that tetanus toxin binds essentially irreversibly to nerve terminals before there is onset of neuromuscular blockade (14). Simpson has shown that the 50,000 dalton carboxyterminus of the heavy chain appears to mediate binding (40). This fragment will bind to and occlude receptors, thus affording protection against the neuroparalytic effects of the parent molecule.

The literature dealing with the binding of tetanus toxin to brain tissue is more extensive, and most of the reports predate those on neuromuscular transmission. The entire field of study began with the work of Wassermann & Takaki, who provided evidence that brain tissue has receptors for the toxin (41). They found that the residual toxicity in supernatants of solutions incubated with homogenates of brain was diminished, apparently because the toxin was absorbed by the brain. In the intervening years investigators have done radioligand studies to characterize the toxin receptor in brain synaptosomes. They have fractionated the toxin molecule to identify the binding component and have made a number of attempts to clarify the nature of the receptor.

Two different groups have reported that there is a high affinity tetanus toxin receptor in rat and bovine brain membranes. The reported dissociation constants were in reasonably good agreement and both were in the nanomolar range (17, 42). The binding of radiolabeled toxin to brain membranes was antagonized by unlabeled toxin, and radioligand that was already bound could be displaced by unlabeled material. A fragment representing the 50,000 dalton carboxyterminus of the heavy chain antagonized the binding of free toxin and displaced the binding of pre-bound toxin (16, 17).

The pharmacological studies on the neuromuscular junction (see above) and the ligand binding studies on brain membranes gave qualitatively similar results, but the quantitative aspects differed significantly. The apparent dissociation constant for tetanus toxin binding to the phrenic nerve-hemidiaphragm was about 100-fold higher than that reported for brain. This may be a reflection of the fact that a viable neuromuscular junction, unlike brain membranes, has permeability barriers and may also have mechanisms for sequestering toxin. It should be noted that there is a similar quantitative disparity between affinity constants for botulinum neurotoxin binding to brain membranes and neuromuscular junctions, and the underlying problems may be the

same. The data suggest that the affinity constants obtained with brain membranes are authentic, but those obtained with functioning neuromuscular junctions are complicated by factors inherent in using a viable tissue. It is hoped that a paradigm will ultimately be developed that gives equivalent findings on inert membranes and on functioning tissues.

The receptor for tetanus toxin has not been unequivocally identified, but van Heyningen deserves credit for having isolated a component from nerve tissue that has receptor-like qualities. Using the original Wassermann-Takaki observations as the basis for his studies, van Heyningen proceeded to isolate the material(s) from brain responsible for adsorbing toxin. A series of reports (43–46) culminated in the discovery that certain classes of gangliosides could bind to and inactivate tetanus toxin. Later work has in some respects tended to support the idea that gangliosides could be tetanus toxin receptors (e.g. 47–51). For example, the 50,000 dalton carboxyterminus of the molecule, which is thought to mediate binding, has a high affinity for gangliosides, and gangliosides compete with brain membranes for binding of toxin (16, 17). Proteolytic agents such as trypsin and chymotrypsin and protein modifying agents such as iodoacetamide, ethoxyformic anhydride, *N*-ethylmaleimide, and *N*-bromosuccinimide do not abolish toxin binding sites, and these data support the belief that the toxin receptor is a lipid or complex lipid (i.e. ganglioside) as opposed to a protein (42). Finally, gangliosides can be inserted into non-neural membranes such as those of human erythrocytes, and the modified membranes bind tetanus toxin in a way that mimics nerve membranes (52, 53).

In spite of this suggestive evidence, compelling data that prove gangliosides are receptors have not been published. The various interpretations that were given to the interaction between botulinum neurotoxin and gangliosides are equally valid when discussing tetanus toxin and gangliosides. For both toxins there is evidence for an initial, nontoxic binding step, and there is evidence that the 50,000 dalton carboxyterminus of the heavy chain governs binding. Additional work is needed to isolate and definitively characterize the receptor.

Internalization Step

Expansion of the two-step model for botulinum neurotoxin action (e.g. binding step, poisoning step) to a three-step model (e.g. binding step, translocation step, poisoning step) was based on a specific observation. When phrenic nerve hemidiaphragms were incubated with toxin at 4° C and binding was allowed to go to completion, the membrane-bound toxin remained accessible to neutralizing antibody. However, when tissues were warmed and there was a short period of nerve stimulation, the toxin rapidly disappeared from accessibility to neutralizing antibody. This occurred long before onset of paralysis. These findings prompted the author to propose that a membrane translocation step was interposed between binding and poisoning (1, 2).

There are a variety of substances that cross membranes by the process of receptor-mediated endocytosis, and the process itself has been relatively well characterized (54–57). Those studies dealing with endocytosis of Semliki Forest virus and with diphtheria toxin may be most instructive in terms of providing an analogy. For both the virus and the toxin, there is an initial binding step at the plasma membrane. The receptor-bound material is internalized by structures variously referred to as endocytic vesicles or receptosomes. In most cases, these vesicles migrate toward lysosomes. A proton pump progressively lowers the vesicular pH to a value of 4.0 or lower. This fall in pH triggers a conformational change in the virus/toxin, the result of which is injection of material through the membrane and into the cytoplasm.

Three pieces of evidence support the idea that a pH-dependent step underlies the penetration of endosome membranes (58–62). First, drugs that raise the pH of endosomes and lysosomes (e.g. lysosomotropic agents, such as chloroquine) greatly diminish the activity of Semliki Forest virus and diphtheria toxin. Second, a pH gradient that is artificially created across the plasma membrane can cause direct injection of virus/toxin through the plasma membrane. As expected, lysosomotropic agents do not antagonize virus/toxin that penetrates directly into the cytoplasm and bypasses the endosome-lysosome pathway. Third, and of particularly relevance to diphtheria toxin, experiments on lipid bilayers show that a pH gradient causes the toxin to insert into the artificial membrane and form large channels (19, 63–66). Interestingly, these channels may be large enough to accommodate the passage of the distended form of a polypeptide.

The findings on Semliki Forest virus and on diphtheria toxin have been used to construct a hypothetical sequence that could account for internalization of botulinum neurotoxin and tetanus toxin (Figure 2). A number of recent discoveries encourage a belief that the model is correct. Pharmacological experiments on the mouse phrenic nerve-hemidiaphragm preparation have shown that drugs known to inhibit the actions of internalized substances (e.g. ammonium chloride and methylamine hydrochloride) also inhibit the neuromuscular blocking actions of clostridial toxins (67). In addition, at least one lysosomotropic agent has been shown to antagonize botulinum neurotoxin (e.g. chloroquine), although it did not antagonize tetanus toxin (68). The reason for this discrepancy is not clear.

Studies on lipid vesicles and on lipid bilayers have shown that the heavy chains of botulinum neurotoxin and tetanus toxin form pH-dependent channels (18, 19). More precisely, it is the 50,000 dalton aminoterminus of the heavy chains that is responsible for channel formation. This is a particularly interesting finding, because the aminoterminus of the tetanus toxin molecule has a hydrophobic domain, which could play a role in toxin insertion into membranes, and this hydrophobic domain is not exposed except at low pH (69).

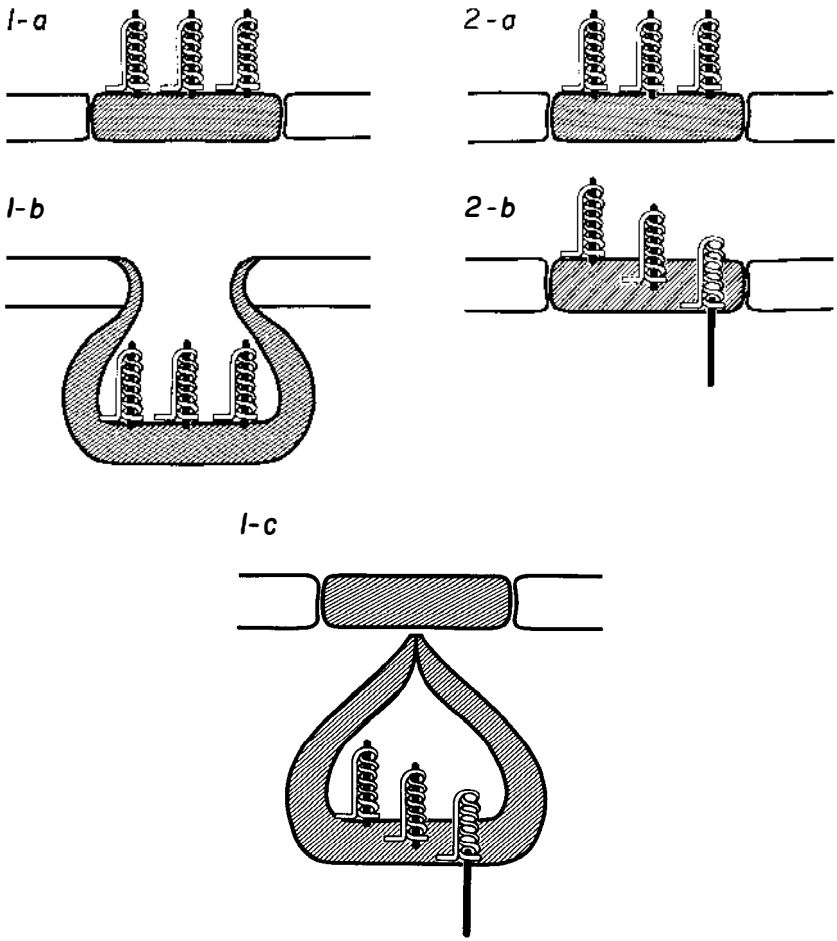

Figure 2 A mechanism for clostridial neurotoxin entry into cells can be built by analogy with other substances, such as Semliki Forest virus and diphtheria toxin. The proposed mechanism for translocation of neurotoxin involves two sequential events. The toxin binds to a specific class of receptors on the plasma membrane of cholinergic nerves (*1-a*). Either at that site or at some other specialized site (viz., caping), the toxin is internalized by the process of receptor mediated endocytosis (*1-b*). This represents the first half of the entry mechanism. The endocytic vesicle becomes progressively more acidic as it approaches the lysosome, and the fall in pH triggers a conformational change in the toxin molecule. A portion of the molecule, probably the aminoterminus, partitions into the membrane and forms channels. The passage of the light chain through the pH-induced channels may represent the second half of the entry mechanism (*1-c*).

By continuing the analogy with Semliki Forest virus and diphtheria toxin, one can propose an experimentally induced mechanism for clostridial neurotoxin entry into cells. This alternate mechanism begins with toxin binding to the plasma membrane (*2-a*). When the tissue is exposed to a medium with low pH, this triggers pH-dependent channel formation. The light chain can then be injected through the plasma membrane and directly into the cytoplasm (*2-b*).

The first study describing the channel forming properties of botulinum neurotoxin provided some comparisons between clostridial toxins and diphtheria toxin (19). Planar lipid bilayers were used to separate two compartments in which pH could be varied independently. In the presence of a symmetric pH of 7.0 or a symmetric pH of 4.0, botulinum neurotoxin, tetanus toxin, and diphtheria toxin fragments possessing the aminoterminus of the heavy chain had only modest channel forming activity. When a pH gradient was created that was equivalent to that across a prelysosomal membrane (i.e. 4.5 to 7.0), the toxin fragments were dramatically more active in forming channels. This result was obtained only when the toxin fragment was on the side of the membrane having a low pH; reversing the pH gradient effectively blocked channel formation.

Perhaps the most intriguing aspect of these channels is that sizing experiments suggest they are large enough to accommodate the passage of a polypeptide across a membrane. This could mean that the aminoterminus of the heavy chain acts like a tunnel protein through which the light chain might pass to reach the cytoplasm. But it is also possible that the channels are epiphenomena that are secondary to some other mechanism for toxin passage through the membrane.

The pharmacological and biophysical experiments pertaining to endocytosis and later penetration of an endosome membrane have been nicely complemented by the histological studies of Dolly and associates (29, 30). Using electron microscopic autoradiography of iodine-labeled toxin, they have shown that a substantial fraction of the material initially bound to cholinergic nerve endings is internalized. The internalization process is energy dependent and is inhibited by low temperature and by agents such as sodium azide and dinitrophenol. Ammonium chloride and methylamine hydrochloride antagonized the internalization process, and chloroquine appeared to trap the toxin inside vesicles. When taken in conjunction with the neuropharmacological studies, these histological data provide convincing evidence that botulinum neurotoxin must be internalized to produce its effects on neuromuscular transmission.

Comparable types of studies to show the internalization of tetanus toxin at the neuromuscular junction have not been published. However, work has been reported on other tissues, and the data are supportive of the concept of endocytosis (70).

The current state of the literature can be summarized in two statements reflecting the belief that internalization involves at least two events. There is widespread acceptance of the idea that clostridial neurotoxins must cross the plasma membrane, and receptor-mediated endocytosis is the most plausible mechanism to account for this. It is also thought that toxin molecules must reach the cytoplasm to exert their poisoning effect, but there is uncertainty about the underlying process. One possibility is that pH-induced channels act like tunnel

proteins that permit the passage of active material into the cytoplasm, but other possibilities deserve to be considered.

Intracellular Poisoning Step

Botulinum neurotoxin and tetanus toxin block acetylcholine release from the neuromuscular junction (7, 71). Early studies on botulinum neurotoxin suggested a relatively simple outcome, in which the toxin blocked all transmitter release. Subsequent studies have provided a more complex picture.

Botulinum neurotoxin blocks nerve stimulus-induced release of transmitter, and the effect is virtually complete (26, 72). The toxin also blocks spontaneous quantal release, but the effect is not complete (73, 74). Even in severely poisoned tissues there is a residual population of spontaneous miniature endplate potentials (mepp's). These residual mepp's have a mean amplitude that is less than that seen at normal junctions. The effect of the toxin on spontaneous non-quantal release of acetylcholine is not clear. Two groups have reported that the toxin blocks non-quantal release (75, 76), while one group has reported no effect (77).

The action of the toxin on nerve stimulus-induced and on spontaneous quantal release of acetylcholine, and the putative action of the toxin on spontaneous non-quantal release, are all acute phenomena. Experiments on tissues that are chronically poisoned reveal an additional effect (78, 79). Several days after onset of toxin-induced blockade, there occurs with increasing frequency a spontaneous class of mepp's that are large in amplitude. The origin and mechanism of release of these unusually large mepp's is not known.

The effects of tetanus toxin at the mammalian neuromuscular junction are similar to those of botulinum neurotoxin (13–15, 80). Tetanus toxin blocks nerve stimulus-evoked release of acetylcholine, and it greatly diminishes the frequency of spontaneous mepp's. The effects of the toxin on spontaneous non-quantal release have not been reported. There are also no reports that describe the effects of chronic poisoning on a population of large amplitude mepps.

Some attention has been drawn to the fact that there are subtle differences between the effects of botulinum neurotoxin and tetanus toxin on transmitter release at the neuromuscular junction (e.g. 15, 80). These differences have been interpreted to mean that the two toxins have separate and distinct mechanisms for poisoning transmitter release. For example, Dreyer et al have proposed that tetanus toxin acts to influence the movement of synaptic vesicles toward the active zones, whereas botulinum neurotoxin acts at or within the active zones (80). This is an interesting idea that may ultimately prove to be true, but for the moment one should be cautious. In truth, the proposal that there are different mechanisms of action stems entirely from a comparison of botulinum neurotoxin type A and tetanus toxin. Comparisons with the six other

botulinum neurotoxins have not been undertaken. This may be a critical issue, because data now show differences among the various botulinum neurotoxins (81, 82). It may be wise not to draw conclusions until all seven botulinum neurotoxins and tetanus toxin have been examined by the same experimental techniques. Such work may reveal that the seven botulinum neurotoxins are distinctly different from tetanus toxin, or it may reveal that all eight substances are merely variations on a common molecular theme.

No well-defined mechanism to account for the ability of clostridial toxins to block transmitter release has been advanced. A recent study hypothesizes that botulinum neurotoxin stimulates the intracellular metabolic systems that normally remove calcium from the vicinity of the active zones (83). One group has reported that tetanus toxin blocks calcium channels in nerve membranes (84); other groups have reported that neither botulinum neurotoxin nor tetanus toxin acts on calcium channels (85, 86).

The author has proposed that botulinum neurotoxin is an enzyme (2). This proposal was based on several clues, including the remarkable potency and long duration of action of the toxin, which are difficult to explain by a nonenzymatic mechanism. It was also based on a systematic comparison of botulinum neurotoxin with other toxins. The purpose of this comparison was to identify substances, sufficiently similar in structure and/or biological activity to botulinum neurotoxin, to serve as models. The outcome of this search was that no well-known neurotoxin could be found that was fundamentally similar in its structure-function relationships to botulinum neurotoxin. However, diphtheria toxin, a substance not ordinarily associated with the nervous system, appeared to share many features with botulinum neurotoxin. And it can now be added, there are many similarities between diptheria toxin and tetanus toxin as well.

As summarized in Table 1, the clostridial neurotoxins and diphtheria toxin (for reviews, see 87–89) are microbial in origin. The genetic material for each is thought to be in a virus particle or something derived from a virus, such as a plasmid. In the immediate post-translational stage, they are all single chain polypeptides that possess little toxicity (and see Figure 1). These inactive precursors are nicked by proteolytic cleavage to yield dichain molecules, with one heavy chain and one light chain. The tissue targeting domain is known to be in the carboxyterminus of the heavy chain, and the channel forming domain is in the aminoterminus of that chain. For diphtheria toxin, the light chain is an enzyme that has ADP-ribosylating activity.

The similarities between clostridial neurotoxins and diphtheria toxin are too numerous to overlook. It is noteworthy that all proceed through the same sequence of three steps in producing their poisoning effects. Each has a binding domain in one portion of the heavy chain and a channel forming domain in the other portion. It is difficult to avoid speculation that the clostridial neurotoxins, like diphtheria toxin, have an enzymatic domain in the light chain. This

Table 1 A Comparison of diphtheria toxin and clostridial neurotoxins

Characteristic	Diphtheria toxin	Botulinum neurotoxin	Tetanus toxin
Origin	Microbial *(Corynebacterium diphtheriae)*	Microbial *(Clostridium botulinum)*	Microbial *(Clostridium tetani)*
Source of genetic material	Virus	C&D: virus A,B,E,F&G: unknown	Plasmid
Post-translational structure	Single chain polypeptide	Single chain polypeptide	Single chain polypeptide
Active structure	Dichain polypeptide	Dichain polypeptide	Dichain polypeptide
Molecular weights of chains	Light ~ 20,000 Heavy ~ 40,000 (1:2 ratio)	Light ~ 50,000 Heavy ~ 100,000 (1:2 ratio)	Light ~ 50,000 Heavy ~ 100,000 (1:2 ratio)
Location of binding domain	Carboxyterminus of heavy chain	Carboxyterminus of heavy chain	Carboxyterminus of heavy chain
Location of channel forming domain	Aminoterminus of heavy chain	Aminoterminus of heavy chain	Aminoterminus of heavy chain
Location of enzyme domain	Light chain	?	?

speculation is especially hard to avoid when viewed in the context of recently published studies on the botulinum binary toxin.

The Mechanism Of Action Of The Binary Toxin

Efforts to isolate and characterize the eight botulinum toxins led to the discovery that one of them is unique. The serotype designated C_2 was found to be composed of two independent polypeptide chains (20, 21). There is a heavy chain and a light chain, but they are not linked by any covalent bonds. Neither of the chains possesses substantial toxicity, but the combination is very toxic (21, 22, 90, 91). In deference to the structural and toxicological data, the two chains can be regarded as a true binary toxin (22).

The botulinum binary toxin differs in the spectrum of its pharmacological actions from the botulinum neurotoxins (22, 90–92). The latter act rather exclusively on nerve endings to block release of transmitter. The binary toxin acts on a host of tissues, including brain, liver, lung, intestine, and vasculature.

When administered in vivo, the binary toxin and the neurotoxins produce respiratory failure, but the underlying mechanism is not the same. The neurotoxins block transmission between motoneurons and the muscles of respiration. The binary toxin evokes an array of cardiopulmonary effects, including increased vascular permeability, effusive secretions into the airway, pulmonary edema and bleeding, collection of fluids in the thoracic cavity, and extreme hypotension. Most of these effects appear to be related to the movement of fluids across membranes.

Although the manifestations of binary toxin and neurotoxin poisoning appear unrelated, the structure-activity relationships are quite similar. Studies involving chain-specific antibodies suggested that the heavy chain mediated binding and the light chain mediated poisoning (22). This proposed scheme has been verified by experimental evidence. Ohishi has published pharmacological experiments that implicate the heavy chain in binding (90, 91), and more recently he has complemented the work with histological experiments (23). Using fluorescent-labeled derivatives, he has convincingly shown that the heavy chain binds to the plasma membrane of vulnerable cells, and in the absence of this binding the light chain does not become associated with cells.

The light chain has been tested for a variety of enzymatic actions, and the result of this work has been a determination of the molecular basis for the action of the binary toxin. Two groups have shown that the light chain is an enzyme with ADP-ribosylating activity. Leppla (personal communication) has discovered that the chain ADP-ribosylates a protein found in Chinese hamster ovarian cells. The author has reported that the chain has ADP-ribosylating activity in two systems (24). In a model system, the toxin ADP-ribosylates homo-poly-L-arginine. In a system involving endogenous substrates, the toxin ADP-ribosylates a protein found in numerous eucaryotic cells.

The binary toxin is the first of the botulinum toxins for which a mechanism of action has been determined. Even so, there is a considerable amount of work remaining to be done. The substrate has yet to be isolated and characterized, and the role of the substrate in cell function is still unknown. These matters need to be resolved before a link can be made between the molecular action of the toxin and the cellular and systemic actions of the toxin.

The data on the binary toxin naturally raise the question whether botulinum neurotoxin or tetanus toxin have ADP-ribosylating activity. The author is aware of at least six laboratories that have tested this idea; although most of the results have not been published (but see 93), everyone is in agreement that the neurotoxins do not possess such activity, or if the neurotoxins do possess such activity it must be very difficult to demonstrate. The data on diphtheria toxin and on the binary toxin provide clues that the neurotoxins are enzymes, but the precise nature of that enzyme activity remains to be determined.

THE RIDDLE OF ORIGINS

There is one aspect of the study of botulinum toxin and tetanus toxin that has been extremely difficult to resolve. No one has been able to provide an explanation for the origin or the function of these molecules. Indeed, the question of origin and function has proved so baffling that no major article addressing the issue has been written during this century.

It may be helpful to clarify why these matters have been viewed as a riddle. Most neurotoxins of biological origin can be envisioned as serving one of two purposes: they are used by predators to immobilize prey (e.g. alpha-bungarotoxin), or they are used by potential prey to ward off predators (e.g. tetrodotoxin). In some cases it is possible to deduce the origin of a toxin used for these purposes. For example, certain components of snake venoms are enzymes (e.g. phospholipase A2 neurotoxins), and these neurotoxic enzymes may have evolved from digestive enzymes.

One can easily see why the clostridial neurotoxins are viewed as puzzling. To begin with, they do not serve any obvious function, either to advance predation or to ward off predation. And furthermore, there are no obvious predecessors from which they might have evolved. To make matters even more disconcerting, it must now be pointed out that this is only half the riddle.

Although clostridial toxins are named in accordance with the microorganisms that produce them, this practice results in what might be called misnomers. At least two of the botulinum neurotoxins (C and D) are encoded by genetic material that is not native to the host bacteria. The genetic material is found in a virus particle that enters the bacteria (94–96). Clostridia that are infected by the bacteriophage are capable of producing toxin, but when cured of the bacteriophage they lose the ability to make toxin.

The prevailing belief is that the genetic material responsible for synthesis of botulinum neurotoxin (94–96) and tetanus toxin (97) is found either in virus particles or in extrachromosomal elements that may have come from virus particles (e.g. plasmids). Aside from the fact that this raises questions about names like botulinum toxin and tetanus toxin, this information seems to make more obscure the reason for the existence of the molecules. On the one hand, the genetic information for the toxins is found in virus particles that infect procaryotes. But on the other hand, the toxins themselves affect eucaryotes, and in particular those highly developed eucaryotes that have a peripheral nervous system. By any standard of reasonableness, this is a puzzling state of affairs.

One advantage of the proposed model for clostridial neurotoxin action is that it has helped to explain the nature of the interaction between two remarkably potent toxins and the nervous system. It has served as a stimulus to move research from a phenomenological level, which describes toxin action in terms

of outcome, to a mechanistic level, which describes toxin action in terms of cellular, subcellular, and even molecular events. Another advantage of the model, though perhaps less obvious at first, is that it provides tentative clues about the origin of clostridial neurotoxins. By combining certain elements of the molecular pharmacology of the toxins with certain principles of molecular biology, one can construct a hypothetical scheme to account for the origin of these substances.

THE CONCEPT OF SUPERFUNCTION

It must be stressed that the model discussed above is not unique to the clostridial toxins. As implied by the discussion and the data in Table 1, it is equally applicable to diphtheria toxin. In fact, the model is to varying degrees true for a host of bacterial toxins (e.g. *Pseudomonas aeruginosa* exotoxin, cholera toxin, *E. coli* enterotoxin, pertussis toxin) and for a number of plant lectins (e.g. abrin and ricin). In fairness to the accomplishments of others, one should note that many aspects of the model were developed before more recent work indicated that it could encompass the clostridial toxins (25).

An important implication arises from the finding that many protein toxins behave in accordance with the three-step model. The implication is that investigators should be seeking a general theory to account for the origin and function of a dozen or more protein toxins rather than a narrow explanation that pertains only to clostridial toxins. To be more precise, investigators should try to deduce the origin of a class of molecules that has two major properties: (*a*) The molecules are synthesized in one type of cell but act on another and remote type of cell, and (*b*) the molecules possess a receptor binding domain, a translocation domain, and an enzyme domain.

Pharmacologists will be quick to note that these two major properties are largely shared by another class of molecules that are ubiquitous among eucaryotes. Much of the terminology used to describe the protein toxins needs to be modified only slightly to become acceptable terminology for describing messenger systems. The most well understood messenger systems are those that have three components. (*a*) A first messenger or signal component (e.g. neurotransmitter, hormone) is synthesized in one cell type but is secreted to act on a remote cell type. (*b*) A transduction component is interposed between the extracellular first messenger and an intracellular second messenger. Typically, transduction can be attributed to the opening of ion channels and/or to the induction of an enzyme. (*c*) The intracellular enzyme (e.g. adenylate cyclase) or its product (e.g. cyclic-AMP) is a third component that initiates a cascade of events culminating in an organotypic response.

The similarities between toxins and messenger systems may be much more than merely semantic. To substantiate this claim, the author will introduce a

novel term that may help to explain the relationships between protein toxins, messenger systems, and other complex molecular systems. The term is a corrollary of one that has been used to describe the immunoglobulin family; the term is superfunction.

Investigators studying the origin and genetic correlates of immunoglobulin proteins have coined the term supergene (98). Stated simply, a supergene is a set of individual genes or gene families that have sequence homology but that do not necessarily encode proteins with the same function. By contrast, one can envision superfunction as a set of individual functions or families of functions that are similar in terms of expressed activity but that do not necessarily stem from the same genes. Thus, there are messenger systems that involve a variety of transmitters and hormones as signals, a variety of ion channels or allosteric proteins as transducers, and a variety of enzymes and products as intracellular mediators; these messenger systems compose one functional family. The dozen or so protein toxins with binding, translocation, and enzyme domains compose another. These two families as well as others (see below) can be grouped together under the concept of superfunction.

The relatedness between toxins and messenger systems is far more extensive than can be considered here. However, three specific illustrations may help to make the point. There are examples of toxins whose tissue binding domain recognizes the same cell surface receptor as the signal component of messenger systems (99, 100). Another similarity is that toxins and messenger systems have been highly conservative with their intracellularly acting components. The former makes considerable use of ADP-ribosylating enzymes, and the latter makes considerable use of adenylate cyclase. Perhaps most provocatively, the two have numerous points of convergence. Cholera toxin ADP-ribosylates a regulatory protein that stimulates adenylate cyclase, and pertussis toxin ADP-ribosylates a regulatory protein that inhibits adenylate cyclase (101). Certainly the most striking example of convergence is anthrax toxin. The enzyme component of this toxin does not merely alter adenylate cyclase; the enzyme component of this toxin is adenylate cyclase (102).

Returning to the concept of superfunction, there is one last family that must be considered here. To do so begins to make clear the possible origin of botulinum and tetanus toxins. Many virus particles have components or can encode components that are essential to infection and that resemble the models discussed above. Of necessity, these particles must have a binding domain that can attach to receptors on the cell surface. They also have domains that mediate translocation. As discussed above, the mechanism of membrane translocation used by Semliki Forest virus has served to guide research on diphtheria toxin and clostridial toxins. And finally, virus particles encode the information for numerous enzymes that profoundly alter the behavior of infected cells.

Again, there are many examples that can be cited to show the relatedness

between virus function and toxin function, but one is particularly illustrative in the present context. The multiple domain structure of viruses and toxins implies that all the domains must be present to obtain full expression of activity. In the absence of any particular domain, the virus or toxin loses its ability to exert its effects. Uchida et al have reported an interesting experiment in which an incomplete virus was mixed with an incomplete toxin to create a novel and completely functional unit (103). First, virus ghosts were produced by emptying Sendai virus particles of their normal contents. Next, a mutant of diphtheria toxin that possessed enzyme activity but lacked cell binding activity was isolated. The incomplete toxin was then loaded into the virus ghost. The newly constituted unit affected only those cells that had receptors for the virus, and it acted intracellularly only on those molecules susceptible to the ADP-ribosylating effects of diphtheria toxin.

This experiment sets the stage for putting forward two possible ideas to account for the origin of clostridial toxins. The first of these is rather straightforward and easy to grasp. Clostridial toxins may not have arisen *de novo* as unusual molecules synthesized in one cell and acting on another. Instead, the toxins may be variants on the proteins that viruses ordinarily synthesize. Virus particles are evolutionarily ancient, and therefore they could have served as the precursor for the functional property of originating in one place but acting remotely in another place. In addition, virus particles have or encode for binding domains, translocation domains, and enzyme domains. This means they could have been precursors for molecules such as toxins that possess similar domains. The clostridial toxins may be proteins encoded by nucleic acid that at one time served as a template for virus domains, but which through mutations and other modifications now serve as a template for toxin domains.

The second proposal is a variant on the first, and it attempts to address the fact that substrates for many of the potent protein toxins are found mainly or exclusively in eucaryotic cells. The essence of the proposal, which is illustrated in Figure 3, is as follows. Eucaryotic cells carry the genetic information for the binding, transduction, and enzyme-messenger functions, and virus particles carry the information for binding, translocation, and enzyme functions. It is conceivable that a recombinant event could have allowed a virus to capture a eucaryotic gene segment encoding an enzyme that participated in a messenger system. Or to make the proposal broader, the recombinant event could have resulted in capture of almost any eucaryotic enzyme. The product would be a gene segment of non-eucaryotic origin that encodes binding and translocation domains linked to a gene segment of eucaryotic origin that encodes an enzyme domain.

These two proposals certainly should not be viewed as rigid. To the contrary, they are conceptual models that are representative of the two most logical sources of the toxins, i.e. a modified viral origin, or a modified viral and

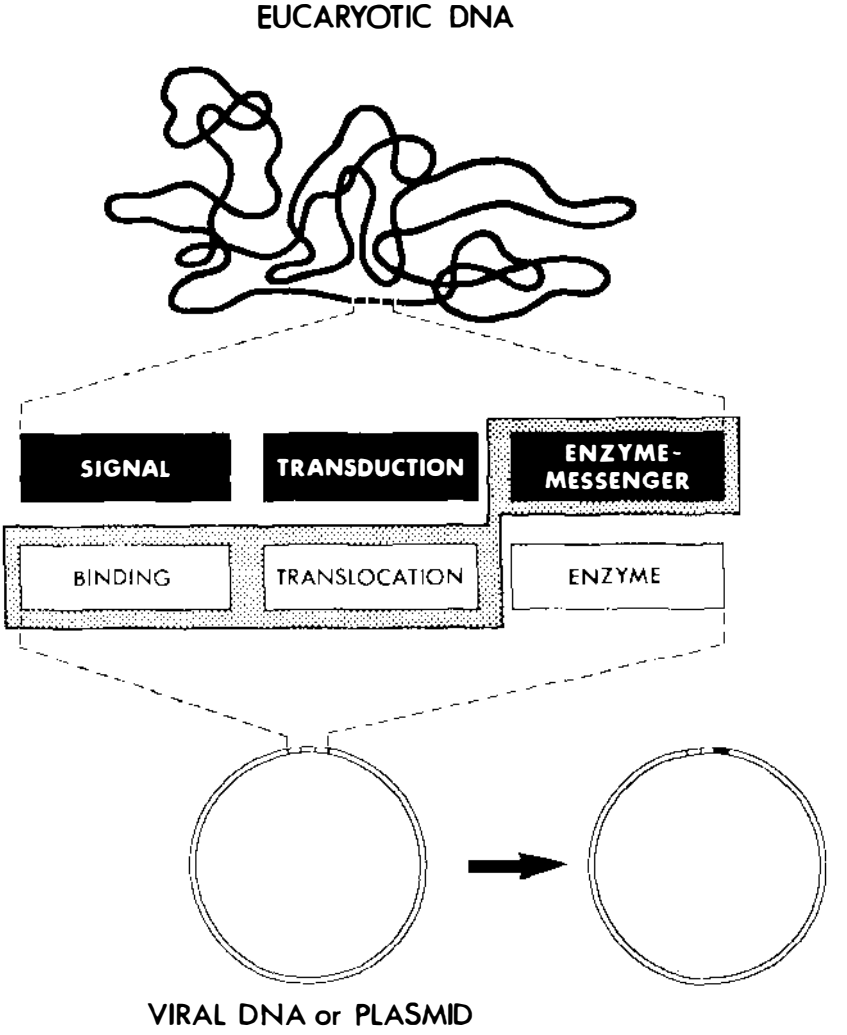

Figure 3 One possible explanation for the origin of clostridial toxins involves the mechanism of recombination. Eucaryotic genes carry the information for signal, transduction, and enzyme components of messenger systems. Viral or other extrachromosomal elements carry the information for binding, translocation, and enzyme components associated with infectivity. Through a recombinant event, viral gene segments for binding and translocation may have become linked to a eucaryotic gene segment for enzyme activity. This newly constituted element could encode a multiple domain molecule similar to a protein toxin that acts on substrates in eucaryotic cells.

eucaryotic origin. Either of these origins, when viewed in the context of superfunction, could explain the two major properties of protein toxins (e.g. different sites for synthesis and activity; multiple domains).

For reasons that are clear, it is difficult to test the idea that a gene encoding the structural proteins of a virus evolved into a gene encoding a protein toxin. There is however a method for deducing whether the enzyme component of a protein toxin is the same as or similar to an enzyme normally occurring in eucaryotic cells. Investigators can search eucaryotic cells to determine whether there are enzymes that are indistinguishable from those in toxins. Only a limited amount of work has been done, but it is interesting to report that eucaryotic sources of cholera toxin-like (104, 105) and diphtheria toxin-like (106) enzymes have been reported. The workers who have made these exciting discoveries are continuing their efforts to compare enzymes in toxins and in nucleated cells.

Much more work must be done before one can say with certainty whether the two proposals just described can explain the origin of clostridial toxins. An important ingredient will be continued effort to determine the function of the light chains of botulinum neurotoxin and tetanus toxin. In the meantime, work is already in progress to locate a eucaryotic source of the enzyme found in the light chain of the botulinum binary toxin.

MERGING MOLECULAR PHARMACOLOGY AND MOLECULAR BIOLOGY: UTILIZING THE CONCEPT OF SUPERFUNCTION

The concept of superfunction implies that when a functional domain from one complex molecule is substituted for the equivalent domain in another molecule, the newly created structure may be biologically active. It would be desirable to obtain evidence to support this idea from naturally occurring events, but the chances of detecting a spontaneous recombination are rare. There is one exception to this rule. Botulinum neurotoxin occurs in at least seven serotypes. Individual types are usually synthesized by individual strains of bacteria, but there are strains that produce more than one type. A single strain that produces more than one botulinum neurotoxin greatly increases the possibility of detecting recombination.

In a series of interesting papers, a Japanese group has provided data that are indicative of a recombinant event (107–109). They have isolated a botulinum neurotoxin that behaves much like a hybrid, with a heavy chain apparently derived from one serotype and a light chain derived from another. Neurotoxin

molecules with hybrid qualities possessed the same potency as non-hybrid molecules. The group that did the work has itself speculated that the hybrid toxin could be the result of recombination (107).

An alternate method to genetic recombination can be used to show that functional domains may be exchangeable between molecules that belong to the same family. Posttranslational products can be dissociated into their respective components, and then these components can be reassociated into a novel hybrid. There are examples of this that are especially relevant to the clostridial toxins. Botulinum neurotoxin (BNT) and tetanus toxin (TT) are composed of heavy (H) and light (L) chains linked by a disulfide bond (S–S). Thus, botulinum neurotoxin type A could be written as BNT_A(H)–S–S–BNT_A(L), and tetanus toxin would be written as TT(H)–S–S–TT(L). The concept of superfunction implies that hybrid molecules such as BNT_A(H)–S–S–BNT_B(L) or BNT_F(H)–S–S–BNT_G(L) should produce neuromuscular blockade. Even more provocatively, the concept implies that BNT(H)–S–S–TT(L) and TT(H)–S–S–BNT(L) should also be biologically active. The proposed model for clostridial toxin action suggests that low doses of the former should be tissue targeted to cholinergic nerve endings, where it will produce flaccid paralysis, and the latter should enter the central nervous system, where it will produce spastic paralysis. Three laboratories are working collaboratively to make these posttranslational hybrids and to use them to test current thinking about clostridial toxin action (J. R. Robinson, Vanderbilt University; B. R. DasGupta, University of Wisconsin; and the author).

This is but a narrow application of an idea that many investigators have already grasped. Hybrid toxins are immensely valuable for studying toxin action, but they may also be useful as therapeutic agents and as pharmacological tools. Readers who are not familiar with the field of hybrid toxin research may wish to consult the reviews by Olsnes & Pihl (110), Vitetta et al (111), and Thorpe & Ross (112).

The clostridial toxins are among those multiple domain molecules whose constituent parts could be used to form hybrids that would be therapeutic agents or pharmacological research tools. A hybrid molecule that retained the heavy chain of botulinum neurotoxin but that replaced the light chain with an anti-light chain antibody might well be a clinically useful drug for entering nerve endings and arresting the effect of internalized toxin. Conversely, hybrid molecules that retained the light chains but replaced the heavy chains with drugs that are tissue targeted for specific types of nerve endings (e.g. dopaminergic, serotonergic, enkephalinergic) might well be a family of substances for blocking mediator release from all chemically transmitting nerves. In these and other settings, hybrid clostridial toxins could become valuable drugs in the disciplines of experimental and clinical pharmacology.

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