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IDENTIFICATION OF THE MAJOR STEPS IN BOTULINUM TOXIN ACTION

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■ **Abstract** Botulinum toxin is a uniquely potent substance synthesized by the organisms *Clostridium botulinum*, *Clostridium baratii*, and *Clostridium butyricum*. This toxin, which acts preferentially on peripheral cholinergic nerve endings to block acetylcholine release, is both an agent that causes disease (i.e., botulism) as well as an agent that can be used to treat disease (e.g., dystonia). The ability of botulinum toxin to produce its effects is largely dependent on its ability to penetrate cellular and intracellular membranes. Thus, toxin that is ingested or inhaled can bind to epithelial cells and be transported to the general circulation. Toxin that reaches peripheral nerve endings binds to the cell surface then penetrates the plasma membrane by receptor-mediated endocytosis and the endosome membrane by pH-induced translocation. Internalized toxin acts in the cytosol as a metalloendoprotease to cleave polypeptides that are essential for exocytosis. This review seeks to identify and characterize all major steps in toxin action, from initial absorption to eventual paralysis of cholinergic transmission.

INTRODUCTION

Botulinum toxin is generally regarded as the most poisonous of all poisons (1). Indeed, its potency is so great that no one has yet quantified the minimum concentration, or minimum number of molecules, needed to disrupt function of a vulnerable cell. This remarkable level of potency is made even more impressive by the fact that the toxin must progress through a lengthy and complex sequence of events to reach its site of action, the peripheral cholinergic nerve ending.

Botulinum toxin is encountered in a wide variety of clinical settings. To begin with, it is the etiologic agent responsible for the disease botulism (2, 3). This disease can occur naturally as a form of accidental food poisoning, but it can also occur unnaturally as a product of malice, such as in bioterrorism and biologic warfare (4–6). Another setting in which the toxin is encountered is the treatment of disorders in which there is excessive and uncontrolled peripheral cholinergic nerve activity (e.g., dystonia). The toxin can be injected locally in doses that diminish but do

not abolish neuronal activity, with the result that normal function is approximated or even attained (7–10). Yet another setting in which the toxin is encountered is experimental therapeutics (11). Botulinum toxin, or more accurately polypeptide fragments derived from the toxin, are being evaluated as potential carrier molecules in the construction of oral and inhalation drugs. These polypeptide fragments may become the basis for an entirely new generation of medical products, such as oral and inhalation vaccines.

To understand why botulinum toxin is found in such diverse settings, one must first understand the complex sequence of events that underlie toxin action. Whether acting as an agent that causes disease or as an agent to relieve disease, the toxin must penetrate a series of cellular and intracellular membrane barriers. Botulinum toxin does not typically cause cell death, and, therefore, it does not cross membrane barriers by killing cells in its path. Instead, the toxin has the ability to recognize and exploit transmembrane and transcellular transport processes that have evolved to serve some other purpose. Two of these processes have been studied in some detail. Botulinum toxin utilizes a transcytosis pathway in epithelial cells to cross from the outside world to the general circulation (12, 13). The toxin subsequently utilizes an endocytosis and translocation pathway to enter the cytosol of cholinergic nerve endings where it exerts its neuroparalytic effects (14–19).

In this review, an effort has been made to identify and characterize all of the major steps that are essential to toxin action. This information will serve to highlight those areas in which there has been substantial progress as well as those in which there is still much to be learned.

ORIGIN AND MECHANISM OF ACTION

Botulinum toxin is a microbial product synthesized by the organisms *Clostridium botulinum*, *Clostridium baratii*, and *Clostridium butyricum* (20). *C. botulinum* produces all seven known serotypes (A to G), whereas *C. baratii* and *C. butyricum* produce only one serotype each (F and E, respectively).

Botulinum toxin is synthesized as a single-chain polypeptide with a molecular weight of approximately 150 kDa (for reviews on toxin structure, see References 21–25). Most organisms that manufacture the toxin possess a protease that nicks the molecule to create a dichain structure in which a heavy chain (approximately 100 kDa) is linked by a disulfide bond to a light chain (approximately 50 kDa). The dichain molecule is the active form of the toxin that poisons cholinergic transmission. Certain strains of Clostridia release the toxin in a single-chain form, but this does not necessarily diminish the potential for disease. Proteases in the gut can nick the toxin and therefore convert it to a fully activated form.

Botulinum toxin is released from bacteria as part of a noncovalent multimeric complex. The auxiliary proteins that are best characterized are hemagglutinins (HA) and a nontoxin, nonhemagglutinin (NTNH). Neither of these proteins plays a role in toxin-induced blockade of cholinergic transmission. Indeed, it is unlikely

that the intact multimeric complex even reaches nerve endings. The complex spontaneously dissociates in solutions of physiological pH and ionic strength, which means there should be substantial dissociation in the general circulation. In addition, the number of HA binding sites on red blood cells is so large that this receptor pool should serve to retain HA in blood.

Although auxiliary proteins play no meaningful role at the site of toxin action, this does not mean that they are unimportant. To the contrary, the complex of toxin, HA and NTNH is critical to the process of oral poisoning. When these proteins are intertwined, they are unusually resistant to the harsh conditions of low pH and proteolytic enzymes in the gut. Thus, auxiliary proteins are helpful companions in the journey that will ultimately deliver toxin to vulnerable cells.

Although the overwhelming majority of all reported cases of botulism have an oral etiology, the disease can have other origins (26). Botulism can be due to inhalation of toxin, contaminated puncture wounds, accidental trauma, and surgery. There is evidence that auxiliary proteins are not essential to inhalational poisoning (13). This may simply reflect that the respiratory system, unlike the gastrointestinal system, has no harsh conditions of low pH and proteolytic enzymes. By extension, one may assume that auxiliary proteins are not essential for poisoning that is secondary to penetrating wounds.

When the toxin enters the lumen of the gut or airway, it must cross membrane barriers to reach the general circulation. A likely mechanism for this phenomenon has been described (12). Botulinum toxin binds to the apical surface of epithelial cells, undergoes receptor-mediated endocytosis and transcytosis, and is delivered to the basolateral surface of cells. This could well account for the ability of the toxin to reach blood and lymph (Figure 1).

Botulinum toxin that reaches the general circulation must exit the vasculature to reach the extracellular space in the vicinity of its target organs, which are cholinergic nerve cells. The toxin certainly escapes the peripheral vasculature because peripheral cholinergic nerve endings are its principal site of action. However, there is not a single study in the modern era that describes toxin egress from the vasculature. The phenomenon could be an active transcellular process or it could simply be paracellular movement. On the other hand, it is well known that botulinum toxin does not penetrate the blood-brain barrier. Thus, the toxin has little ability to impair central cholinergic transmission in intact organisms, although it does block transmission in tissue slices, isolated cells, and isolated synaptosomes of central origin (27).

When the toxin reaches peripheral cholinergic nerve endings, there is again a sequence of membrane-penetrating events (for reviews on mechanism of action, see References 16–19, 28). Initially, the toxin binds to the surface of plasma membranes, and this is followed by receptor-mediated endocytosis and pH-induced translocation across the endosome membrane. When the toxin reaches the cytosol, it acts as a zinc-dependent endoprotease to cleave polypeptides that are essential for exocytosis. Blockade of transmitter release accounts for the flaccid paralysis and autonomic dysfunction that are characteristic of the disease botulism (Figure 1).

Although the toxin acts preferentially on cholinergic nerve endings, it does have the ability to block exocytosis from other nerve endings as well. One can produce blockade of transmitter release from nerve endings that utilize norepinephrine, serotonin, and a host of other mediators merely by increasing the concentration of toxin (27). Toxin action can even be extended to nonneuronal cells. For example, inserting molecular mimics of true membrane receptors can render glandular cells sensitive to toxin action. In all these cases, there is still the general sequence of binding, receptor-mediated endocytosis, pH-induced translocation, and cytosolic metalloendoprotease activity.

The substrates for the seven toxin serotypes, as well as the specific cleavage sites, have been determined (16, 17). Botulinum toxin types A and E act on synaptosomal-associated protein of 25 kDa (SNAP-25); serotypes B, D, F, and G act on vesicle-associated membrane protein (VAMP), also known as synaptobrevin; and serotype C acts mainly on syntaxin, although it can also cleave SNAP-25.

The duration of action of the seven toxin serotypes varies, although serotype A is known to have the most sustained action (29–31). Whether measured in laboratory animals or in human beings, the duration of serotype A action can be many weeks to many months. This impressively sustained action may be a function of the substrate cleavage product, the intraneuronal half-life of the toxin, or a combination of the two. It is noteworthy that no one has yet determined how the toxin is metabolized or otherwise eliminated from nerve endings.

ABSORPTION FROM THE GASTROINTESTINAL SYSTEM

General Characteristics of the Transport Process

The various etiologies of botulism can be divided into two broad categories: (*a*) primary intoxication and (*b*) primary infection followed by secondary intoxication. In primary intoxication, the patient ingests a food that is already tainted with botulinum toxin. This happens when a food contaminated with Clostridia is prepared and/or stored in a way that allows organisms to grow and multiply. In essence, the contaminated food serves as a culture medium for bacteria. When the organisms mature and undergo autolysis, they release toxin. Thus, an unwitting victim consumes the toxin merely by consuming food.

In primary infection, the patient ingests food that harbors Clostridial spores that may germinate and colonize in the gut. In the process of growth, division, and autolysis, organisms release toxin in situ, and this leads to secondary intoxication. Consumption of Clostridial spores is probably somewhat common, although the incidence of secondary intoxication is low. This can be attributed to several factors, with the most important being the inability of *C. botulinum* to compete for a niche in healthy guts that have a normal flora. This explains the fact that primary infection with secondary intoxication occurs almost exclusively in young infants (e.g., the normal flora has not developed) (32–35) or in older persons who have

been treated with certain antibiotics (e.g., the normal flora has been reduced or eliminated) (36).

Regardless of whether poisoning is due to primary or secondary intoxication, these two broad categories of etiology have one key feature in common: Toxin in the lumen of the gut must penetrate membrane barriers to reach blood and lymph. Toxin molecules, and certainly progenitor complexes of toxin plus HA and NTNH, are too large for any significant rate of paracellular diffusion. Furthermore, mechanisms such as pinocytotic uptake and transport seem too inefficient for a toxin that is so potent. It is more likely that the toxin binds exploitatively to a receptor that is linked to an efficient transport process.

Two groups have tried to more clearly define the mechanism for toxin escape from the gut. Both groups agree that the process is initiated by receptor binding to epithelial cells. However, there is disagreement between the groups about the location of the binding domain. Oguma and his associates have argued that the ligand binding property is found in HA (37–39), whereas this author and his colleagues have proposed that the ligand binding moiety is found in the toxin itself (12, 40). With regard to the latter proposal, it should be noted that (*a*) homogeneous toxin administered by itself can escape the gastrointestinal system and produce peripheral neuromuscular blockade (40) and (*b*) at least one outbreak of human botulism has been attributed to an organism that does not carry the gene for HA (E.A. Johnson, personal communication). These two observations mean that botulinum toxin poisoning by the oral route does not have an absolute requirement for HA. On the other hand, these observations do not rule out the possibility that there could be both toxin-mediated and HA-mediated processes for binding and transport.

Studies aimed at clarifying the role of the toxin in mediating escape from the gut have utilized immortalized human gut epithelial cell lines (T-84 and Caco-2) (12). These cells possess the desirable quality of forming polarized monolayers in which apical surfaces are oriented toward the medium and basal surfaces adhere to the support. Therefore, polarized monolayers with patent tight junctions can be used to study toxin migration from the *cis* to the *trans* side of human gut epithelial cells (Figure 2).

Studies with iodinated botulinum toxin have shown that serotypes typically associated with human poisoning (e.g., A and B) can bind to the apical surface of cells, undergo transcytosis, and be released on the basolateral side (12). Toxin that undergoes transport is released in a form that is structurally and functionally indistinguishable from that originally added to cells. Thus, the neuromuscular blocking activity of native toxin and that of an equimolar amount of toxin that has crossed human epithelial cells are the same.

Not all serotypes have the ability to penetrate gut epithelial cells efficiently. In contrast to serotypes A and B, serotype C (which has never been shown to cause human illness) and tetanus toxin (which is structurally related to botulinum toxin but has never been implicated in oral poisoning) display little ability to cross gut epithelial cells. Similarly, not all polarized monolayers have the ability to transport botulinum toxin. For example, Madin-Darby canine kidney cells (MDCK), which

have been shown to bind and transport other ligands, do not transport botulinum toxin in an efficient manner.

Interestingly, T-84 cells can transport botulinum toxin types A and B in both the apical to basolateral direction and in the reverse direction. The latter is unlikely to be of clinicopathological importance. Toxin that is transported from the lumen of the gut to the general circulation is immediately and dramatically diluted so there should be little if any reverse transport. Nevertheless, the observation that the toxin can be ferried in both directions may ultimately prove useful in efforts to isolate and characterize the toxin receptor.

A limited amount of structure-function analysis has been done in an attempt to identify the minimum domain needed for binding and transcytosis (41). This work has demonstrated that the single-chain unnicked molecule and the dichain nicked molecule are transported at similar rates. When the dichain molecule is reduced and the light chain is removed, the residual heavy chain retains the ability to penetrate epithelial monolayers. Expression products that represent 88%, 66%, 50%, and 45% of the heavy chain—all with the original carboxyterminus—can bind and cross gut epithelial cells. However, expression products that have lost only short segments from the carboxyterminus have greatly diminished activity. Apparently, the minimum essential domain is located in the carboxyterminal half of the heavy chain, and this domain requires residues that are close to the carboxyterminus itself.

Experiments have been done to label toxin with fluorescent dye and then visualize its movement by serial x-y confocal imaging (42). This work demonstrated binding that appeared to be distributed randomly across the apical surface of epithelial cells. Internalization was rapid and occurred within approximately 5 min, and transport across the cell with eventual release was prominent within 10 to 20 min. Visualization of the transcytosis pathway revealed one interesting feature that could not be detected merely by collection of labeled material on the basal surface: Toxin that was endocytosed was distributed across the crown of cells, but transport vesicles carrying the toxin moved to the periphery and remained there until reaching the base of cells. This migratory pattern was a byproduct of the anatomy of human gut epithelial cells. There is a large nucleus that occupies the central portion of each cell, and, therefore, transport vesicles are displaced to the periphery.

Identification of the Transport Cell

One of the most important characteristics of the transport process is the identity of the cells that actually carry toxin. The two logical choices are absorptive epithelial cells and M cells. Both cell types, along with intercellular tight junctions, serve the purpose of creating a barrier between the lumen of the gut and the general circulation. However, both cell types have the ability to capture certain molecules or macromolecular complexes and transport them. In the case of absorptive cells, the transport process typically does nothing more than ferry molecules across structural barriers. In the case of M cells, the transport process can be more involved. The M cell Peyer's Patch complex can capture and process molecules, including partial metabolic breakdown, to present potential antigens to the mucosal immune system.

Work to assess the respective contributions of absorptive cells and M cells to the transport of botulinum toxin has recently been completed (43). Two approaches were used and both pointed to a predominate role for absorptive enterocytes. In one line of investigation, absorption of botulinum toxin was examined in a knockout strain of mice that has been engineered to be deficient in gut M cell Peyer's Patch complexes. Interestingly, botulinum toxin was equally active as an oral poison in both the knockout strain and in the wild-type strain from which the mutant animal was derived. This result indicates that the major route utilized by toxin is transcytosis across absorptive cells.

In the second line of study, monolayers of absorptive cells were transformed into M cells by coculture with Raji lymphocytes (43). This in vitro transformation has been shown to produce enormous increases in the rate of transport of large entities, such as labeled beads (44). When transformed cells were used to gauge botulinum toxin type A transport, there was indeed an increase in the rate of movement from the apical surface to the basolateral surface (approximately fivefold). Although this may at first seem impressive, one must place the data into a meaningful context. In the guts of mammals, the number of absorptive enterocytes outnumbers M cells by orders of magnitude. This means that M cells could outweigh the contribution of absorptive cells only if their transport rates were vastly higher. A fivefold increase is numerically significant but far below what would be necessary to be functionally significant. Thus, the monolayer work appears to support the in vivo work in implicating absorptive cells as the major participant in botulinum toxin transport.

CREATING AN ORAL VACCINE

The botulinum toxin molecule is immunogenic and can be used to evoke systemic resistance (45–47). There is currently a parenteral vaccine against the toxin that is administered as an investigational new drug. The toxoid is generated by obtaining an ammonium sulfate precipitate from cells and inactivating it with formalin. The investigational toxoid is used to protect persons who are exposed (e.g., toxin investigators) or may be exposed (e.g., armed forces at risk of biological warfare) to botulinum toxin. A newer generation of injectable toxoid that utilizes recombinant fragments of heavy chain is being evaluated in human clinical trials (47).

It would be highly desirable to have a nonparenteral vaccine that could be administered by the oral or inhalation routes. Such preparations would diminish the need and therefore the cost of medical personnel to inject vaccine, reduce the possibility of accidental needle sticks, and reduce the need to store and eventually dispose of medical waste (needles and syringes).

A measured amount of progress has been made in developing an oral botulinum toxin vaccine. In the initial approach, the techniques of molecular biology were used to generate a nontoxic variant of the parent molecule (48). Site-directed mutagenesis was used to alter the light chain of the toxin so that it could no longer act catalytically to cleave substrates that are essential for exocytosis. This molecule retained the ability to bind and cross gut epithelial cells and it retained the antigenic

character of the heavy chain, but it was devoid of the ability to poison cholinergic transmission. When administered to animals via the oral route, this mutant form of the toxin evoked resistance to native toxin without evoking any obvious adverse effects.

In more recent work, systemic immunity was evoked by oral administration of heavy chain (which was obtained by isolating the protein from clostridia) or polypeptide fragments of heavy chain (which were obtained as expression products). The rationale was simply to eliminate that portion of the molecule that produces blockade of exocytosis (e.g., catalytic light chain; see below). Both the heavy chain and its fragments evoked high levels of resistance to native toxin (A.B. Maksymowych & L.L. Simpson, unpublished data).

ABSORPTION FROM THE RESPIRATORY SYSTEM

The fact that botulinum toxin is an oral poison is widely known; the fact that it is an inhalation poison is not as widely appreciated. A simple explanation for the lack of awareness that botulinum toxin can act via the airway relates to incidence of disease. There are numerous reports of patients who have succumbed to botulism as a form of food poisoning; however, there is only one report of patients who have become poisoned by the inhalation route, and this incident was restricted to laboratory workers (49).

Aside from marked differences in incidence, there is one particularly stark contrast between oral poisoning and inhalation poisoning. For the latter, there is great concern that any large outbreaks of poisoning may not be accidental phenomena. They may instead be the products of malice, as in bioterrorism or biological warfare (4–6).

In the recent past, an effort has been made to examine the underlying basis for inhalation poisoning (13). For understandable reasons, this research has to some extent been modeled after that done on the gastrointestinal system (12, 40). This work has demonstrated that botulinum toxin is active by the inhalation route, but it has also shown that there is one notable difference between oral and inhalation poisoning. When entry is via the mouth, the auxiliary proteins HA and NTNH play the important role of protecting the toxin against metabolism (40, 50–55). Thus, progenitor toxin is more potent than homogeneous toxin when given orally. When entry is via the nose, auxiliary proteins are not essential to protect the toxin (13). Thus, progenitor toxin does not display enhanced potency compared to isolated toxin.

Transcytosis experiments have been done to further clarify mechanisms of inhalation poisoning (13). Both rat primary cells (alveolar) and an immortalized human cell line (Calu-3) were used to measure toxin transport across polarized monolayers of alveolar epithelium. This work has its positive aspects, but it is also subject to one important question. On the positive side, alveolar epithelial cells bind and transport toxin in both the apical to basolateral and basolateral to apical directions. As with gut epithelial cells (see above), alveolar epithelial

cells transport native toxin in an unmodified form and release it in a fully active conformation. However, the entire toxin molecule is not essential to obtain binding and transcytosis. The isolated heavy chain is also carried across alveolar epithelial cells, and the rates of transport are close to those observed with the parent molecule. Apparently, the minimum essential domain for binding and transcytosis across airway cells resides somewhere within the heavy-chain component.

Although these findings are encouraging, there remains at least one important but unanswered question. When the toxin is administered by nose, the exact location of absorption has not yet been determined. It is certainly possible that most uptake occurs in nasal epithelium rather than alveolar epithelium. If this were to be true, it would mean that transport studies on alveolar cells should be seen as models for those on nasal epithelial cells, at least until an immortalized line of human nasal epithelial cells suitable for transcytosis studies is developed.

CREATING AN INHALATION VACCINE

In addition to in vitro work on the transport of toxin and its polypeptide components, in vivo studies have been done to evaluate inhalation absorption of these molecules (13). Experiments were done on toxicity, pharmacokinetics, and induction of immunity. As expected, inhalation of the native toxin produced the characteristic outcome of peripheral neuromuscular blockade (see above). Inhalation administration of the heavy chain, or the carboxyterminal half of the chain, produced no obvious toxicity. When the various proteins were iodinated and their appearance in blood monitored, the time to reach peak plasma concentrations for toxin and for heavy chain were almost identical, although the actual peak plasma concentration for toxin was somewhat higher.

The fact that polypeptide fragments of the toxin were absorbed through the airway raised the obvious possibility of creating an inhalation vaccine (13). To date, several candidates have been tested. The heavy chain, the carboxyterminal half of the heavy chain, and a polypeptide representing approximately 45% of the carboxyterminal half of the heavy chain have all proved useful as vaccines. By contrast, removal of approximately 5% from the carboxyterminus of the heavy chain greatly diminished absorption and subsequent immunogenicity (J.B. Park & L.L. Simpson, unpublished data).

It is noteworthy that the various inhalation vaccine candidates all evoked a strong systemic immunoglobulin (IgG) response. Furthermore, animals were protected not only against inhalation challenge but also against other routes of challenge, including injection. This means that inhalation administration of the vaccine candidates was not simply evoking a local immune response. This conclusion was further supported by studies similar to those described above on knockout mice that are deficient in M cell Peyer's Patch complexes. Inhalation administration of toxin produced equivalent effects in the knockout mice and in the wild-type strain from which they were derived (43). Similarly, the heavy-chain component of the toxin was equivalently active as an inhalation vaccine in both strains. These results

encourage further studies to determine whether an inhalation vaccine suitable for human use would afford protection against all potential routes of accidental or malicious exposure.

ESCAPE FROM THE VASCULATURE

Botulinum toxin that reaches the general circulation can subsequently leave the vasculature, diffuse through the extracellular space, and ultimately reach its target organs. Unfortunately, no one has undertaken a detailed study of the mechanisms that account for the ability of the toxin to cross endothelial barriers. By analogy with epithelial barriers, one might argue that there is binding to receptors that mediate endocytosis and transcytosis. Alternatively, or in addition, the toxin may simply rely on paracellular movement. Large molecules, such as immunoglobulins as well as formed blood elements, can escape the vasculature by diffusing between cells, so it would be hard to rule out passive mechanisms as at least part of the explanation for toxin escape from the vasculature. The role of active mechanisms, if any, remains to be identified and quantified.

In contrast to peripheral vasculature in the vicinity of target organs, the blood-brain barrier is not permeable to the toxin. This accounts for the fact that the signs and symptoms of botulism can be almost wholly explained on the basis of peripheral cholinergic blockade. It is frightening to imagine how devastating the disease would be if botulinum toxin could freely enter the central nervous system and produce widespread paralysis of cholinergic transmission.

The fact that the blood-brain barrier is comparatively impermeable may provide clues to the differences between central and peripheral vasculature. For example, the combination of cells (and messengers) that constitute the blood-brain barrier are known to result in increased patency of tight junctions between endothelial cells. This could create a physical obstacle to paracellular movement. However, it is also possible that something in the complex evokes changes in the expression of endothelial receptors and/or transcytotic mechanisms. Clearly, there is much that remains to be done to clarify the relationship between botulinum toxin and endothelial barriers.

PERIPHERAL NEUROMUSCULAR JUNCTION

Botulinum toxin can block exocytosis at all peripheral cholinergic sites, including neuromuscular junctions, ganglia of the sympathetic and parasympathetic nervous system, postganglionic parasympathetic sites, and those anomalous postganglionic sympathetic sites that release acetylcholine. Of these various sites, the neuromuscular junction is the one that has received the greatest amount of research and clinical attention. Therefore, it can serve as a model to illustrate the steps in toxin action.

Attempts to clarify the neuromuscular blocking properties of botulinum toxin can be divided into two distinct phases. The earlier phase was initiated by the

pioneering work of Burgen and his colleagues (56). They were the first to use isolated neuromuscular preparations to study toxin action, and they were among the first to pinpoint the process of transmitter release as the likely functional process poisoned by the toxin. Their work was truly the stimulus for more than a quarter century of research, mainly by neurobiologists, aimed at finding an explanation for blockade of acetylcholine release.

The second distinct phase began with the publication of a series of experiments and an associated model that delineated each of the major steps in toxin action (14, 15). This represented a departure from earlier work in two respects. First, most prior efforts to localize the site of toxin action had focused on the membrane, due in part to the presumption that a large protein such as botulinum toxin could not reach the cell interior. The new model offered precisely the opposite view, suggesting that the toxin was internalized to act in the cytosol. Second, as noted above, the earlier phase of toxin research was almost wholly the province of neurobiologists, whereas the later phase benefited enormously from the contributions of investigators from many fields.

As originally illustrated, the model showed the toxin progressing through the following steps: binding to the plasma membrane, receptor-mediated endocytosis, escape from the endosome, and expression of toxicity in the vicinity of the plasma membrane. This model was originally described as having three steps, i.e., the binding step, the internalization step, and the intracellular poisoning step. This perspective was governed by the emerging body of work that identified three functional domains in the toxin molecule in which the carboxyterminal half of the heavy chain is crucial to binding, the aminoterminal half of the heavy chain mediates translocation into the cytosol, and the light chain possesses the enzymatic moiety that cleaves substrates essential for exocytosis (see below for details). More recently, the model has been described as having four steps, which is the way the model was originally drawn (14, 15). The four-step model retains the concepts of initial binding and later enzymatic activity, but it divides productive internalization into two events: receptor-mediated endocytosis and pH-induced translocation across the endosome membrane.

Advances in understanding are coming at an ever increasing pace, and, as a result, the three/four-step model is being overwhelmed by new information. Each of the originally envisioned steps is now itself being divided into multiple steps, and some of these "substeps" have themselves become areas of intense investigation. It is not possible to describe in detail the richness of research that has arisen in relation to each step or substep. However, it is possible to convey at least a general sense of the complex phenomenon of toxin-induced neuromuscular blockade.

Binding to the Receptor

The receptor for botulinum toxin at the neuromuscular junction has not been unequivocally identified. The fact that the toxin is so potent, combined with the fact that there is so little tissue at peripheral nerve endings, has made receptor isolation and characterization quite challenging (but see References 57, 58). As a result, two

alternative approaches have been pursued, and the combined data have been used to formulate hypotheses. The first of these does not involve classical techniques of recepter isolation and characterization. Instead, neuromuscular preparations and/or the toxin have been submitted to a variety of manipulations, ranging from pharmacologic pretreatment to genetic modifications, and the effects of these manipulations on toxin-induced blockade have been used to deduce characteristics of receptors. In the second approach, toxin binding has been studied in tissues such as brain, and the results have been extrapolated to peripheral cholinergic nerve endings.

One of the earliest studies that employed pharmacologic pretreatments reached a conclusion almost identical to that of the most recent studies that employed genetic modifications. The earlier study involved pretreatment of toxin with various membrane components on the assumption that any molecule behaving like a receptor would bind to the toxin and diminish its subsequent potency on the neuromuscular junction. This work concluded that a sialic acid–containing molecule, and perhaps a ganglioside, might be implicated in toxin binding (59, 60). Numerous authors using variations on this approach have reached the same conclusion (61–68). In a similar line of study, it has been shown that lectins possessing affinity for sialic acid antagonized binding and onset of paralysis from subsequent addition of botulinum toxin (69).

A more recent approach to the problem has employed much more sophisticated techniques. Two groups have generated knockout mice that lack enzymes essential for biosynthesis of gangliosides (and possibly related molecules as well). Interestingly, neuromuscular preparations excised from these mutant animals were impressively resistant to toxin (70, 71). In a closely related type of study, investigators were able to show that pretreatment of an immortalized neural cell line with a drug that blocks ganglioside synthesis produced marked resistance to the toxin (72).

The authors of all the cited studies agree that a sialic acid–containing molecule, and possibly a ganglioside, is implicated in toxin binding, but there is no consensus on the exact role played by this molecule. The potentially straightforward concept that a ganglioside is the true receptor encounters resistance for a number of reasons. To begin with, gangliosides are widely distributed in cell membranes, but the toxin acts selectively on cholinergic nerve endings. It is unclear how gangliosides could account for this selectivity of action. Next, the affinity constants that describe the interaction between gangliosides and toxin do not appear adequate to account for the dramatic potency of botulinum toxin. One final point to consider is that toxin binding to receptors appears to be serotype-specific (see below). Thus, for example, serotype A and serotype B each bind to apparently unique sites, and, as a result, there is little cross-antagonism of binding. It is not obvious how gangliosides can account for observations on serotype-specific binding (57, 58).

One possible way to reconcile these seemingly disparate data and concepts is to propose that binding may actually be a two-step phenomenon (73). In the first step, toxin associates with the plane of the membrane. Conceivably, a ganglioside could play this role. Next, the low-affinity complex migrates laterally until it

interacts with a high-affinity binding site. The latter interaction would then allow for subsequent events, such as receptor-mediated endocytosis.

This model certainly has appeal, but it cannot be fully evaluated until the high-affinity binding site(s) have been identified. This brings to light the body of work that has been done on toxin binding to neuronal membranes, mainly of central nervous system origin (for reviews of earlier work, see References 27, 74). There are three main themes that have emerged from these studies: (*a*) Botulinum toxin binds with high affinity to isolated membrane preparations; (*b*) the binding domain of the toxin is localized within the heavy chain; and (*c*) with the exception of serotypes C and D, which are closely related, other toxin serotypes each have their own binding sites that are partially or wholly unique. These general concepts have carried through to the present time, although there are some fine points that are still not resolved. One example of this relates to the minimum domain that governs binding. Work done on the isolated neuromuscular junction suggests that the heavy chain is necessary but not sufficient for high-affinity binding to receptors that mediate productive internalization (75). This contrasts with work on spinal cord cultures, which indicates that the carboxyterminal half of the heavy chain is both necessary and sufficient for binding and internalization (76).

More recently, studies of toxin binding to neuronal and nonneuronal membranes have given rise to an hypothesis about the identity of the receptor for serotype B. It has been proposed that synaptotagmin II may be the high-affinity binding site (77–79), and, furthermore, it has been demonstrated that toxin binding to this molecule is enhanced by complex gangliosides (80, 81). This is an interesting idea and there are data to support the hypothesis (77–81), but there are also reasons to be cautious. Perhaps the major concern is one that is reminiscent of earlier work on gangliosides. Synaptotagmin II has been found to be present in every nerve ending in which it has been sought, regardless of neurotransmitter. This raises the question of how botulinum toxin type B can act selectively on cholinergic nerve endings, and it further raises the question of how toxin receptors can be serotype specific.

In spite of these uncertainties, it must be reported that work on identification of the receptor(s) for botulinum toxin is moving forward and, in some cases, with a high level of sophistication. One of the most exciting advances has been the crystallization of botulinum toxin and a determination of its three-dimensional structure, which has been achieved for serotypes A and B (82, 83). This, in turn, has allowed investigators to identify lectin-binding domains in the heavy chain that may be associated with binding to gangliosides. Although this line of investigation falls outside the scope of the present review, this work represents some of the most elegant structure-function analyzes in the history of botulinum toxin research (for representative papers in this field, see References 84–89).

Receptor-Mediated Endocytosis

This step is the one that has been least examined by botulinum toxin workers. Almost by default, investigators in this field have assumed that the process of

receptor-mediated endocytosis of toxin is essentially the same as that of most ligands that are internalized by cells. This might be true, but there is at least one other possibility that warrants serious consideration. Exocytosing nerves have a vigorous and well-developed mechanism for membrane retrieval. This is thought to be part of an overall mechanism in which synaptic vesicle membrane melds with plasma membrane, and this membrane is later retrieved to reform vesicles (90, 91). It is plausible that the retrieval phase of the vesicle recycling mechanism is the route for toxin entry into nerve endings.

There are several lines of investigation that may implicate vesicles, two of which are cited here. The hypothesis that synaptotagmin II serves as a receptor for one or more serotypes would suggest that vesicles are involved because this molecule has a free domain that is exposed in the lumen of vesicles. Therefore, exocytosis would "exteriorize" the molecule for a brief period of time, during which the putative high-affinity binding step would occur, and then both synaptotagmin and the toxin would be internalized during membrane retrieval.

There may already be a good model for this hypothetical process. Investigators have prepared labeled derivatives of synaptotogmin antibodies then demonstrated that these ligands can be used for visual monitoring of membrane retrieval and reformation of intraneuronal vesicles (92). At least hypothetically, the path followed by the antibody and the path followed by the toxin could be the same.

This analogy has appeal, but at the same time, it has given rise to questions. In unpublished studies (S. Kozaki & L.L. Simpson), antibodies against synaptotagmin II were tested as potential antagonists of botulinum toxin type B at the murine phrenic nerve-hemidiaphragm preparation. The results indicated that the antibody afforded no protection. This means that not only does the antibody binding site not overlap with the toxin binding site, but also that the physical obstruction of a large antibody molecule (approximately160 kDa) does not hinder access by the toxin, which is also a large molecule (approximately 150 kDa). This unusual outcome does not necessarily disallow the hypothesis that synaptotagmin II is the receptor, but it does raise questions that must be answered.

The second line of research pertaining to synaptic vesicles has already been mentioned above. There are morphologic studies on toxin internalization by central nervous system preparations that implicate small synaptic vesicles in the process of binding and internalization. To place this work in the context of the discussion above, one must note that (*a*) the hypothesis that synaptotagmin II is the high-affinity receptor necessarily implicates synaptic vesicles in the process of internalization, whereas (*b*) the hypothesis that synaptic vesicles mediate internalization does not necessarily implicate synaptotagmin II as the receptor.

pH-Induced Translocation

The substrates for botulinum toxin are in the cytosol, and thus it is imperative that the catalytically active domain escape the lumen of the endosome. Ironically, the proposed mechanism for escape was deduced before the intracellular site of toxin

action and the true nature of its enzymatic activity were determined. The deduction was based largely on analogy.

There are many substances/agents that enter cells by receptor-mediated endocytosis and shortly thereafter escape endosomes to act locally in the cytosol. Among the first such agents to be identified were viruses, such as Simliki Forest virus, and microbial toxins, such as diphtheria toxin. For these agents, escape is known to be a pH-dependent phenomenon. These viruses/toxins have occult hydrophobic domains that are buried or inaccessible at normal pH but become exposed when pH is lowered. The newly exposed domains interact with the endosome membrane in a manner that promotes translocation of the active region to the cytosol. This mechanism is ideally suited to eukaryotic cells that have early endosomes with a proton pump that rapidly acidifies the lumen. Therefore, endocytosed agents, such as toxins that have the capacity for pH-induced translocation, can gain rapid access to the cytosol (93).

The possibility that this mechanism was applicable for botulinum toxin was tested by pretreating neuromuscular junctions with drugs, such as chloroquine, that have the ability to neutralize endosomal pH (94). This proved to be a highly effective way to antagonize toxin action, presumably because it left toxin "stranded" in the endosome. This concept was confirmed by testing other drugs that mimic chloroquine in neutralizing endosomes (e.g., methylamine) (95), or by using drugs that inhibit the membrane proton pump and thus inhibit acidification of the endosome (e.g., bafilomycin) (96).

The concept that botulinum toxin is productively internalized by pH-induced translocation is now universally accepted, but the exact nature of the membrane-penetrating event has proved somewhat elusive. The first study that sought to address this point utilized artificial membranes and measured changes in resistance as a function of the location of the toxin (*cis* or *trans*) and the pH of the two chambers (97). This work culminated in the discovery that the presence of toxin and low pH on the same side of the membrane produced increases in conductance typical of channel formation. Others have confirmed this work and that it is the aminoterminus of the heavy chain that is crucial to the phenomenon. These findings could be interpreted to mean that the heavy chain can be induced to form channels that are "escape hatches" for the enzymatic domain to reach the cytosol.

Later studies have resulted in a modified version of the channel model. Rather than envisioning a complete and "leak-proof" channel, this work has advanced the idea that the heavy chain forms a cleft. In this incomplete channel model, the light chain nestles against the heavy chain, while both chains expose their hydrophobic regions to the lipid core of the membrane (for a review of the model, see References 16, 18). Although similar in some respects to the channel model, the cleft model does have one major distinction. In the former, the channels that can be detected as increases in conductance are closely analogous to the channels that allow passage of light chain. In the latter, the channels may be an after effect that reflects the residual changes in the membrane produced by the cleft/passage.

In the most recent contribution to this area, a new and interesting perspective has been added. It has been proposed that the most appropriate explanation for the role of the heavy chain is as a dual channel and chaperone (98). In this model, the channel formed by the heavy chain participates in translocation, but the heavy chain must also function in a dynamic role to escort the light chain across the membrane.

Although these various models have their individual distinctions, it is nevertheless true that they all share certain common features. They are in agreement that the translocation step can be fractionated into at least six distinct events. These include (*a*) a pH-induced change in toxin structure that results in exposure of previously occult hydrophobic domains; (*b*) insertion of the toxin into the endosome membrane; (*c*) translocation of the light chain from the lumenal to the cytosolic surface of the membrane; (*d*) reduction of the single disulfide bond that links the heavy chain and light chain; (*e*) uncoupling of the noncovalent forces that bond the heavy and light chains, with subsequent separation of chains; and (*f*) restoration of light-chain structure associated with movement from an acidic environment (endosome) to a more neutral environment (cytosol).

There is yet another sequence of events that may be superimposed on the translocation process. The light chain of the toxin is a zinc-dependent endoprotease (see below). The acid-induced changes in light-chain structure could produce loss of the essential zinc by two mechanisms: protonation of side groups that bind zinc and/or loss of helical structure necessary for positioning of groups that coordinate the binding of zinc. If this were to occur, then there would have to be a reactivation event during which the light chain would capture an endogenous zinc from the cytosol of nerve endings (99).

Intracellular Poisoning

There is a rich history of early neuropharmacologic and neurophysiologic research aimed at defining toxin action. Much of this could be described as an elimination process; in other words, work was done to rule out such possibilities as blockade of nerve impulse propagation, blockade of calcium channels, blockade of acetylcholine synthesis, and blockade of vesicle loading (for reviews of early work, see References 15, 100, 101). This body of work culminated in the realization that botulinum toxin blocks one of the final steps in exocytosis, and this blockade affects both spontaneous and evoked transmitter release.

Neurophysiologic studies originating in a number of laboratories provided the first real insight into toxin action. More precisely, all investigators were in agreement that the various serotypes blocked transmitter release, but several investigators who examined the phenomenon more closely found that the specific nature of the blockade was not the same for all serotypes. Taking serotype A and serotype B as prototypes, the major distinctions were found to be:

- Both block spontaneous quantal release of acetylcholine, but serotype A reduces frequency of spontaneous quanta by approximately two orders of

magnitude, whereas serotype B reduces frequency by approximately one order of magnitude (102–104).

- Both block evoked transmitter release. However, physiologic (e.g., rapid nerve stimulation) and pharmacologic (e.g., high calcium, aminopyridines) techniques that promote exocytosis have a differential effect on the two serotypes. This is due to the fact that these procedures evoke synchronous release of quanta that can sum to produce a postjunctional response in tissues poisoned with serotype A, but these same procedures evoke asynchronous release and therefore fail to produce postjunctional responses in tissues poisoned with serotype B (105–110).
- α-Latrotoxin can evoke explosive release of acetylcholine in quiescent nerves. It retains this action on nerve endings poisoned with serotype A, but not those poisoned with serotype B (111, 112).

As the literature comparing the various serotypes continued to grow, it became apparent that there were two groups: Serotypes C and E appeared to be similar to serotype A, whereas serotypes D and F were similar to serotype B. Although it was not fully appreciated at the time, these careful and thoughtful electrophysiologic studies anticipated later discovery of the biochemical mechanism of toxin action.

The key to unlocking the true nature of the toxin occurred when the gene sequences and deduced amino acid sequences of the several serotypes were determined (22, 24, 113–115). A comparison of these data with those of previously sequenced proteins of known function revealed that all serotypes have a histidine motif that is characteristic of zinc-dependent metalloendoproteases (116). This, in turn, led to the rapid discovery that all seven serotypes cleave at least one of three intracellular substrates that are essential for exocytosis (116–125). Serotypes A and E cleave SNAP-25; serotype C cleaves syntaxin as well as SNAP-25; and serotypes B, D, F, and G cleave VAMP, also known as synaptobrevin. These three polypeptides, as well as other membrane and cytosolic components, interact to form a multimeric complex that mediates transmitter release. Toxin-induced cleavage greatly diminishes the efficacy of the complex.

The combined discoveries that the toxins are endoproteases and that they cleave polypeptides needed for transmitter release represent major advances in the understanding of toxin action, but they also created something of a puzzle. How could seven toxin serotypes that have a common origin, a similar macrostructure, and a shared zinc-dependent endoprotease action have evolved to attack three apparently distinct substrates? This matter became even more puzzling when it was discovered that each serotype cleaves a unique peptide bond in two senses: (*a*) Serotypes that act on the same substrate (e.g., A and E acting on SNAP-25) cleave different peptide bonds (Gln^{197}–Arg^{198} for serotype A; Arg^{180}–Ile^{181} for serotype E), and (*b*) each serotype cleaves only one peptide bond in its substrate even though the sequence of the scissile bond may be repeated elsewhere in the substrate (e.g., the Gln-Arg sequence and the Arg-Ile sequence occur more than once in SNAP-25).

At least a partial answer emerged when work was done to identify the smallest polypeptide that could serve as a fully competent substrate for each toxin serotype. An inspection of these polypeptides revealed that each of them has a common motif. Four such motifs are found in SNAP-25, two are in syntaxin, and two are in VAMP (126). If it is hypothesized that these common motifs are binding sites for toxin, then the work provides two insights into toxin action. First, substrates for the toxin are not as distinct as once thought. The shared structural motif in SNAP-25, syntaxin, and VAMP helps to explain why there can be three substrates for toxin action. Second, it is no longer a mystery why each substrate has only one scissile bond even though there are repeats of this bond. By virtue of binding to common motifs, and perhaps to other sites as well, the light chains of each serotype become oriented in such a way that there is a preferred cleavage site that makes contact with the catalytic site.

Although it is clear that progress is being made in defining the interaction between toxin and substrate, there is still much to be learned. It is not yet known whether all points of contact on each substrate have been identified. Even less is known about the toxin. To date, none of the light chains has been fully mapped for the domains that bind to and then cleave substrate. However, when this mapping of structure and function is complete, it will no doubt point to yet more steps in toxin action (Figure 3).

TERMINATION OF ACTION

The duration of action of botulinum toxin varies with serotype. Basic science studies on laboratory animal models as well as analyses of case reports on human patients indicate that serotype A has the most sustained action. Efforts have been made to determine why serotype A has such a lengthy duration of action (see, for example, References 29–31, 127–130). The information that emerges from this work will be helpful, especially in the context of using botulinum toxin as a therapeutic agent, but there is an even more fundamental issue that has not been resolved. No one yet knows the mechanism that accounts for termination of toxin action. There is no evidence that the light chain is transported across the plasma membrane to reach the extracellular space. Therefore, intracellular disposition of the molecule seems likely. This suggests that diffusion, proteolysis, or a combination of the two contributes to loss of activity.

The nerve terminal region of motor nerves is quite small compared to the total volume of motoneurons, especially those that govern limb function. This means that by merely diffusing out of terminals and into the axon and eventually into the soma and dendrites, the light chain would experience an increase in volume of distribution of orders of magnitude. In fact, unless there is a mechanism or process that "traps" the light chain in terminal boutons, diffusion into other parts of the cell, with an attendant decrease in concentration, must occur.

There may also be a metabolic process for degrading the light chain, such as cytosolic or lysosomal proteolysis. It is unlikely that exoproteases acting in the cytosol could be a significant factor (Figure 4). Aminopeptidases would have

difficulty attacking the light chain because (*a*) the terminal residue is proline (82), which is often resistant to exoprotease attack, and (*b*) the conformation of the light chain hinders access to the aminoterminus (82, 83). The susceptibility of the carboxyterminus cannot be stated with certainty because the exact location in space of the terminal residues has not been determined. However, cytosolic metabolism by a carboxypeptidase is not likely to be important because (*a*) a large segment of the carboxyterminus can be removed without abolishing catalytic activity (131) and (*b*) the conformation of the light chain may hinder the extent of carboxypeptidase action that would be needed to abolish enzyme activity (82, 83).

Endoprotease attack, and particularly that which can occur in the acidic environment of lysosomes, can cause extensive proteolysis and loss of light-chain activity (H. Kouguchi & L.L. Simpson, unpublished data). If this were a contributing factor in the termination of toxin action, it would represent an interesting turn of events. The first step in the lengthy sequence of events that underlies oral poisoning by botulinum toxin is absorption from the gut. One of the major reasons why the toxin is active by the oral route and can be absorbed into the general circulation is that it is synthesized as a part of a complex with auxiliary proteins (HA and NTNH). This intertwined complex is highly resistant to proteases in the gut, which primarily means endoproteases. However, by the time the toxin reaches the cytosol of target nerves it has lost the protective auxiliary proteins and it has probably lost the heavy chain as well. This means that at the first step in toxin action, the molecule is highly resistant to protease attack, but at the final step in toxin action, the residual polypeptide is highly vulnerable to endoprotease attack. This would appear to be a fitting life cycle for such a remarkable molecule.

CONCLUDING REMARKS

Botulinum toxin is a truly unique substance. The fact that the molecule is so remarkably potent contributes to this uniqueness, as does the fact that the toxin must progress through a lengthy and complex sequence of events during the course of natural poisoning.

Substantial progress has been made in characterizing the steps that underlie toxin absorption from the gastrointesinal system and respiratory system. Even more progress has been made in identifying and describing each of the events that account for toxin-induced blockade of transmitter release. However, there are at least two aspects of toxin action that are not well understood. After the toxin has been absorbed, but before it reaches the cholinergic nerve ending, it must escape the vasculature. There is no compelling body of research that explains the mechanism by which the toxin crosses vascular endothelial barriers. Another area of uncertainty pertains to termination of action. The ability of the toxin to produce blockade of transmission is limited in duration, but the specific mechanisms that cause loss of activity have not been clearly established.

When one examines the field of botulinum toxin research, there is one particularly striking message that emerges. Each major advance in our understanding of toxin action has led to major advances in our understanding of cell biology.

Work on toxin absorption has contributed to knowledge of epithelial cell biology, and work on toxin-induced blockade of exocytosis has had a dramatic effect on the study of nerve ending function. This pattern is likely to continue. Thus, efforts to describe peripheral distribution of the toxin will no doubt contribute to an understanding of endothelial cell biology. Similarly, research on termination of toxin action will provide insights into intracellular metabolic processes. In the aggregate, this provides a powerful motive to intensify the effort to identify all the major steps in toxin action.

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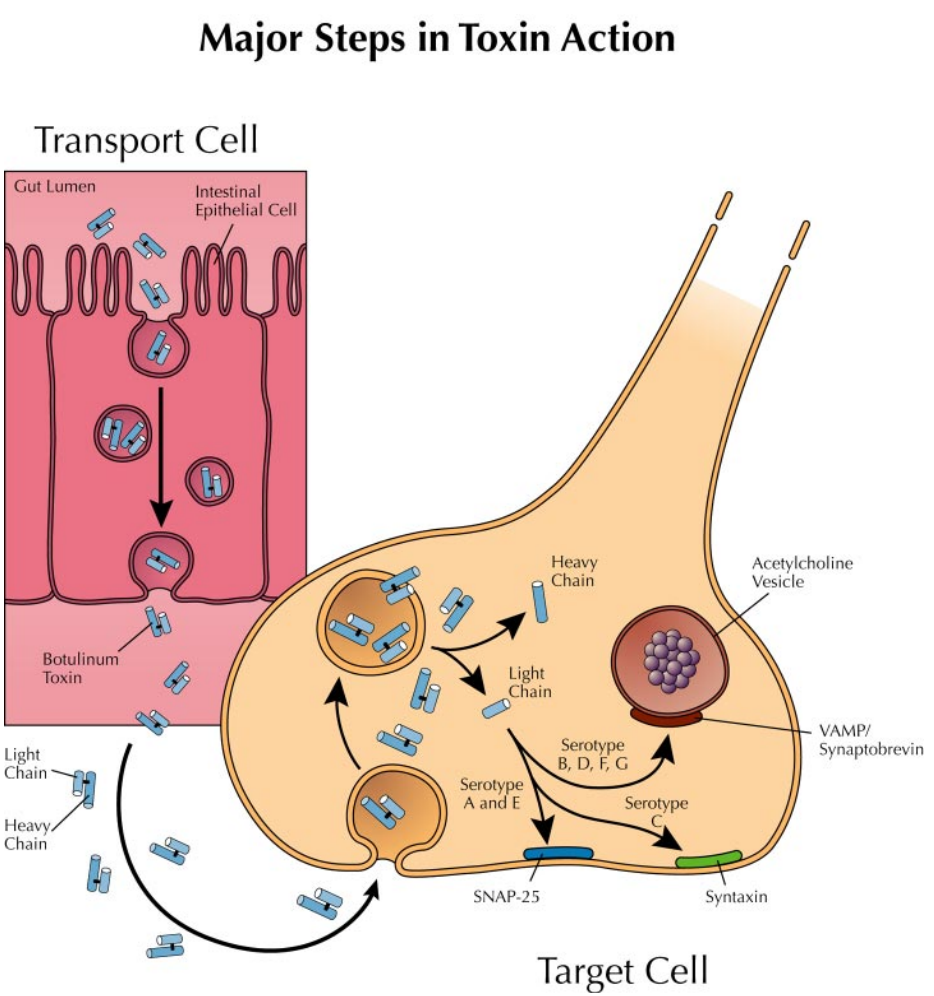

Figure 1 There are a large number of steps in botulinum toxin action, most of which occur at two sites. Epithelial cells, such as those in the gastrointestinal system, can be viewed as transport cells. They bind the toxin and carry it from the lumen of the gut to interstitial fluid and, ultimately, the general circulation. Peripheral cholinergic nerve endings, such as those at the neuromuscular junction, are target cells for toxin action. Botulinum toxin binds to these cells and is productively internalized to reach the cytosol. The light chain of the toxin is an endoprotease that attacks several polypeptides that are essential for transmitter release.

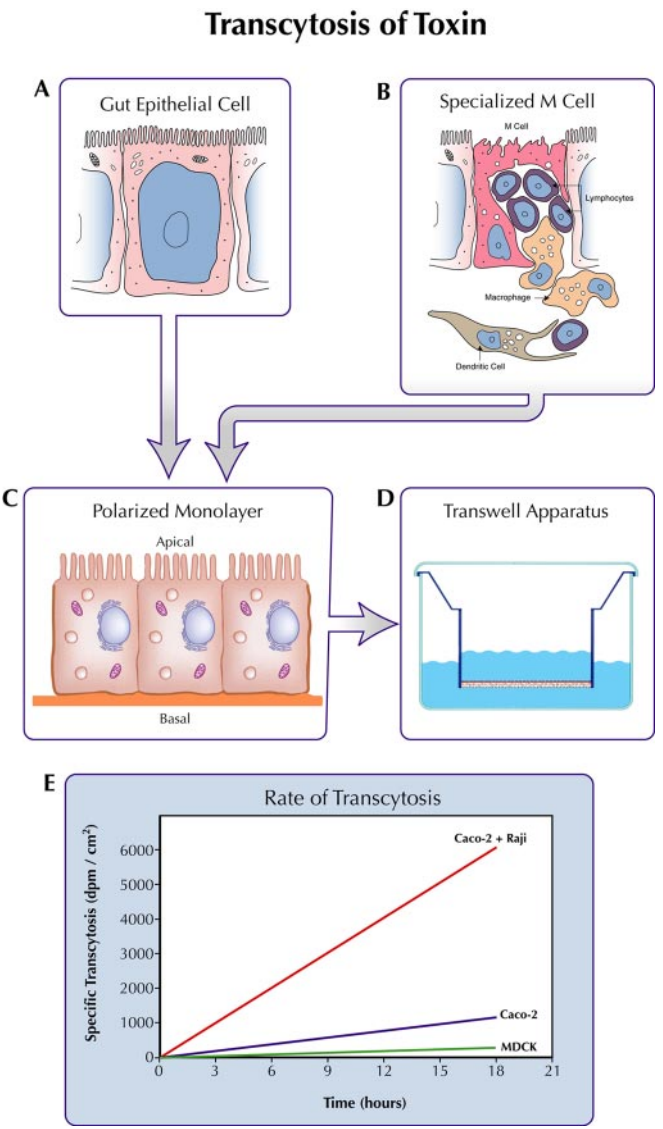

Figure 2 Transcytosis studies with toxin have been done on two types of human gut epithelial cells: (*A*) absorptive enterocytes and (*B*) M cells that are normally part of the Peyer's Patch complex. Normal absorptive cells form polarized monolayers (*C*), and these can be plated in a transwell apparatus to study toxin transport from the apical surface to the basolateral surface or vice-versa (*D*). M cells can be generated by treating normal enterocytes (e.g., Caco-2 cells) with Raji lymphocytes. Polarized kidney cells (MDCK) have relatively little ability to transport toxin (*E*). Normal enterocytes have variable abilities to transport the toxin, from relatively low (Caco-2; illustrated) to relatively high (T-84; not illustrated). When low transport enterocytes are converted to M cells by addition of Raji lymphocytes, the rate of transcytosis is increased (*E*).

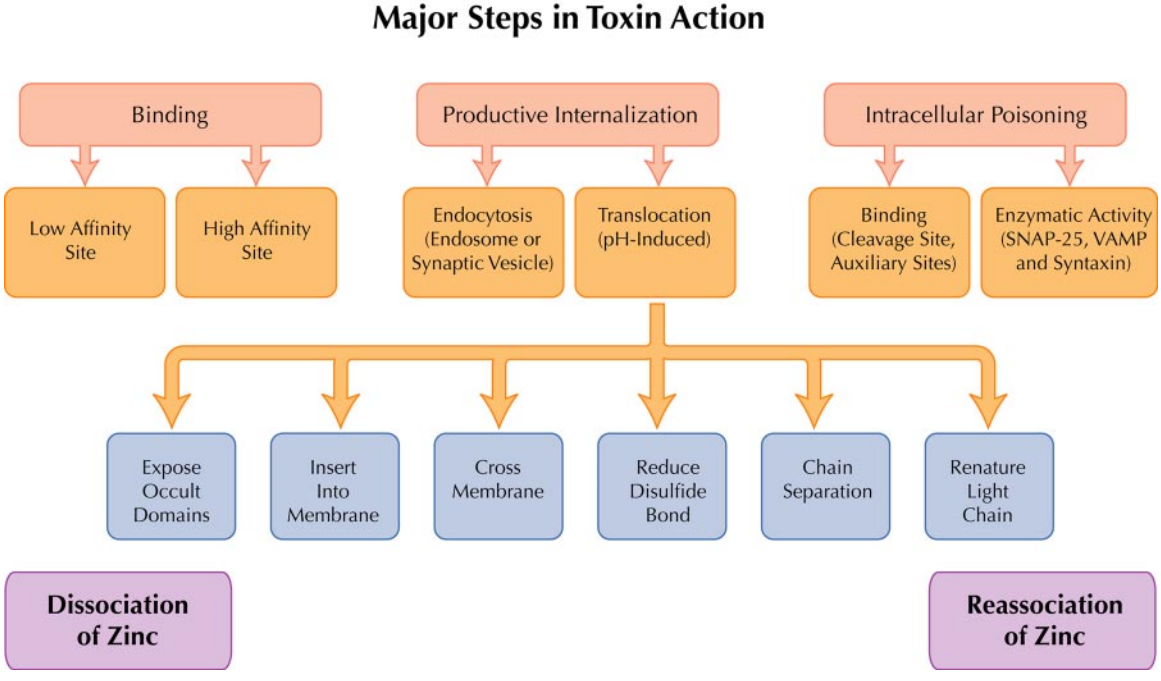

Figure 3 Research on botulinum toxin has led to the discovery of an ever-increasing number of steps in the overall mechanism of action. At the neuromuscular junction, the three main steps are binding, productive internalization, and intracellular poisoning. Each of these main steps can be further subdivided into two steps, and one of these (pH-induced translocation) can itself be subdivided into a series of steps. During the progression of events associated with translocation, there may be an initial loss of zinc owing to pH-induced changes in the structure of the molecule. If this does in fact occur, then the toxin would have to reassociate with zinc that is found in the neuronal cytosol.

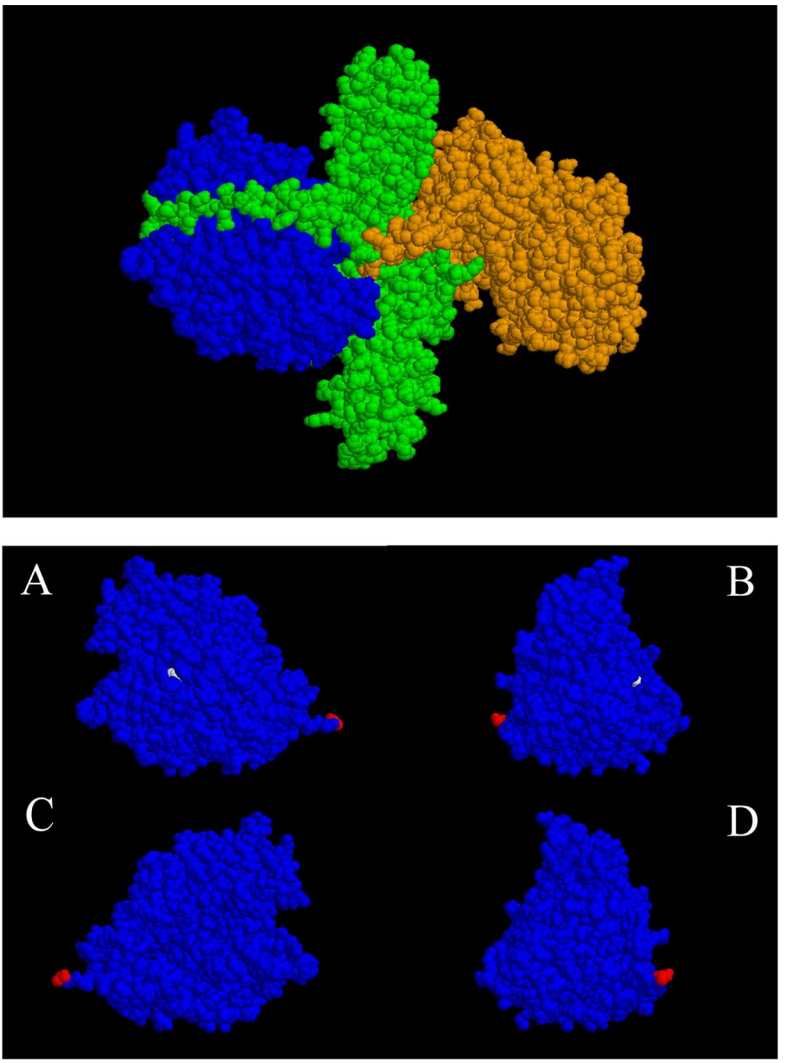

Figure 4 The three-dimensional structure of botulinum toxin type A has been determined (*top*) (82). The molecule is comprised of three functional domains: the carboxyterminus of the heavy chain (*orange*) participates in binding, the aminoterminus of the heavy chain (*green*) participates in productive internalization, and the light chain (*blue*) is a zinc-dependent endoprotease (24). It is possible that the light chain itself can be a substrate for proteolysis, and this proteolytic cleavage could terminate toxin action. It is unlikely that exoprotease attack is significant. The bottom part of the figure shows the isolated light chain in the same orientation illustrated above in the holotoxin (*A*), and then in progressive 90° rotations (*B*, *C*, *D*). As the figure shows, the aminoterminus (*white*) appears shielded from exoprotease attack and the carboxyterminus (*red*) is only partially vulnerable to attack (see text for additional details). On the other hand, the light chain would be susceptible to a variety of endoproteases.