CMLS, Cell. Mol. Life Sci. 56 (1999) 47–61
1420-682X/99/020047-15 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Botulinum toxin as a carrier for oral vaccines

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Received 29 December 1998; received after revision 22 March 1999; accepted 24 March 1999

Abstract. Botulinum toxin is an unusually potent substance that acts on the nervous system to produce the clinical outcome of flaccid paralysis. To produce this effect, the toxin ordinarily proceeds through two separate but essential sequences of events. During the first, the toxin is ingested, it traverses a portion of the gastrointestinal system and then it is transcytosed from the lumen of the gut to the general circulation. During the second, circulating toxin binds to peripheral cholinergic nerve endings, it is endocytosed and then it acts as a metalloendoprotease to cleave polypeptides that are essential for exocytosis. Although botulinum toxin is antigenic, it ordinarily does not evoke an immune response during or after cases of oral poisoning. This is due to the fact that the dose of toxin that produces flaccid paralysis—and potentially death—is less than the dose needed to evoke an antibody response. In the recent past, the techniques of molecular biology have been used to generate an expression product of botulinum toxin that retains the ability to escape the gut and reach the general circulation, retains the ability to evoke an immune response, but has lost the ability to produce neurotoxicity. This modified toxin may have two clinical applications. The expression product itself may have utility as an oral vaccine against botulism. Beyond this, the modified toxin, or a truncation mutant of the toxin, may have utility as a carrier in the construction of other oral vaccines. Both potential applications could lead to the expression of oral vaccines in common foods.

Key words. Botulinum toxin; gastrointestinal system; nervous system; antibodies; oral vaccines; chimeric vaccines; immunity.

Introduction

One of the major challenges of modern medicine is the development of drugs that can be administered by the oral route. Of the various drugs that might be given this way, peptide vaccines have proved to be especially problematic. This is particularly true when the intent is to produce systemic immunity as opposed to mucosal immunity.

The difficulties associated with development of oral peptide vaccines generally fall into three categories, as follows:

1) *Vulnerability*—most peptides undergo degradation when exposed to conditions of low pH and proteolytic enzymes found in the human gut.

2) *Size*—the highly antigenic domains of many agents that produce human illness have been identified, and the size of these domains is typically too large to allow for significant nonspecific diffusion from the lumen of the gut to the general circulation.

3) *Molecular character*—to date, it has proved extremely difficult to design peptide vaccines that will (i) bind exploitatively to receptors in the gut, (ii) undergo

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active transport across the gut wall and (iii) be delivered to the general circulation in a biologically intact form. In spite of these difficulties, considerable effort is being invested in the search for oral vaccines. This effort is justified by the fact that an impressive array of human illnesses are due to agents against which systemic immunity would offer protection or even resistance. These agents range from viruses and bacteria to biological toxins and components of venom. An additional and compelling motive relates to recent accomplishments in molecular biology. Substantial progress has been made in inserting genes that can express exogenous proteins in edible plants. Research of this nature raises the possibility that vaccination could be achieved by ingesting foods that express peptide vaccines.

Although the concept of using an engineered food such as a potato or a banana as a vector for widescale vaccination is appealing, the concept does nothing to overcome the difficulties alluded to earlier. In general, peptides given orally do not have the ability to traverse the gut and reach the general circulation. In fact, the obstacles to success have been so substantial that many authors have described the process of developing oral vaccines for systemic immunity as being purely empirical. In other words, few principles have emerged that have proved to be predictive and have led to the discovery of usable vaccines. Instead, individual techniques have been tested with individual peptide antigens, and these techniques may or may not have worked. Even when they do work for a particular peptide antigen, they typically have little or no utility when applied to other intended vaccines.

The field of research dealing with oral peptide vaccines would be substantially advanced if a reliable and reproducible technique could be found for carrying peptide antigens into the general circulation. Interestingly, there is the possibility that such a technique has been found. The technique is made even more intriguing by the fact that it involves an orally active peptide that is generally considered the most poisonous substance known. This substance is botulinum toxin, which is responsible for a form of food poisoning known as botulism.

Desirable characteristics of an oral vaccine intended to evoke systemic immunity

Before considering the special qualities of botulinum toxin, it may be wise to pause for a moment and consider a broader issue. Posed as a question, this issue is, What are the characteristics that one would expect of an oral peptide vaccine that is administered to evoke systemic immunity? In reality, there may be two sets of characteristics. The first set would apply to a simple vaccine that is administered to evoke immunity against the antigenic component of that vaccine. The second set would apply to a chimeric vaccine composed of (i) a carrier that transports the vaccine from the gut to the general circulation, and (ii) an antigenic component that is intended to evoke systemic immunity.

The properties one would expect of a simple vaccine are as follows:

1) The peptide must be able to survive the harsh conditions of the gastrointestinal system, including the effects of low pH and proteolytic enzymes.

2) The peptide must possess the ability to undergo efficient translocation from the lumen of the gut to the general circulation.

3) The peptide should possess little or no ability to irritate the gastrointestinal system or to cause any form of systemic toxicity.

4) Upon reaching the general circulation, the peptide must retain the conformational and other properties that will allow it to evoke production of neutralizing antibodies.

5) Ideally, the peptide should be one that can be expressed in a common food, thus creating the prospect for an edible and efficacious vaccine.

6) Ideally, the oral vaccine—with no more than minimal alterations—should be usable by other routes of administration, such as parenteral, inhalation or transdermal.

A chimeric vaccine composed of a carrier and an antigenic component should possess all of the properties listed above, but there are additional properties that may be beneficial. The potential importance of these characteristics increases in direct proportion to the potential utility of the carrier as an agent to ferry many antigens from the gut to the general circulation. Thus, the key properties are:

1) The carrier must have relatively little or no ability to evoke mucosal or systemic immunity, and/or

2) The carrier must exist in multiple, nonoverlapping immunogenic states.

3) The carrier must be amenable to conjugating reactions that will allow association between carrier and antigenic components without compromising the ability of the carrier to achieve translocation from the gut to the general circulation and without altering the immunogenicity of the antigen.

There is one final and intriguing possibility to consider. Simple vaccines and chimeric vaccines are not necessarily separate and distinct entities. To the contrary, a simple vaccine (or some portion of a simple vaccine) may be able to function as the carrier component of a chimeric vaccine. Interestingly, this appears to be a distinct possibility with botulinum toxin. A modified version of the native toxin has already been shown to act as a simple oral vaccine [64]. And there is reason to believe that a portion of this molecule could function as the carrier component in chimeric vaccines. This would

be a novel and exciting use for a toxin that is now viewed as the most poisonous of all poisons.

General characteristics of botulinum toxin

Botulinum toxin is a remarkably potent substance produced by the organisms *Clostridium botulinum*, *Clostridium butyricum* and *Clostridium baratii* [1, 2]. *C. botulinum* produces seven different types of toxin, designated A, B, C, D, E, F and G, whereas *C. butyricum* and *C. baratii* produce only one serotype each (E and F, respectively). Regardless of origin, botulinum toxin is synthesized as a relatively inactive single-chain polypeptide with a molecular weight of ca. 150,000 (fig. 1). To become fully active, the toxin must undergo posttranslational processing, during which the molecule is cleaved by a protease ('nicked') to yield a dichain structure in which a heavy chain (ca. 100,000 Da) is linked by a disulfide bond to a light chain (ca. 50,000 Da). The dichain molecule with an intact interchain disulfide bond is the holotoxin that causes the disease botulism [4].

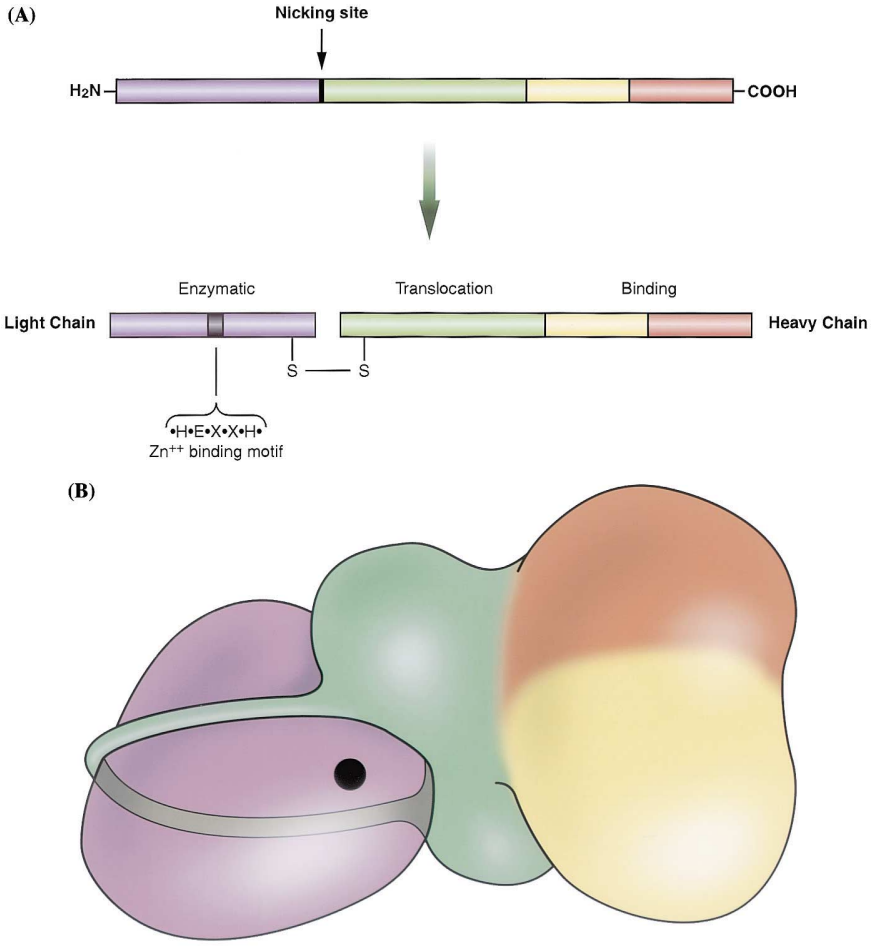

Figure 1. Schematic linear structure (*A*) and three-dimensional structure (*B*) of botulinum toxin. As indicated in *A*, the toxin is synthesized as a single chain polypeptide (ca. 150 kDa). This molecule undergoes proteolytic cleavage ('nicking') to yield a heavy chain (ca. 100 kDa) and a light chain (ca. 50 kDa) linked by a disulfide bond. Structure-function analyses suggest that the carboxy terminus of the heavy chain plays a major role in binding, the amino terminus of the heavy chain is involved in translocation and the light chain is a zinc-dependent metalloendoprotease. *B* illustrates the three-dimensional relationship of light and heavy chains. The purple region represents the light chain with a single catalytic zinc molecule (black sphere). The green portion of the structure represents the translocation domain with its beltlike encirclement of the light chain, whereas the yellow and the orange regions represent, respectively, the amino-terminal and carboxy-terminal portions of the binding domain. This illustration was modeled upon the three-dimensional structure of type A toxin [3].

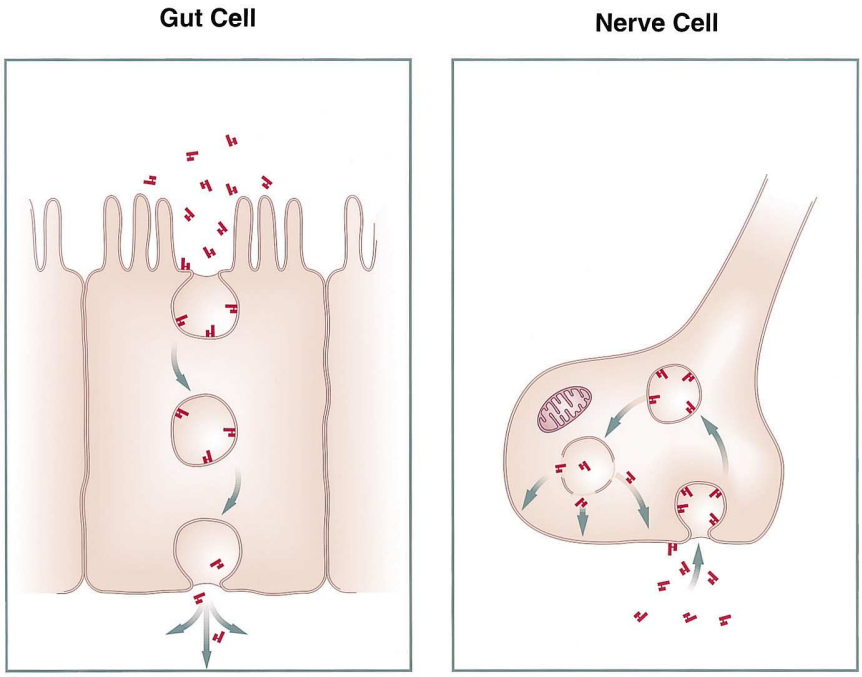

Figure 2. The toxin progresses through two separate but essential sequences of events in oral botulism. During the first, ingested toxin binds to receptors in the lumen of the gut (apical surface of gut cells), is transcytosed across these cells, then released into the general circulation (basal surface of gut cells). During the second sequence of events, the toxin binds to surface receptors on cholinergic nerve endings, undergoes receptor-mediated internalization and pH-induced translocation out of the endosome, then enters the cytosol. Light chain of the toxin that reaches the cell interior acts as an enzyme to cleave substrates that are essential for the process of exocytosis.

Although there are seven immunologically distinct toxin types, only three of these are typically associated with human illness (types A, B and E). Each of these serotypes has its own set of distinguishing characteristics, but nevertheless all three adhere to the same general scheme of events in causing human illness. This scheme carries with it the requirement that the toxin be able to penetrate a series of membranes. The membranes of principal interest are those in the gut and those in the nervous system (fig. 2).

The overwhelming majority of cases of botulism are due to oral poisoning. Patients can either ingest preformed type A, B or E toxin, or they can ingest organisms that manufacture the toxin while in the gut. In either case, toxin escapes from the gut to reach the general circulation. Escape from the gut requires penetration of epithelial membranes.

The target organs for toxin action are peripheral cholinergic nerve endings, and particularly the cholinergic neuromuscular junction. Enormous strides have been made in our understanding of toxin action on nerve cells, including discovery of the precise mechanism that accounts for poisoning. Botulinum toxin is a zinc-dependent metalloendoprotease that acts in the cell interior to cleave peptides that are essential for exocytosis. For the toxin to exert this action, it must penetrate the membranes of peripheral cholinergic nerve endings.

To date, relatively little work has been done to characterize the mechanism by which the toxin crosses membranes in the gut. The large size of the toxin molecule is a strong indicator that passive diffusion between cells is unlikely. The remarkable potency of the toxin strongly indicates that passive transport (viz. bulk phase diffusion) is also unlikely. The most plausible mechanism for toxin penetration of gut cells is specific binding followed by transcytosis.

During the recent past, the authors have found that botulinum toxin binds specifically to receptors on the mucosal side of polarized gut cells grown in monolayer. Bound toxin is actively transported across cells and is delivered intact and unmodified on the serosal side of monolayers. Thus, transcytosed toxin is lethal when administered to animals, and it causes neuromuscular blockade when added to phrenic nerve-hemidiaphragm preparations. As explained more fully in the next section, this is likely to be the route followed by the toxin as it migrates from the gut to the general circulation [5].

In contrast to our knowledge of toxin interaction with gut cells, there is a substantial body of information on toxin interaction with nerve cells. Most of this information relates to a model of toxin action proposed by the authors [1, 2, 6]. According to this model, botulinum toxin proceeds through a sequence of three steps to produce its poisoning effects. The initial step involves binding of the toxin molecule to receptors on cholinergic nerve membranes. This step, which is mediated by the carboxy-terminal half of the heavy chain, has no observable effect on cell function. Following binding, the toxin is productively internalized and eventually reaches the cell interior. This complex process, which requires the hydrophobic amino-terminal half of the heavy chain, also produces no adverse effects on cell function. During the final step, the light chain of the toxin acts in the cytosol to modify substances that are essential for exocytosis.

This model for botulinum toxin action is accepted by all workers in the field, and as a result current research focuses on efforts to define more clearly the three steps in toxin action. For example, the binding step has been studied by using iodinated toxin and nerve membrane preparations of central nervous system origin [7–13]. This work has produced kinetic data (e.g. K_d and B_{max} for high-affinity and low-affinity sites), and it has produced two descriptive findings. First, the various serotypes of botulinum toxin do not share the same receptor; instead, each serotype appears to have its own unique receptor, or set of receptors [14]. Second, clostridial toxin receptors appear to have at least one property in common: each receptor has an exposed sialic acid residue [15]. These kinetic and descriptive findings on receptors are useful, but there is much that remains to be learned. There is a need to isolate and characterize receptors, to clarify the role of these molecules in normal membrane physiology and to describe the molecular aspects of the interaction between receptors and toxin.

There has been considerable progress in the study of toxin internalization by nerve endings, which has resulted in the finding that internalization is a progression of two events. During the first, the toxin is internalized by receptor-mediated endocytosis. This allows the toxin to penetrate the plasma membrane, but it leaves the toxin trapped within a membrane-delimited structure. Next, the toxin penetrates the endosome membrane to reach the cytosol. This second event reflects a remarkable ability on the part of the toxin to exploit normal cell biology. Neuronal endosomes, like all eucaryotic endosomes, possess a proton pump that lowers intralumenal pH. The botulinum toxin molecule—and more precisely the amino terminus of the heavy chain—has a 'pH sensor' that detects low pH. When intralumenal pH falls to levels of 5.5 and lower, the pH sensor induces a conformational change. The most striking consequence of the induced structural change is that the toxin exposes a previously occult hydrophobic domain. This portion of the molecule inserts into the endosome membrane and promotes translocation to the cytosol.

There is a considerable amount of evidence to show that botulinum toxin relies on receptor-mediated endocytosis to cross the plasma membrane and an acid-dependent mechanism to cross the endosome membrane. The authors provided the first data to support the concept of receptor-mediated endocytosis. This involved studies with antibodies as research tools [16] and drugs that block receptor-mediated endocytosis [17, 18]. The work was nicely complemented by the morphologic studies of Black and Dolly, who used electronmicroscopic autoradiography to show internalization of labeled toxin [19, 20]. A number of laboratories have reported that botulinum toxin undergoes pH-induced changes in conformation and that these changes cause the toxin molecule to insert into membranes. The initial study, reported by the authors and their collaborators, involved electrophysiological techniques to monitor pH-induced toxin insertion into lipid bilayers [21]. This work was subsequently reproduced by several laboratories [22–24]. In another approach to the problem, both Montecucco and his associates [25, 26] and the authors and their colleagues [27] have used biochemical probes to demonstrate that low pH induces changes in toxin conformation and exposure of occult hydrophobic domains. This work has produced a consensus among investigators that endosomal acidification triggers the process that allows the toxin to escape to the cytosol.

The final step in botulinum toxin action is intracellular blockade of exocytosis. The authors proposed that this step was likely to be enzymatic in nature [6, 28], and this triggered almost a decade of intense research to identify some form of catalytic activity associated with the molecule. The culmination of this work was the discovery that botulinum toxin is a zinc-dependent metalloendoprotease [29, 30]. It has subsequently been discovered that the substrates for toxin action are SNAP-25 (synaptosomal-associated protein of 25 kDa), synaptobrevin (also know as VAMP, or vesicle-associated membrane protein) and syntaxin. These three polypeptides act cooperatively to form a multimeric complex that is essential for exocytosis. Thus, botulinum toxin cleavage of any one of these molecules blocks release of chemical mediators from cells, such as cholinergic neurons. Blockade of exocytosis produces the flaccid paralysis that is characteristic of the disease botulism.

The picture that is emerging is that botulinum toxin must proceed through two separate and independent sequences of events. During the first sequence of events, ingested toxin escapes from the lumen of the gut to

reach blood and lymph. During the second sequence of events, the toxin moves from the outside to the inside of cholinergic nerve endings. Toxin movement across gut cells is not known to produce any adverse effects on cell function, but toxin movement into nerve cells has a profound effect on cell biology. In the latter case, the toxin poisons the process of neurotransmitter release.

Botulinum toxin and the gastrointestinal system

If botulinum toxin is to be usable either as a simple vaccine or as a chimeric vaccine, there are certain characteristics of the molecule that must be retained and others that must be removed. Among those properties that must be retained, the molecule must be able to survive the harsh conditions of the gut, and it must be able to translocate from the lumen of the gut to the general circulation. Certainly, the single most important property that must be removed is the ability of the molecule to cause poisoning.

Absorption of toxin from the gut is essential to onset of disease, so it is obvious that there must be an efficient mechanism for toxin to cross gut membranes. However, surprisingly little is known about specific mechanisms that contribute to absorption [31]. Characterization of the mechanism of toxin absorption and transcellular movement has been complicated by the fact that neurotoxin by itself rarely if ever causes disease. Naturally occurring botulism is caused by toxin that is part of a complex with auxiliary proteins, including a family of hemagglutinins and a single nontoxic nonhemagglutinin protein [32–34]. It is the inclusion of auxiliary proteins in a complex with the toxin, coupled with the ability of these proteins to protect the toxin from degradation, that has been the focus of much work in the past.

Examination of the literature reveals that, over history, three types of toxin preparation have been studied. These preparations are (i) bacterial culture supernatants, which represent toxin in its crude form; (ii) toxin complexes, which are formed by noncovalent association with other proteins (see above); and (iii) pure toxin purified from complexes.

In the early 1900s, work originating from the laboratory of G. M. Dack examined the absorption of crude toxin preparations from the gastrointestinal systems of a variety of animals (rats, guinea pigs, rabbits, hogs, mice, monkeys) [35–39]. In animals that were susceptible to intoxication, absorption of toxin was described as being from the upper gastrointestinal system. Early studies by Bronfenbrenner [40, 41] demonstrated that crude toxin was relatively resistant to acid conditions such as those in the gut and to gastric proteases. Subsequent work by Lamanna, Littauer and Halliwell supported the earlier observations [42–44].

In the middle 1940s, toxin-auxiliary protein complex was isolated, and physicochemical characterization was undertaken [42, 45–49]. Recognition that the complex—or at least the toxin—needed to be absorbed from the intestinal lumen into the bloodstream or lymphatic system, however, did nothing to explain how this very large protein complex was able to penetrate membranes of the gastrointestinal tract. The actual size of the protein (toxin, toxin complex or 'particle') that finds its way into the circulation was not addressed until later [50–52].

Early studies with toxin complexes suggested that the upper small intestine might be the primary site of toxin absorption [35–40, 51, 53, 54]. In particular, May and Whaler concluded that toxin was much more readily absorbed from the upper small intestine, and that the lymph delivered absorbed toxin to the systemic circulation [54].

Subsequent studies attempted to correlate oral toxicity relative to the molecular size of botulinum toxins [55–57]. Ohishi and colleagues [58] demonstrated in mice that the potency of toxin serotypes A, B and F increased as the molecular size of the administered complex increased. Further studies from Sakaguchi's laboratory demonstrated that it was stability of the larger molecular weight toxin complexes with respect to the gastrointestinal environment (low pH and proteases) that contributed to their greater oral toxicities [59–62]. Simply stated, wrapping the neurotoxin molecule in the other complex-associated proteins protected toxin from the harsh gastrointestinal environment.

Virtually all of the work that has been completed to date indicates that auxiliary proteins probably play a role in protecting toxin from pH extremes and proteolytic enzymes found in the gut. Unfortunately, until recently there was almost no work addressing the role of these auxiliary proteins in the process of toxin absorption [5, 63–65].

Maksymowych and Simpson [5] have recently demonstrated that differentiated, polarized human colon carcinoma cells (T-84 and Caco-2) specifically bound and transcytosed pure botulinum neurotoxin serotype A. This work supported three conclusions: (i) human gut cells can bind and transport certain clostridial neurotoxins, (ii) the toxins are transcytosed in a biologically active conformation that causes illness and (iii) the ability to bind and transcytose toxin is not a generalized property of all differentiated and polarized cells that form tight junctions. Passive mechanisms such as diffusion between cells did not appear to play a significant role. These combined data showed that human intestinal cells can bind and transcytose pure toxin in a form that is structurally and functionally intact. With respect to using the botulinum toxin molecule to create oral

vaccines, the single most important finding is that pure neurotoxin, in the absence of auxiliary proteins, can be absorbed from the gut.

Botulinum toxin and the immune system

Botulinum toxin is a large protein, and thus it is intuitively obvious that the molecule is antigenic. However, as the term 'serotype' implies, each of the seven botulinum toxins (A–G) is relatively or absolutely antigenically distinct. One consistent finding both in historic terms (e.g. antisera) and in contemporary terms (e.g. monoclonal antibody) is that no antibody has been found that produces neutralization of all serotypes.

There is a substantial body of scholarly work dealing with the immunological properties of botulinum toxin, and several reviews of the field are available [66, 67]. Attention here will be focused only on those topics that pertain to oral vaccines. Even more precisely, the goal will be to focus on issues that impact on the way in which a useful simple vaccine or chimeric vaccine should be constructed.

A simple vaccine (i.e. one in which a modified version of the toxin is used as an antigen to evoke production of antibodies that neutralize native toxin) is easy to envision. The most straightforward approach to the construction of such a vaccine is to make the smallest change necessary to render the molecule nontoxic, and in doing so retain most or all of the epitopes that elicit neutralizing antibodies. As explained in more detail below, this feat has already been accomplished. Site-directed mutagenesis was used to alter the zinc binding motif that governs metalloendoprotease activity [64]. The product possessed all the qualities of an oral vaccine, including the essential quality of being nontoxic.

The immunological issues surrounding construction of a chimeric vaccine are more complex. Ideally, a structure-function analysis of the toxin molecule would reveal that there is a minimal domain (viz. carboxy-terminal half of heavy chain) that mediates binding and transcytosis across gut cells. This minimal domain would have few if any epitopes that trigger production of neutralizing antibodies. Therefore, this minimal domain could be linked to an antigen of interest (viz. antigenic portion of tetanus toxin, diphtheria toxin, pertussis toxin etc.) to create a chimeric oral vaccine.

Although all of the key features of a structure-function analysis of the toxin have not yet been determined, the data that are available strongly suggest that the ideal chimeric vaccine may not be attainable. This conclusion stems from the following observations. To begin with, structure-function analyses of toxin action at the neuromuscular junction indicate that the heavy chain, and particularly the carboxy-terminal half of this chain, is the location of a binding domain. The binding domain may not be completely localized to this region, but nevertheless this portion of the molecule is certainly necessary. Thus, a fragment of the toxin that contains the amino-terminal half of the heavy chain linked to the light chain does not bind efficiently to nerve endings. Comparable structure-function analyses of toxin binding to gut cells have not been performed, although preliminary evidence does suggest that the heavy chain plays an important role [Maksymowych and Simpson, unpublished data]. However, there are as yet no data to indicate whether the carboxy terminal, the amino terminal or both are essential, so it is premature to suggest that the binding domain for nerve cells and the binding domain for gut cells are absolutely the same.

The fact that the heavy chain appears integral to the process of binding suggests that, in the context of constructing a chimeric vaccine, it would be desirable if this region possessed few, if any, epitopes. Unfortunately, there is a considerable literature showing that the opposite may be true. The preponderance of work published to date indicates not only that the binding domain is antigenic, but that it may be the most antigenic portion of the molecule. Shortly after the two-chain structure of the toxin molecule was deduced, work began to characterize the immunologic properties of the individual chains. In a representative study, Kozaki and colleagues [68] demonstrated both that the heavy chain was more antigenic than the light chain and that antibodies against the heavy chain were more likely to have neutralizing activity than those directed against the light chain.

In another representative study from Kozaki and colleagues [69], the greater propensity of the heavy chain to evoke neutralizing antibodies was confirmed, and in addition one of the critical mechanisms that account for antibody-induced loss of toxicity was demonstrated. Certain antibodies were shown to bind to the heavy chain, and the antibody ~ toxin complex was shown not to bind to nerve membranes. Presumably the antibody ~ antigen complex occluded the binding domain or created steric hindrance that blocked toxin association with nerve membranes.

It is important to note that blockade of binding is not the only mechanism for antibody-induced loss of toxicity. Simpson and colleagues [70] showed that botulinum toxin which was bound to the cell surface continued to expose certain epitopes. Furthermore, certain antibodies that recognized these epitopes could produce substantial loss of toxicity. In view of the fact that cell surface binding had already occurred, the antibody ~ antigen complex must have impeded a step in the intoxication process that follows binding (viz. endocytosis or translocation).

Similar types of experiments have been done with permeabilized cell preparations or materials injected directly into cell interiors. Bartels and colleagues [71], using a permeabilized chromaffin cell preparation, showed that antibodies could be used to relieve the blockade of catecholamine release produced by botulinum toxin. Toxin in the cell interior was beyond the steps of binding, internalization and translocation, so the effects of antibody were either specific to enzymatic activity or were producing a precipitation effect. In the same vein, Cenci Di Bello and colleagues [72] injected antibodies directly into nerve cells, and these antibodies blocked the action of toxin applied to injected cells.

The message that emerges from this work is that there are multiple epitopes in the toxin molecule, and there are multiple mechanisms by which antibody ~ antigen reactions can produce loss of toxicity. And, as indicated earlier, the heavy chain is the location of most of the antigenic domains in the molecule. No one yet claims to have quantified every epitope in the toxin molecule, let alone mapped these sites, but a recent study by Chen and colleagues [73] may provide a good quantitative insight. In an attempt to generate a large library of monoclonal antibodies to botulinum toxin, they were able to produce 40 that recognized the intact molecule. Of these, 25 recognized the heavy chain and 15 recognized the light chain. Of those that mapped to the heavy chain, 22 recognized the carboxy-terminal half.

These findings may seem to discourage the concept that botulinum toxin can be used as a carrier component in the generation of chimeric oral vaccines. The administration of one such vaccine, which could lead to the generation of either mucosal or systemic antibodies, might seem to preclude subsequent administration of similar vaccines. However, there are important factors that substantially counterbalance this apparent drawback. As will be explained shortly, the existence of multiple serotypes, the finite lifetime of immunity, and the combinatorial possibilities for building and administering chimeric vaccines add together to produce a large number of authentic therapeutic possibilities.

Botulinum toxin and the nervous system

If botulinum toxin were to be used as a simple vaccine, it would have to retain its ability to translocate from the gut to the general circulation and also retain its ability to evoke systemic production of neutralizing antibodies. If the toxin were to be used as the carrier component of a chimeric toxin, it would still have to retain its ability to translocate, but its ability to evoke antibody production may or may not be essential (see below). Regardless of whether the intended use is as a simple vaccine or a chimeric vaccine, the toxin would have to be devoid of the ability to poison the nervous system. The goal, then, is to retain certain features of the toxin while at the same time removing its most distinguishing characteristic, which is the ability to block neuromuscular transmission.

As reviewed earlier, the mechanism of botulinum toxin action on the nervous system can be envisioned as a sequence of events. Several of these events pertain to the toxin gaining access to its site of action (e.g. binding to receptors, penetration of the plasma membrane and penetration of the endosome membrane), whereas the final event pertains to blockade of exocytosis (e.g. metalloendoprotease activity). Altering the toxin molecule so that it does not bind, cannot penetrate the plasma membrane, cannot penetrate the endosome membrane and/or fails to express endoprotease activity would result in loss of toxicity. However, as indicated above, this is only part of the challenge. The toxin must be stripped of its ability to poison nerves, but not stripped of its ability to translocate from the gut to the general circulation.

There is a highly suggestive literature indicating that the carboxy-terminal half of the heavy chain is essential for toxin binding to nerve terminals. This might at first be taken to mean that removal of this portion of the molecule would lead to generation of a vaccine candidate. Unfortunately, the issue is not so simple. To begin with, the concept that the binding domain resides solely in the carboxy-terminal half of the heavy chain could be an oversimplification. Binding may in reality depend on portions of both the heavy chain and the light chain. Removal of a complex binding domain whose components are in tertiary proximity but not linear proximity could be a daunting task. And this may be the lesser obstacle to overcome.

There is a distinct possibility that some or all of the structural elements that govern toxin binding to nerve endings also participate in toxin binding to receptors on the lumenal side of the gut. If this were true, efforts to abolish toxicity would simultaneously abolish vaccine status. Until there is more information about the structure-activity relationships that govern toxin binding to gut cells and nerve cells, attempts to convert the toxin into a vaccine by removing its purported nerve binding domain may yield little of value.

After the initial binding event, toxin disposition in gut cells and in nerve cells is fundamentally different. In gut cells, the toxin is transported in an unmodified form from one cell surface to the other, where it is released to the cell exterior. In nerve cells, the toxin is transported to the interior of the nerve ending, where it undergoes pH-induced translocation to reach the cytosol. At some point in this migration, the toxin is modified (viz. reduction of the interchain disulfide bond) so that the enzymatic light chain will become fully active.

An analysis of these cell-specific schemes suggests that there may be two ways to alter toxin disposition in nerve cells without significantly altering disposition in gut cells. One possibility would be to block pH-induced translocation of toxin from endosomes to the neuronal cytosol. There are pharmacological agents that produce this effect, either by blocking acidification of endosomes or by neutralizing the acidity of endosomes (e.g. methylamine, bafilomycin). However, these drugs are not totally effective in blocking translocation, and they are not desirable agents for human administration.

Another approach would be to alter the molecule by site-directed mutagenesis so that the light chain cannot be loosened from the heavy chain. Replacing the disulfide bond with a different type of interchain linkage may allow the toxin to retain its ability to bind while losing its ability to translocate and/or express enzymatic activity. This is a speculative possibility that may warrant consideration.

The final step in toxin action is the one that appears most amenable to a desired form of alteration. The light chain possesses a zinc binding domain and a substrate binding domain. The former has been well characterized, and several laboratories have used the techniques of molecular biology to express a protein that has mutations in this domain and that consequently fails to exert endoprotease activity. The latter has not been well characterized, and a molecule that fails to bind substrate has not yet been generated.

One of the exciting features of a toxin molecule that has mutations in the zinc binding domain is that it continues to possess those features that are essential for a simple vaccine. Indeed, a molecule of this nature has been made, and it was shown to be an oral vaccine against botulism [64]. The potential this molecule may have as a carrier component of a chimeric vaccine remains to be determined, but there is reason to be optimistic.

The toxin as a simple vaccine

In the recent past, a modified version of botulinum toxin has been constructed that possesses the minimum essential characteristics of a simple vaccine (fig. 3). More precisely, the modified toxin can be administered orally, it translocates to the general circulation where it induces production of neutralizing antibodies, but it has no ability to produce neuromuscular blockade or other obvious signs of toxicity.

The original work, which was done with botulinum neurotoxin type C [64], involved site-directed mutagenesis and subsequent expression in a bacterial system. Three amino acid mutations ($H^{229} \rightarrow G$; $E^{230} \rightarrow T$; and $H^{233} \rightarrow N$) were introduced into the zinc binding motif of the light chain. The gene for the modified light chain, as well as the message for the remainder of the toxin molecule, was reconstructed in vector pQE-30 and expressed in *Escherichia coli*. The resulting expression product migrated identically to native toxin in polyacrylamide gel electrophoresis, and it was recognized by serotype-specific antibodies. However, the expression product did not block neuromuscular transmission, nor did it act as an endoprotease to cleave substrate. Furthermore, the expression product was devoid of systemic toxicity, even when administered at doses that were orders of magnitude higher than the lethal dose of native toxin.

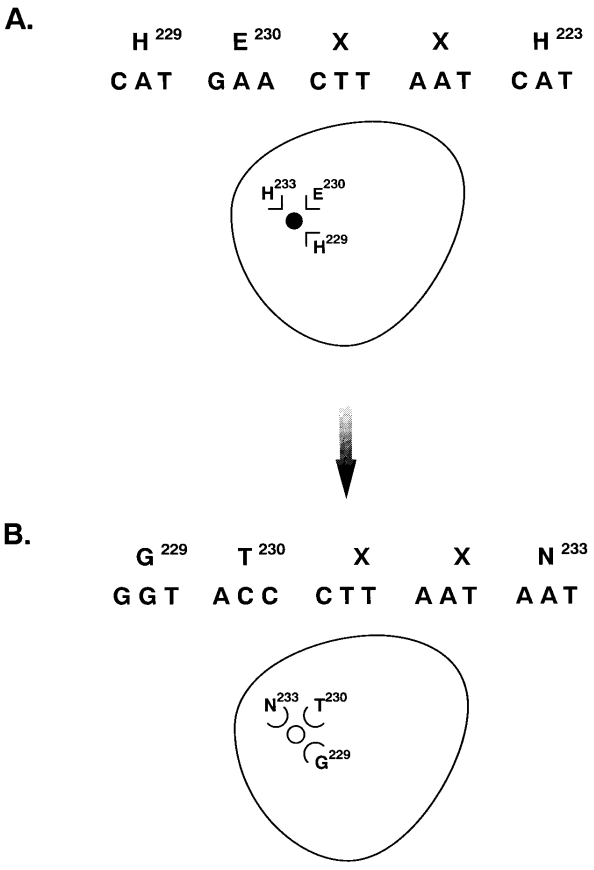

Figure 3. Schematic representation of the zinc-binding domain from toxin serotype C (*A*) and the mutated nonbinding domain (*B*). *A* illustrates native toxin amino acids that represent the metalloprotease consensus sequence H-E-X-X-H. This linear sequence is followed by a three-dimensional representation of this domain coordinating a zinc molecule (black sphere) within the light chain of the toxin. *B* illustrates amino acids in this domain that were mutated. This linear sequence is followed by a three-dimensional representation of the altered site. The region of the light chain which a zinc molecule would have occupied is indicated by the empty black circle.

Experiments designed to test the potential of the recombinant molecule as a vaccine produced promising results. Both the recombinant holotoxin as well as the recombinant light chain were administered either orally or parenterally (subcutaneously). As expected, parenteral administration of the recombinant molecules, both of which are large proteins (holotoxin ~ 150 kDa: light chain ~ 50 kDa), led to production of systemic antibodies. However, oral administration led to a differential outcome. Recombinant holotoxin retained the ability to elicit production of circulating antibodies that recognized both the recombinant toxin and the native toxin. By contrast, recombinant light chain did not elicit production of circulating antibodies.

Certainly the most exciting result to stem from the work was the finding that systemic antibodies evoked by oral administration of the nontoxic recombinant molecule neutralized native toxin. This outcome, which was obtained with serotype C, is now being reproduced with other serotypes.

Botulinum toxin as a component of a chimeric vaccine

The concept of using a modified version of botulinum toxin as a simple vaccine could hold promise in several clinical settings. For example, botulism continues to be a source of concern in various areas of veterinary medicine, and the availability of an easily administered, oral vaccine could ease these concerns. Another example, and one that is more troubling in nature, relates to human medicine. Botulinum toxin is a potential agent of biological warfare. Should a need arise for active immunity in a large population of persons really or potentially vulnerable to biological attack with the toxin, the availability of an oral vaccine could prove to be of enormous value.

Interestingly, the role of the toxin in simple vaccines may ultimately turn out to be only one of its therapeutic benefits, and perhaps not even the most important benefit. The toxin may have a far greater role in the construction of chimeric vaccines. More precisely, the toxin molecule might have utility as a carrier that could be used to ferry many different antigens into the body. The ideal chimeric construct would be one that contains (i) a fragment of the toxin molecule that retains the ability to bind and undergo transcytosis across gut cells and (ii) an antigenic fragment from a pathologic agent of clinical concern (viz. tetanus toxin, diphtheria toxin, pertussis toxin etc.).

To date, a chimeric oral vaccine has not been tested (although the authors are in the process of doing this). Nevertheless, there is reason for measured optimism about the outcome because many of the criteria enumerated earlier for an ideal vaccine seem quite plausible. Thus, it seems reasonable that a binding and translocation domain can be identified, it seems reasonable that a carrier peptide—or an antigenic peptide attached to it—will have little toxicity and there are many techniques available for linking potential carrier peptides to potential antigenic peptides. The only two obstacles that appear to merit concern are the possibility that the chimeric agent will be subject to metabolism, and the possibility that evoked immunity (muscosal or systemic) to the carrier domain will seriously limit the prospect for repeated administration of that carrier. Fortunately, neither of these concerns is insurmountable.

Metabolic degradation

In an earlier section there was a listing of the three broad categories of obstacles that have hindered the development of oral peptide vaccines. Stated briefly, these limitations are: (i) susceptibility of peptides to metabolic degradation, (ii) inability of peptides to diffuse efficiently from the gut to the general circulation and (iii) inability of peptides to bind specifically and be transported across gut cells.

The concept of a chimeric vaccine inherently addresses the second and third points. Botulinum toxin is highly efficient at binding and being transcytosed across gut cells. Indeed, it would be hard to imagine anything else. Botulinum toxin is an oral poison, and it is the most potent biological substance known. These facts essentially mandate that the toxin must be highly efficient at escaping the gut and reaching the general circulation. If a binding and transcytosis domain of the toxin were used as a carrier, this would mean that the chimeric agent would not have to diffuse between cells; instead, the carrier would transport it through cells and into the general circulation. Therefore, the second and third points are effectively addressed.

This then leaves unresolved the first point. To what extent must a carrier ~ vaccine construct be resistant to metabolic degradation in order to be an effective oral vaccine? In addressing this question, it may be helpful to start with an elementary observation about gastrointestinal physiology. The gut is not a perfect machine for degrading proteins. Therefore, the real challenge is not to overcome a completely perfect metabolic machine. The challenge is to create circumstances under which a reasonable fraction of an oral dose of vaccine will escape an imperfect metabolic machine and reach the general circulation. There is a compelling basis for arguing that merely by attaching an efficient carrier domain to the vaccine one may have met the challenge.

Consider for a moment the simplified scheme in figure 4. A protein that enters the gastrointestinal system will

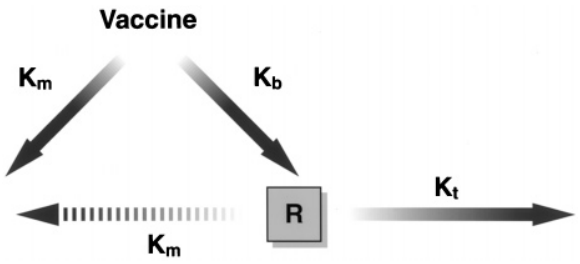

Figure 4. Theoretical kinetics for oral vaccine absorption from the gastrointestinal system. The passage through the gut and subsequent absorption of an oral vaccine do not require that it overcome a perfect metabolic machine. Only a reasonable portion of an oral dose of vaccine needs to escape degradation and reach the general circulation. A protein that enters the gastrointestinal system is expected to be subject to metabolism (m), which can be represented by some overall rate constant (K_m). A toxin-based oral vaccine is expected to demonstrate high-affinity binding to gut receptors (K_b). There may be some dissociation from these receptors, which would once again make the vaccine susceptible to metabolism (K_m); however, a majority of the bound protein would be internalized and transcytosed at a rate constant represented by (K_t).

normally be subject to metabolism (m), and therefore one might envision some overall rate constant that summarizes the entire metabolic process (K_m). For the majority of proteins, this is the only rate constant that will apply, and the molecule will undergo partial or complete degradation.

There are a small number of proteins for which there can be a different fate. These proteins associate with receptors, and this means that there is a rate constant for binding to receptors (K_b). In the case of some proteins, there may be dissociation from receptors, and if so, the released protein would join unbound protein for metabolism and/or elimination. However, the majority of the bound protein would be transcytosed, and this would be done according to the rate constant K_t.

The argument being advanced here is that a usable and effective oral vaccine can be created by attaching a carrier that binds to receptors in the lumen of the gut and has a rate constant for binding that is comparable to or exceeds the rate constant for metabolism. As it turns out, this is not a speculative argument. It is an observation for which there are many examples, one of which is a particularly appropriate analogy for the concepts being advanced here.

The site of action of botulinum toxin is the neuromuscular junction. Healthy nerve endings at these junctions normally function by releasing the neurotransmitter acetylcholine. Transmitter that is released from nerve endings can undergo one of two fates. One possibility is that it can be metabolized by the enzyme acetylcholinesterase. This is a unique enzyme, because it possesses one of the highest turnover rates of any known enzyme (i.e. number of substrate molecules cleaved per unit of time). The other possibility is that acetylcholine can bind to nicotinic cholinergic receptors to evoke a muscle response. It is highly instructive that released acetylcholine can trigger a response, given that it does this in an environment containing one of the most active metabolizing enzymes known. It is able to do this because there are high-affinity receptors in the same geographic region as the enzyme, and the rate constant for association with receptors rivals that for association with the enzyme.

The beauty of botulinum toxin is not merely that it can be modified to act as a carrier. More than this, it binds to receptors that are in the same vicinity as metabolic processes, and these receptors mediate a transport process that completely removes the molecule from metabolic systems. Furthermore, the extraordinary potency of native toxin is highly suggestive evidence that the rate constant for association with receptors will compare favorably with the rate constant for metabolism of any attached antigen. Both evidence and deduction indicate that the modified toxin could be a superb device for creating orally active drugs.

Undesirable immunity

The potential utility of botulinum toxin as a carrier might theoretically be limited by the undesirable ability of the peptide to evoke immunity to itself. This theoretical concern would be paramount if there were only one botulinum toxin or if there were only one setting in which the toxin could be employed, but neither of these is true. To the contrary, the combinatorial possibilities of serotypes and settings substantially overcome any theoretical limitation. This is true both for monovalent and for polyvalent vaccines.

A construct that contains a single carrier (viz. minimal domain of botulinum toxin) and a single vaccine (viz. minimal domain of tetanus toxin) is properly referred to as a monovalent vaccine. In this particular construct there is by definition only one possible vaccine component (e.g tetanus toxin). However, there are in theory seven possible carriers (e.g. the minimal domains of botulinum serotypes A–G).

Almost all outbreaks of human botulism are due to serotypes A, B and E. This brings to mind an interesting clinical possibility. The primary goal of administering a conjugate is to provide immunization against the vaccine component. However, the carrier component of each conjugate will also elicit antibody formation. Therefore, a botulinum toxin type A carrier ~ tetanus toxin vaccine conjugate could achieve the primary goal of immunizing against the disease tetanus, but as a

secondary consequence it would also immunize against the disease botulism. This argues strongly in favor of evaluating serotypes A, B and E as carriers.

It is important to note that a potential advantage in one context can be a potential limitation in another. If a patient develops immunity to serotype A that is used as a carrier for a tetanus vaccine, this would mean that serotype A would have little or no future utility as a carrier for other vaccines—at least for the interval when there may be mucosal or serum antibodies against this particular carrier. This might seem to suggest that botulinum toxin can be used to create only seven vaccines, i.e. one each for the seven serotypes. Even if this were true, it would still mean that the botulinum toxin carrier could have an enormous worldwide public health impact. Imagine, for example, oral vaccines against tetanus, diphtheria, pertussis, rubella and cholera, plus the various serotypes of botulinum toxin. This alone is more than adequate justification to evaluate the carrier concept, but it is in truth a 'worst-case scenario'. There are at least two highly viable approaches that could be used to substantially expand the number of vaccines that could be created. Both approaches are conceptually simple, and the first is also technically simple (fig. 5).

One way to minimize the impact that neutralizing antibodies may have when a carrier is given in sequential settings is simply to administer more than one monovalent vaccine in the same setting. Thus, one might test the idea of giving a serotype A carrier ~ tetanus vaccine construct and a serotype A carrier ~ diphtheria vaccine construct simultaneously. For reasons that are obvious, one would be administering each construct at a dose that is well within the range needed to evoke a healthy immune response. It has been a general observation in immunology that increasing the amount of immunogen that is already well within the range needed to evoke a protective immune response does little if anything to alter the rate of appearance of neutralizing antibodies. Therefore, the possibility of simultaneous administration of monovalent vaccines that utilize the same carrier is worthy of experimental testing. If this strategy worked, and if one administered only two monovalent vaccines in each setting, there would be the potential for creating a theoretical maximum of 14 vaccines (7 serotypes × 2 monovalent vaccines per setting). The real maximum will not be known until research is done to determine the number of serotypes that translocate across the gut wall.

The second approach that could be used is one for which the authors cannot claim credit. They have merely borrowed an idea that has been advanced and now widely tested by investigators developing parenteral vaccines [74, 75]. This approach utilizes agents known as polyvalent vaccines.

In the context of this article, a polyvalent vaccine is one that has a single carrier that is linked to two or more antigenic peptides. Thus, a single toxin serotype carrier could be linked to a string of antigens, such as tetanus and/or diphtheria and/or pertussis. Strings of three or more epitope domains have become increasingly common in the field of parenteral vaccine synthesis. There is no obvious reason why the idea should not be tested with an oral carrier.

One must be cautious about speculating whether this strategy will or will not succeed. Nevertheless, it does seem appropriate to say that, if the technique does work, it has a compounding advantage. First, one could create polyvalent vaccines that have two, three or conceivably more antigenic peptides attached to a single carrier. Second, polyvalent vaccines that utilize a single carrier, like monovalent vaccines with a single carrier, could be given simultaneously. In practical terms, this may mean that there are a large number of vaccines that could be constructed with just seven potential carriers, and this could be done in a way that largely circumvents the potential limitation of any individual serotype triggering production of neutralizing antibodies. Indeed, it may be that the ability of the immune system to respond to simultaneous vaccinations will ultimately be a bigger limitation than the ability of investigators to create oral vaccines using botulinum toxin as a carrier.

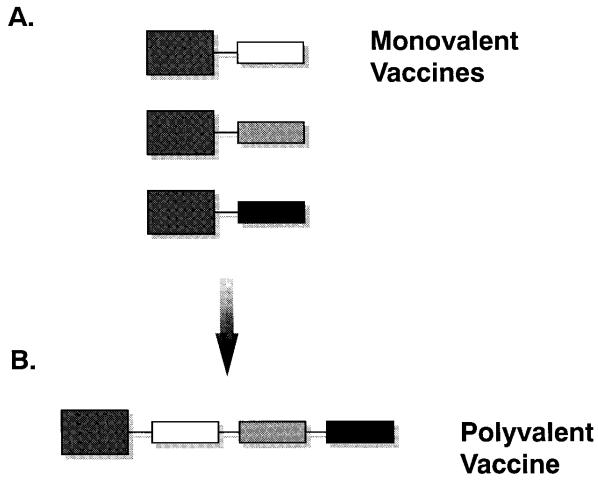

Figure 5. Use of botulinum toxin to create oral vaccines. There are two potential ways in which botulinum toxin can be used to create chimeric oral vaccines. In one case, the carrier domain of toxin can be linked to a single antigenic peptide to create a monovalent vaccine. As illustrated in this figure, the same carrier could be used to create a family of monovalent vaccines that could be administered simultaneously (*A*). In the other case (*B*), the carrier could be linked to multiple antigenic domains to create a polyvalent vaccine.

Therapeutic conflict

There are several layers of potential benefit in the proposed clinical use of botulinum toxin, but there is also a cautionary note. One must bear in mind that botulinum toxin is already an approved medication that is administered to patients with certain types of dystonia. Difficulties can arise when attempting to utilize the toxin in another therapeutic realm, especially if the therapeutic gains in the two settings are potentially incompatible.

Botulinum toxin has been used for more than a decade in the treatment of dystonias such as blepharospasm and strabismus [76]. By definition, a dystonia is a neurological problem characterized by excessive and involuntary efferent activity. Neither the origin nor the underlying mechanism for any dystonia has been determined. The only thing that has been unequivocally established is that the final common path for all dystonias is the cholinergic nerve ending. In response to this fact, local injection of botulinum toxin has been introduced—and in some instances is the treatment of choice—for diminishing excessive release of transmitter. This in turn relieves the signs and symptoms of dystonia.

At the moment, botulinum toxin type A is the only serotype that has been approved for use in the United States. Four other serotypes are being evaluated in clinical trials, and it is possible that some of these will be approved. If the strategy for generating oral vaccines proposed in this article should work, the same sequence would probably prevail. Botulinum toxin type A, which is the most well characterized of the serotypes, will be evaluated first, and the results will establish a context in which to evaluate other serotypes.

It is absolutely essential that several serotypes be examined, because it is not possible to utilize the same serotype as a treatment for dystonia and as a carrier for oral vaccines. The problem arises from the fact that the botulinum toxin fragment in the carrier ~ vaccine conjugate would elicit antibody formation not only against the vaccine component but also against the carrier component. This in turn would reduce or abolish the antidystonia effect of botulinum toxin in any patient who had previously received the carrier ~ vaccine conjugate.

Fortunately, both the nature of the problem and the nature of the solution are easy to grasp. By consensus within the medical community, at least one serotype must be reserved for treatment of dystonia, and the remaining serotypes can be exploited to develop carrier ~ vaccine conjugates. The fact that nature has evolved so many serotypes should allow physicians and scientists to exploit the toxin in different therapeutic settings.

Acknowledgements. This work was supported in part by NIH grant NS22153.

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