Journal of Toxicology

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Guest Editors: Martha L. Hale, Shuowei Cai, and S. Ashraf Ahmed

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Editorial Board

Contents

Hindawi Publishing Corporation
Journal of Toxicology
Volume 2012, Article ID 678963, 2 pages
doi:10.1155/2012/678963

Editorial

Medical Applications of Clostridia and Clostridial Toxins

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Received 4 September 2012; Accepted 4 September 2012

The bacterial genus *Clostridium* produces a diverse array of protein toxins, many of which have played an important role in disease and medicine. Although initially known for their ability to do harm, many clostridial toxins have been shown to have therapeutic value for a diverse array of diseases. The articles included in this special issue provide insight into some of that diversity and show that bacterial toxins remain a valuable storehouse for development of new therapeutic measures.

Perhaps the most known toxin therapeutics derived from the clostridial species are neurotoxins secreted by *Clostridium botulinum*. Treatment using BTX is remarkable in that the amount of toxin required is small and the beneficial effects are dramatic, particularly considering the fact that many of the conditions, such as dystonias and muscle spasms, do not respond to other pharmacological treatments. Over the last few decades, BTX has also become cosmetically important and has created significant markets for the drug. Most commercial BTX products are not the pure toxin, but a combination of the toxin and toxin-associated proteins. While these progenitor toxins are safe, a purified form of the toxin would eliminate extraneous proteins and offer a safer drug. Clinical investigations conducted by Y. Matsuka and coworkers (J. Toxicol., 12, 2012) show that BTX purified over a lactose column was an effective treatment for urinary incontinence and prostatic hyperplasia. Their studies provide further data confirming that purified BTX can be used in lieu of progenitor toxins for treatment of many spasmodic conditions.

Although the focus of most BTX treatment has centered upon using the toxin as a local paralytic to relieve spasm-related illness, new studies show also that BTX aids in the treatment of other difficult-to-treat conditions. D. Intiso (J. Toxicol., 12, 2012) provides an excellent review showing the positive effects of BTX as a supplement in treatment strategies in which the patient requires neurorehabilitative intervention. In treating conditions such as cerebral palsy, stroke, sialorrhea, and other neuromuscular diseases, BTX permits the individual to benefit from physical therapy which is often extremely difficult without some muscle relaxation. New uses of BTX are not limited to rehabilitative intervention, but as suggested by F. j. Lebeda and colleagues (J. Toxicol., 12, 2012), BTX treatment may enhance the processes of wound healing. By performing a systematic search of the literature, the authors developed a qualitative model that shows the possibility of using BTX therapy in wound healing. BTX therapy disrupts muscle spasms. Muscle relaxation around the wound would help to reduce pain and inflammation so that the healing process can proceed more quickly. This work and future studies show that many future treatments for a diverse array of diseases and conditions may benefit from the therapeutic use of BTX.

While BTX may be the best known, other clostridial toxin-based therapeutics are either currently available or in developmental phases, and three examples are included in this issue. G. Krautz-Peterson et al. and B. Gao and A. McClane utilize clostridial toxin cell binding components as delivery vehicles for therapeutic agents. As with many clostridia toxins, *C. difficile* toxins have a cell binding component and an enzymatic toxic component. These toxins are somewhat unique in that they also contain a protease within the toxin. G. Krautz-Peterson and colleagues show studies that support the ability to link the cell binding region of *C. difficile* toxin B (TcdB) with a therapeutic agent.

When the agent is endocytosed, the protease cleaves the drug from the TcdB and is free to act. Additionally, the cell receptor region of TcdB may be replaced with another cell binding receptor, such as the BTX cell receptor region (Hc), thus limiting the cell types to which the TcdB may bind. By combining a BTX inhibitor to the TcdB-Hc, the authors indicate that this drug would provide a BTX inhibitor that is needed to treat botulism.

Another avenue for utilizing clostridia toxins as therapeutics has been advanced by the investigations of B. Gao and A. McClane (J. Toxicol., 12, 2012) in their development of a therapeutic using *C. perfringens* enterotoxin (CPE). CPE binds to claudin receptors found in tight junctions and cells expressing large amounts of the claudin receptor are particularly susceptible to the cytolytic CPE. Because cancer cells usually express large quantities of claudin on their cell surface, the cells are fairly sensitive to CPE intoxication and CPE provides a natural cytolytic agent for many cancer cells. The primary problem associated with CPE therapy is the fact that the toxin is a protein and, as such, may induce antibodies against CPE which could reduce its effectiveness *in vivo*. B. Gao and A. McClane solve this problem by using the primary receptor domain (C-CPE) to enhance permeability and delivery of chemotherapeutic agents. The truncated size of C-CPE reduces immunogenicity problems while providing a claudin-binding component forthe agent.

The abundance of clostridial toxins with their specific substrates provides a wealth of new pharmacological agents. Combined with the ability to engineer clostridia or its toxins to target specific cells and tissues, the possibilities seem endless. However, the utility of clostridia in medicine does not end with the clostridial proteins. As reviewed by B. Umer et al., the spores of many clostridia species have been developed as cancer therapies. Solid tumors pose a problem to current therapies because the therapeutic cannot be evenly distributed throughout the tumor, partly due to hypoxia and necrotic areas of the tumor. Clostridia spores are strictly anaerobic and will only colonize areas devoid of oxygen and therefore seek out the tumor environment. The spores have been modified to improve their tumor cytolytic capabilities. This review gives an excellent overview of the field and show the possibilities for using clostridia spores in therapy.

As shown by the diverse subjects presented in this special issue, the same characteristics that make Clostridia excellent pathogens have also made the Clostridia an abundant source for development of pharmacological agents. I would like to thank those that contributed to this special issue. Their investigations have given us more insight into the many medical applications of Clostridia and its toxins. Their work provides the foundation for many new and innovative ways in which Clostridia may be harnessed to develop more effective treatments for many diseases and medical conditions.

Acknowledgments

The views expressed in this article are those of the author and do not necessarily reflect the official policy or position of the Department of the Army, the Department of Defense, or the US Government.

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Hindawi Publishing Corporation
Journal of Toxicology
Volume 2012, Article ID 648384, 8 pages
doi:10.1155/2012/648384

Clinical Study

Application of Purified Botulinum Type A Neurotoxin to Treat Experimental Trigeminal Neuropathy in Rats and Patients with Urinary Incontinence and Prostatic Hyperplasia

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Received 25 July 2011; Revised 15 February 2012; Accepted 10 April 2012

Academic Editor: S. Ashraf Ahmed

Type A neurotoxin (NTX) of *Clostridium botulinum* was purified by a simple procedure using a lactose gel column. The toxicity of this purified toxin preparation was retained for at least 1 year at −30°C by supplementation with either 0.1% albumin or 0.05% albumin plus 1% trehalose. When purified NTX was used to treat 49 patients with urinary incontinence caused by either refractory idiopathic or neurogenic detrusor overactivity, 36 patients showed significant improvement in symptoms. These beneficial effects were also observed in cases of prostatic hyperplasia. The results obtained with NTX were similar to that of Botox. The effects of NTX on trigeminal neuralgia induced by infraorbital nerve constriction (IoNC) in rats were also studied. Trigeminal ganglion neurons from ipsilateral to IoNC exhibited significantly faster onset of FM4-64 release than sham-operated contralateral neurons. Intradermal injection of NTX in the area of IoNC alleviated IoNC-induced pain behavior and reduced the exaggerated FM4-64 release in trigeminal ganglion neurons.

1. Introduction

In this manuscript, three main topics are described: (1) the structure of type A progenitor toxin and purification of type A neurotoxin by a lactose gel column based on progenitor toxin characteristics; (2) treatment of patients with urinary incontinence and prostate hyperplasia with this purified neurotoxin; (3) application of the purified neurotoxin for treating trigeminal neuralgia in a rat model. Introduction including the purpose of these three topics is described at the beginning of each paragraph.

2. Structure of Type A Progenitor Toxin and Purification of Type A Neurotoxin

Clostridium botulinum strains produce immunologically distinct neurotoxins (NTX; types A to G) with molecular masses (M_r) of approximately 150 kDa. In an activated form, the NTXs have a cleavage site at the one third position of their N terminus conjugated with an S–S bond, which can be cleaved by cellular proteases or exogenously added trypsin. In culture fluid and in acidic foods, the NTXs associate with nontoxic components and form large complexes designated

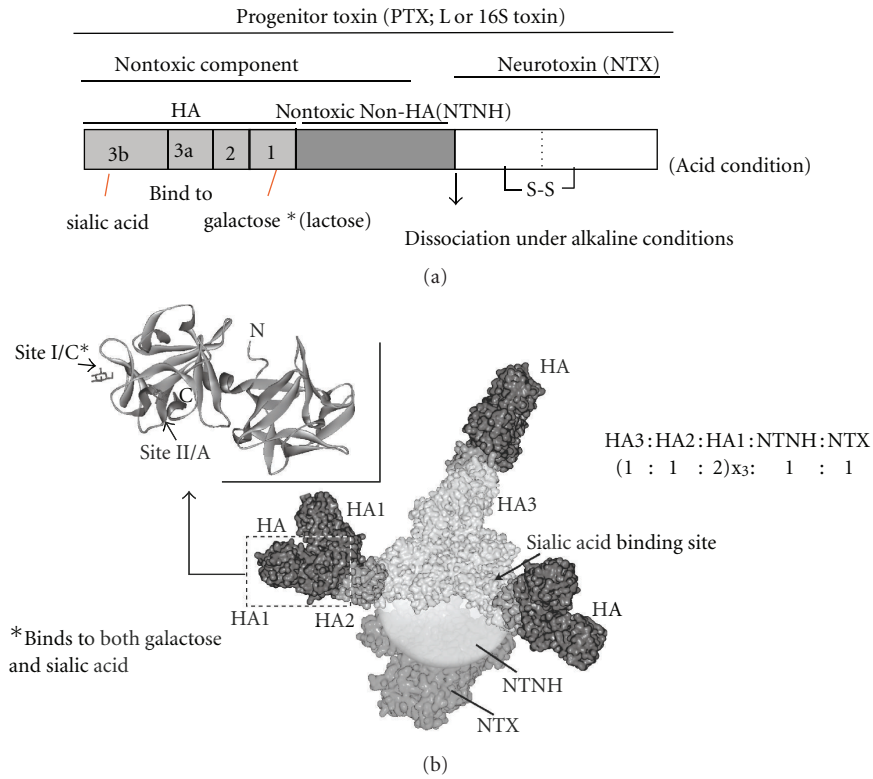

Figure 1: Progenitor L toxin structure. (a) Scheme indicating that (1) under alkaline conditions PTXs dissociate into an NTX and nontoxic components; (2) fully activated NTX is cleaved at a site in the N-terminus; (3) HA1 and HA3b bind galactose and sialic acid, respectively. (b) Predicted tertiary structure of type C and D L toxins and molar ratio of their (sub)components. Model is based on the structural data of Hasegawa et al. [8] and Nakamura et al. [7]. Three dimensional structure of HA1 and its binding sites for sugars are also shown. Both type A and C HA1 showed similar structures consisting of two β-trefoil domains conjugated by an α-helix. Two sugar-binding sites (I and II) on C-terminal β-trefoil domain are predicted and A-HA1 and C-HA1 binds to galactose via sites II and I, respectively. In the case of type C, HA1 also bound to sialic acid via site I [6].

progenitor toxins (PTXs). PTXs are found in three forms with M_r of 900 kDa (19S, LL), 500 kDa (16S, L), and 300 kDa (12S, M); later, it became clear that the actual M_r of the L and LL toxins is much larger than 500 and 900 kDa, respectively (see below). The M toxin is composed of an NTX and a nontoxic component having no hemagglutinin (HA) activity (designated nontoxic non-HA; NTNH), whereas the L and LL toxins are composed of an NTX(s), an NTNH(s), and HAs. The type A strain produces three forms of toxin (M, L, and LL), while types B, C, and D produce M and L toxins. In alkaline conditions in the absence of gastric juices, the PTXs dissociate into the NTX(s) and nontoxic components, L and LL toxins (designated HA-positive PTXs) dissociate into an NTX(s), and a nontoxic component (complex) of an NTNH(s) and HAs, and the M toxin dissociates into an NTX and an NTNH [1].

We found that (1) LL toxin is a dimer of L toxins; (2) cleavage or deletion is detected in the N-terminal region of the M toxin NTNH; (3) HA consists of three to four subcomponents designated HA-1, -2, and -3 (-3a, -3b); (4) HA1 and HA3b bind to galactose and sialic acid, respectively, present on the surface of erythrocytes and small intestine epithelial cells [2–4]. Recently, we also analyzed the crystal structures of type A and C HA1, and type C HA3 to describe their sugar binding sites [5–7]. Furthermore, by investigating the tertiary structure of type C and D L toxins with electron microscopy, the molar ratio of NTX : NTNH: (HA1 : HA2 : HA3) was predicted to be $1 : 1 : (2 : 1 : 1)_{x3}$, and the actual L toxin M_r was calculated to be approximately 750 kDa. The LL toxin M_r was, therefore, estimated to be about 1,500 kDa because it is a dimer of the L toxin, although its tertiary structure remains unclear [7, 8]. Some of these data are summarized in Figure 1.

Type A and B PTXs have been used to treat patients with strabismus, blepharospasm, nystagmus, facial spasm, spastic aphonia, and many other dystonias including cervical dystonia, hemicrania (migraine), and some urinary disorders [9–16]. For these treatments, PTXs are mainly used because they are easily obtained and are more stable than NTXs. While these treatments are very effective, they can present serious problems in some patients in that anti-PTX, including anti-NTX, antibodies are sometimes produced after several injections. We think that the use of NTX alone may be better than using PTX because both HA1 and HA3b of PTX have immunoenhancing activity, although there are no definitive data for this immunoreactivity in humans [17]. Therefore,

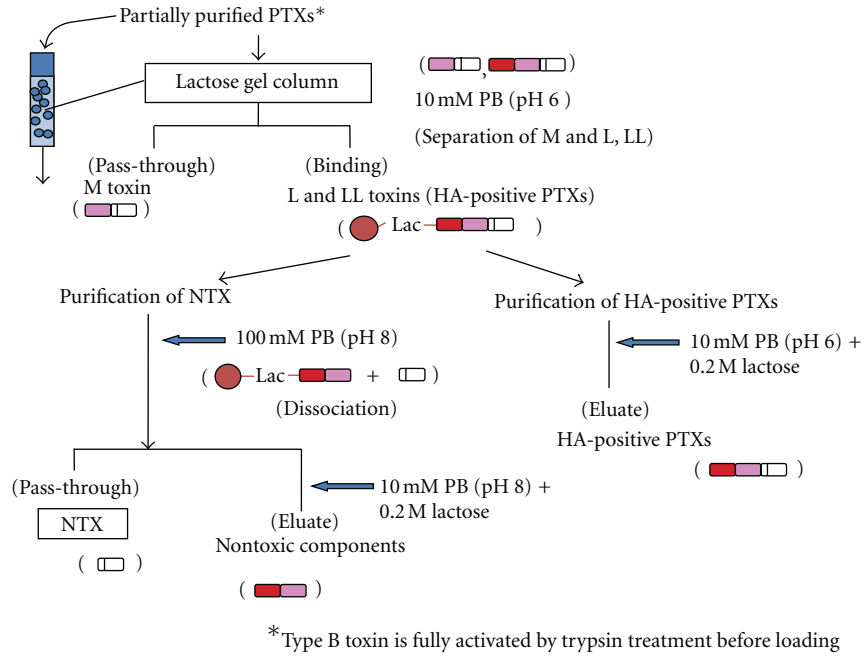

Figure 2: Simple procedure for purifying type A and B NTXs. Partially purified type A HA-positive PTXs (L and LL) or trypsin-treated (fully activated) type B L toxin was layered on a lactose gel column under acidic conditions (pH 6.0). After washing, the pH was adjusted to 8.0 to obtain the NTX. The bound HA-positive PTX(s) and nontoxic components were eluted with buffer supplemented with 0.2 M lactose.

we established simple procedures for purifying type A NTX and for long-term storage of type A NTX at −30°C without reductions in toxicity.

As shown in Figure 1, the HA-positive PTXs bind to galactose via HA1 under acidic conditions, and the conjugation of NTX and nontoxic components dissociates under alkaline conditions. Therefore, to isolate only the HA-positive PTXs, partially purified PTXs were first subjected to an aminophenyl beta-lactose gel column equilibrated with 10 mM sodium phosphate buffer (pH 6.0). From this column, purified preparations of HA-positive PTXs, NTX, or HA plus NTNH could be obtained by washing the column with different buffers (Figure 2). This procedure is simpler than previous protocols that used several different columns and conditions. Moreover, the NTX preparation purified by a lactose gel column showed the same toxicity per mg protein and SDS-PAGE banding profile as those obtained with previous procedures. We also found that NTX toxicity can be fully maintained for at least one year when albumin (0.05%), trehalose (1%), and Tween-20 (0.01%) were added to the storage solution [18]. The NTX, albumin, and trehalose were diluted in endotoxin-free isotonic sodium chloride solution. Finally, this purified type A NTX preparation was used as a therapeutic agent for urinary incontinence and prostate hyperplasia and as an analgesic for trigeminal neuralgia in a rat model.

Prior to clinical use, the safety and quality of the NTX preparation and albumin were checked using NTX concentrations that were 100 folds greater than those used in treatment and albumin at 50 mg/mL. Cytotoxicity was evaluated using IMR-32 (human, abdominal), Neuro-2a (mouse, spinal cord), U937 (human, pleural effusion), NOMO-1 (human, peripheral blood), HT-29 (human, colon), Caco-2 (human, colon), HeLa (human, cervix), and Vero (African green monkey, kidney) cell lines. A mutagenicity assay of NTX and albumin was performed by an Ames test according to the preincubation technique of Yahagi et al. [19]. Prion and endotoxin contamination was assayed with a Prionics-Check Western kit (Prionics AG) and a Limulus amoebocyte lysate assay (BioWhittaker), respectively, and that of AIDS and hepatitis viruses was assayed by PCR by a commercial company (SRL, Tachikawa, Japan). In addition, approval from the ethics committees of both Okayama University and Kawasaki Medical School for this clinical trial and patient informed consent were obtained.

3. Clinical Trials

3.1. Refractory Urgency Incontinence. A preliminary study by Schurch and colleagues on 31 patients with neurogenic detrusor overactivity (NDO) demonstrated a significant increase in the mean maximum bladder capacity (296 to 480 mL, $P < 0.016$) and a significant decrease in mean maximum detrusor voiding pressure (65 to 35 cmH_2O, $P < 0.016$) in patients injected with Botox (the main toxin used was likely LL) [14]. Seventeen of 19 patients were completely continent and very satisfied with the procedure. Interestingly, baseline improvement of urodynamic parameters and incontinence persisted in 11 patients after a 36-week follow-up period. Cruz and associates recently reported the results of a multicenter, international, randomized, double-blind,

and placebo-controlled phase III clinical trials of Botox for NDO patients. Patients received 30 intradetrusor injections of either 200 U ($n = 92$) or 300 U ($n = 91$) Botox, or placebo ($n = 92$). By week 6, either 200 U or 300 U Botox significantly reduced the number of incontinence episodes per week (-21.8 and -19.4, resp.), significantly increased maximal cystometric capacity, and significantly improved quality of life scores compared to placebo. The median time to patient request for retreatment was the same for both Botox doses (42.1 weeks) [20].

Intradetrusor Botox injections have extended beyond the realm of neurogenic bladders to patients with nonneurogenic voiding and storage disorders [14, 15]. Dmochowski and associates obtained favorable effects with intradetrusor Botox injections in a double-blind, placebo controlled, randomized study that evaluated several Botox doses (i.e., 50, 100, 150, 200, and 300 U) versus placebo in 313 patients with idiopathic detrusor overactivity (IDO) [16]. Patients who experienced urinary urge incontinence with at least eight episodes/week and eight or more micturitions/day were included in the study. The primary endpoint was weekly urinary urgency incontinence episodes at 12 weeks. The authors observed a significant difference in the number of urgency incontinence episodes/week at many time points for Botox -treated patients compared to placebo patients.

Our group performed a clinical trial using NTX to treat patients with urgency incontinence caused by NDO or by IDO. A total of 200 U (for NDO), 100 U (IDO for women), and 50 U (IDO for men) NTX were injected into the bladder wall in a minimally invasive outpatient technique using flexible cystoscopy. All patients with NDO performed clean intermittent catheterization before the treatment. Urodynamic maximum cystometric capacity and maximum detrusor pressure were evaluated before and one month after treatment by filling cystometry. Subjective and objective measures included frequency of voids and number of incontinence episodes per 24 hours from a 3-day bladder diary before treatment and 1, 3, and 6 months after treatment. A total of 16 patients with spinal NDO and a mean age of 42.3 years (range 20 to 75 years, 2 females and 14 males) and 33 with IDO and a mean age of 71.9 years (range 50 to 83 years, 15 females and 18 males) were treated [18]. NTX treatment produced an increase in the mean maximal cystometric capacity from 138.2 ± 13.8 mL at baseline to 358.8 ± 25.2 mL at one month ($P = 0.0036$) in NDO patients and 171.8 ± 10.2 mL at baseline to 319.8 ± 19.4 mL at one month ($P < 0.0001$) in IDO patients. The mean maximal detrusor pressure in detrusor overactivity decreased from 65.9 ± 6.5 cmH_2O at baseline to 27.3 ± 4.8 cmH_2O ($P = 0.0025$) one month after treatment in NDO patients and 63.5 ± 5.0 cmH_2O at baseline to 32.3 ± 4.4 cmH_2O ($P = 0.0001$) at one month for IDO patients. The mean number of daily urinary incontinence episodes decreased from 4.67 ± 0.7 to 1.07 ± 1.0 ($P < 0.0001$) at 1 month and to 2.07 ± 0.4 ($P = 0.0002$) at 3 months for NDO patients. The mean number of episodes of urinary incontinence daily decreased from 4.46 ± 0.5 to 1.49 ± 0.4 ($P < 0.0001$) at 1 month, to 1.82 ± 0.4 ($P = 0.0002$) at 3 months, and to 2.28 ± 0.7 ($P = 0.0051$) at 6 months for IDO patients. Ten of 16 NDO patients (62.5%) and 15 of 33 IND patients (45.5%) obtained complete continence within 1 month of NTX injection. NTX treatment halved the number of urinary incontinence episodes for thirteen NDO patients (81%) and 23 (69.7%) IDO patients. The mean duration of efficacy was 3 to 10 months (median 4 months) in NDO patients and 4 to 12 months (median 6 months) in IDO patients. Ten of 33 (30.3%) IDO patients had no subjective improvement. Although 5 patients had improved cystometric findings, they did not have subjective improvement due to the increase of residual urine volume and voiding dysfunction. Nineteen (57.5%) of 33 IDO patients had residual urine volumes >100 mL at 1 month posttreatment and 4 male patients required clean intermittent catheterization for a few weeks. No serious side effects occurred (Table 1).

In our study, NTX injection therapy achieved excellent results within one month of injection. Of the 49 patients analyzed, 36 patients reported a decrease or absence of incontinence. Followup analysis of these patients showed that these effects lasted 3 to 12 months after the single treatment. While the optimum injection dose has not yet been determined, in our study, 50–100 U for IDO and 200 U for NDO were supposed to be suitable treatment doses. These results demonstrated that NTX has almost the same activity as Botox.

3.2. Benign Prostatic Hyperplasia. The first off-label use of Botox to treat benign prostatic hyperplasia (BPH) in humans was reported by Maria et al. in 2003 [21]. In a randomized, placebo-controlled study, 30 men with symptomatic BPH were randomized to receive either saline or 200 U Botox, which produced clinical improvement within 1 month of treatment. By 2 months, 13 patients in the treatment group (87%) versus 3 patients in the control group (10%) reported subjective relief of BPH symptoms ($P = 0.00001$). At 12 months, the International Prostate Symptom Score decreased by 62% for the Botox-treated group, maximum urinary flow rate increased by 85%, postvoid residual urine volume decreased by 85%, and prostatic volume decreased by 61%. The prostatic-specific antigen values were also reduced by 38%. No urinary incontinence or systemic side effects were reported. In other reports of Botox treatment for prostatic hyperplasia, the increase in maximum urinary flow rate (40–121%) and the decrease in International Prostate Symptom Score (48–65.5%) were statistically significant [11–13]. There was a statistically significant reduction in prostatic volume that varied from 13.3% to 68%.

We treated 10 male patients (mean age 70.0 years, range 61–79 years) with unsatisfactory response to alpha1-adrenoceptor blockers with 200 U (prostate volume > 30 mL) or 100 U (prostate volume < 30 mL) NTX injected into the prostate using a minimally invasive outpatient technique. Evaluation included uroflowmetry, postvoid residual urine volume, prostate volume, and International Prostate Symptom Score and was carried out at baseline and 1, 3, 6, and 12 months posttreatment. Prostate-specific antigen was measured at baseline, 6 months, and 12 months after injection. Seven out of 10 patients noted improvement within

Table 1: Change of parameters before and after intradetrusor NTX treatment of NDO and IDO patients.

		Baseline	1 month	3 months	6 months
NDO	MCC (mL)	138.2 ± 13.8	358.8 ± 25.2*	—	—
	Pdetmax (cmH_2O)	65.9 ± 6.5*	27.3 ± 4.8*	—	—
	Frequency	7.53 ± 0.7*	5.71 ± 0.3*	6.31 ± 0.4*	—
	Incontinence (times/day)	4.67 ± 0.7*	1.07 ± 1.0*	2.07 ± 0.4*	—
IDO	MCC (mL)	171.8 ± 10.2	319.8 ± 19.4*	—	—
	Pdetmax (cmH_2O)	63.5 ± 5.0*	32.3 ± 4.4*	—	—
	Frequency	13.7 ± 0.6*	11.4 ± 0.7*	11.2 ± 0.7*	10.5 ± 0.9*
	Incontinence (times/day)	4.46 ± 0.5*	1.49 ± 0.4*	1.82 ± 0.4*	2.28 ± 0.7*

* Compared with baseline, $P < 0.05$.
MCC: maximum cystometric capacity; Pdetmax: maximum detrusor pressure at detrusor overactivity.

Table 2: BPH Patient profiles and results after intraprostatic NTX treatment.

	Baseline	1 week	1 month	3 months	6 months	9 months	12 months
Number of patients	10	10	10	10	9	9	8
IPSS	23.8 ± 7.0	19.4 ± 9.3*	16.3 ± 10.3*	14.9 ± 8.2*	13.8 ± 7.5*	13.8 ± 7.6*	16.9 ± 7.3*
Storage	8.7 ± 4.8	7.2 ± 4.5	6.7 ± 5.4	5.3 ± 3.8*	4.4 ± 3.5*	5.4 ± 3.5*	5.2 ± 4.3*
Voiding	11.8 ± 3.4	9.5 ± 4.4	6.9 ± 5.0*	7.4 ± 4.8*	7.0 ± 4.2*	6.8 ± 4.5*	7.4 ± 4.6*
QOL score	5.2 ± 1.0	4.3 ± 1.6*	3.4 ± 1.6*	3.3 ± 1.8*	3.2 ± 1.6*	3.7 ± 1.4*	4.3 ± 1.5*
Qmax, mL/s	6.3 ± 3.1	5.5 ± 2.0	6.1 ± 2.6	8.8 ± 2.9*	6.8 ± 3.9	6.2 ± 2.2	7.2 ± 4.0
Residual urine (mL)	99.5 ± 94.5	84.9 ± 83.1	101.5 ± 97.0	65.3 ± 73.6*	52.9 ± 62.5*	86.3 ± 100	72.4 ± 58.2
Prostatic volume (mL)	47.8 ± 21.2	45.9 ± 22.3	40.2 ± 18.2*	39.2 ± 19.5*	40.2 ± 19.2*	42.9 ± 23.2	41.0 ± 17.0
PSA (ng/mL)	4.30 ± 3.0				3.75 ± 2.2		4.35 ± 2.8

* Compared with baseline, $P < 0.05$.

1 month. The mean International Prostate Symptom Score decreased from 23.8 ± 7.0 to 16.3 ± 10.3 ($P = 0.0093$) at 1 month, to 14.9 ± 8.2 ($P = 0.0074$) at 3 months, and to 16.9 ± 7.3 ($P = 0.018$) at 12 months after injection. The mean prostate volume decreased from 47.8 ± 21.2 to 39.2 ± 19.5 mL ($P = 0.0076$) at 3 months. Postvoid residual urine volume improved at 3 and 6 months posttreatment. The mean prostate-specific antigen did not change during the observation period (Table 2).

Although our study achieved only an 18% reduction in prostate volume, this outcome was similar to that obtained by Kuo and Chuang et al. [11, 12]. Botox effects appeared during the first week to one month after treatment and these benefits were maintained for 6 to 12 months [11–13]. In our study, the beneficial results were evident within 1 week of treatment and continued for 12 months. The maximum effects were from 3 to 9 months and the prostatic volume reached a minimum level at 3 to 6 months. Silva et al. [13] also reported that the prostate volume decreased gradually to a minimum level at 6 months. Therapeutic efficacy relates to the injection dosage, diffusion of toxin within the prostate, the ratio of epithelium and stromal components, and detrusor function. The improvements in International Prostate Symptom Score, maximum urinary flow rate, reduction of prostate volume, and duration of effect seen for our cases were almost the same as previous reports for Botox [11–13].

4. Application of NTX in a Rat Model of Trigeminal Neuropathy

Trigeminal neuropathic pain is characterized by recurrent episodes of intense, lancinating facial pain [22, 23]. Management of trigeminal neuropathic pain remains a major therapeutic challenge with antiepileptic drugs currently being the main treatment choice [24, 25]. However, since some patients cannot tolerate these drugs because of side effects (e.g., dizziness, drowsiness), more effective and safer drugs are required to treat trigeminal neuropathic pain. Botulinum toxin is one such candidate, and open-label trials with trigeminal neuralgia patients showed botulinum toxin efficacy [26, 27]. Animal studies reported that botulinum toxin injection into the peripheral tissue decreases leg neuropathic pain induced by sciatic nerve ligation or transduction [28–30]. However, a systematic review indicated that there is a lack of sufficient data that would suitably recommend botulinum toxin as an evidence-based treatment for secondary headaches or cranial neuralgias [31]. Most of these clinical trials used commercially available botulinum PTXs and did not use NTX. Since the true effects of NTX are currently unclear, continued studies on the effect of NTX treatment for trigeminal neuropathic pain animal models are needed. Here, we evaluated the effect of type A NTX in trigeminal neuropathic pain animal models.

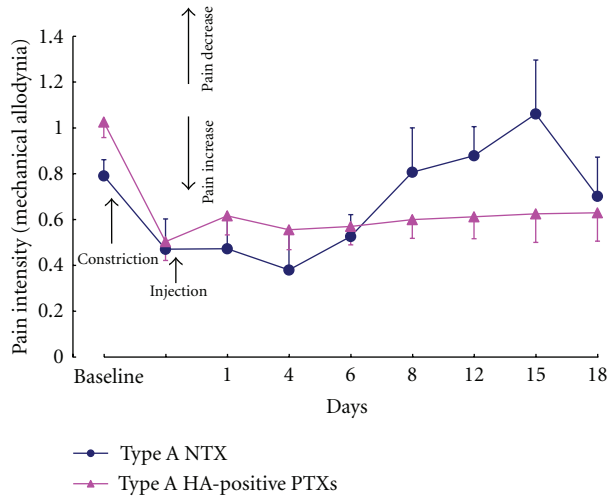

Figure 3: Mechanical allodynia after injection with type A NTX or PTXs in a rat hindpaw neuropathy model. Mechanical allodynia was tested with an electric von Frey filament and the hindpaw withdrawal thresholds measured. The data were divided by the contralateral naïve hindpaw data. After sciatic nerve contraction, the ipsilateral hindpaw threshold was decreased. Ipsilateral injection with NTX or HA-positive PTXs produced the same toxicity (10 U/0.1 mL) in mechanical allodynia. The effects of NTX on decreasing pain levels were longer lasting than HA-positive PTXs. $n = 4$; data represent the mean ± SEM.

Kitamura et al. [32] reported that a large decrease in head withdrawal threshold was observed after ipsilateral infraorbital nerve (branch of trigeminal nerve) constriction (IoNC) by stimulating the face with an electric von Frey filament. This result showed that the rats had tactile allodynia, which trigeminal neuropathic pain patients usually show clinically and which is considered to be a pain response. The intradermal ipsilateral injection of type A NTX (100 pg or 10 U) significantly increased the head withdrawal threshold compared to saline-injected trigeminal neuropathic pain rats, suggesting that NTX injection decreased rat trigeminal neuropathic pain. These results are consistent with those of Filipović et al. [33], who also found thatunilateral injection of Botox reduced IoNC-induced allodynia with the effect lasting more than 2 weeks. Next, we compared the analgesic effect of type A NTX and HA-positive PTXs (a mixture of L and LL toxins obtained using a lactose gel column as shown in Figure 2). Figure 3 shows that, compared to PTX, NTX decreased pain levels for longer periods.

Kumada et al. [34] tested freely moving rats and found that type A NTX attenuated IoNC-induced thermal hyperalgesia. These effects were dose dependent (2–200 pg) and statistically significant at 100 and 200 pg ($P < 0.05$). Offsite (neck) injection of type A NTX did not relieve thermal hyperalgesia and coinjection of type A NTX with a neutralizing antibody in the area of infraorbital nerve innervation prevented relief of thermal hyperalgesia. These results strongly suggest that intradermal injection of type A NTX in the area of infraorbital nerve innervation alleviates IoNC-induced thermal hyperalgesia.

The detailed mechanisms of the type A NTX effects on trigeminal neuropathic pain in animal models are not clear. Some studies suggest that the botulinum toxin undergoes axonal transport. Antonucci et al. [35] reported that, after injection of type A NTX into rat whisker muscles, cleaved synaptosomal-associated protein of 25 kDa (SNAP-25) appeared in the facial nucleus, indicating that the toxin migrated along the axons and underwent neuronal transcytosis. Matak et al. [36] found that Botox truncated SNAP-25 in the medullary dorsal horn (spinal trigeminal nucleus) was evident 3 days following the peripheral treatment. Filipović et al. [33] reported that unilateral Botox injection reduced-IoNC induced bilateral dural extravasation while Kitamura et al. [32] observed that neurons isolated from trigeminal ganglion ipsilateral to infraorbital nerve constriction exhibited significantly faster onset of FM4-64 dye release in dissociated trigeminal ganglion neurons compared to neurons in contralateral sham surgery. Intradermal injection of type A NTX in the area of infraorbital nerve innervation reduced the exaggerated FM4-64 dye release in trigeminal ganglion neurons from these rats.

In this manuscript, we demonstrated that type A NTX purified with a lactose gel column could be used clinically and was as effective as commercially available PTXs. Recently, preparations of type A NTX alone and type B PTX (probably L toxin) have been made commercially available. The production of anti-NTX Ab in repeatedly injected patients as well as the efficacy of the toxin must be monitored. Usually, type B toxin is not fully activated but can be activated following trypsin treatment. Our procedure is suitable for purifying fully activated type B toxin with partially purified type B PTXs first fully activated by trypsin just prior to loading on the lactose gel column. After binding of the L toxin to the column, trypsin was washed out and the fully activated L toxin or NTX can then be obtained similarly to type A toxins (Figure 2) [37]. We also found that the

toxicity of type B NTX could be maintained at −30°C for long periods by adding 0.05% albumin. We plan to evaluate fully activated type B NTX for clinical usage in the future.

Abbreviations

NTX: Neurotoxin
PTX: Progenitor toxin
HA: Hemagglutinin
NTNH: Nontoxic non-HA
NDO: Neurogenic detrusor overactivity
IDO: Idiopathic detrusor overactivity
IoNC: Infraorbital nerve constriction.

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Hindawi Publishing Corporation
Journal of Toxicology
Volume 2012, Article ID 862764, 8 pages
doi:10.1155/2012/862764

Review Article

Clostridial Spores for Cancer Therapy: Targeting Solid Tumour Microenvironment

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Received 23 December 2011; Accepted 27 January 2012

Academic Editor: Martha L. Hale

Solid tumour accounts for 90% of all cancers. The current treatment approach for most solid tumours is surgery, however it is limited to early stage tumours. Other treatment options such as chemotherapy and radiotherapy are non-selective, thus causing damage to both healthy and cancerous tissue. Past research has focused on understanding tumour cells themselves, and conventional wisdom has aimed at targeting these cells directly. Recent research has shifted towards understanding the tumour microenvironment and it's differences from that of healthy cells/tissues in the body and then to exploit these differences for treatmeat of the tumour. One such approach is utilizing anaerobic bacteria. Several strains of bacteria have been shown to selectively colonize in solid tumours, making them valuable tools for selective tumour targeting and destruction. Amongst them, the anaerobic *Clostridium* has shown great potential in penetration and colonization of the hypoxic and necrotic areas of the tumour microenvironment, causing significant oncolysis as well as enabling the delivery of therapeutics directly to the tumour *in situ*. Various strategies utilizing *Clostridium* are currently being investigated, and represent a novel area of emerging cancer therapy. This review provides an update review of tumour microenvironment as well as summary of the progresses and current status of Clostridial spore-based cancer therapies.

1. Introduction

Cancer is currently a major cause of morbidity and mortality internationally and poses a significant burden on both patients and their families and the healthcare system as a whole. Solid tumours, in particular, account for 90% of all cancers. A solid tumour is composed of a complex mix of tumour cells and nontumour cells, including supporting stromal and infiltrating blood cells, immune cells, and various molecules in proximity to these cells. This collection of cells and their metabolism is referred to as the tumour microenvironment. This unique tumour milieu not only allows for growth and metastasis, but additionally aids in the resistance of cancer cells to current chemotherapy and radiotherapy, thereby hindering their success. Consequently, there is an urgent demand for more suitable and effective treatment options for those suffering from solid tumours.

2. Tumour Microenvironment

Research in the past has typically focused on understanding the molecular and genetic aspects of cancer cells, which led to treatment options geared towards killing the cancer cells themselves. Recent advances in the field of oncology have lead to a greater understanding of tumour pathology. Currently, a greater research emphasis has been placed on comprehending the unique environment of solid tumours, referred to as the tumour microenvironment. Developments in this field have lead to the possibility of pursuing methods of treatment which serve to control this milieu in such a way that will enable us to manage the growth and metastasis of the tumour itself. This has generated the potential for new therapeutic targets and treatment options for cancer patients. *Clostridium*-spore-based bacterial therapy is one such a novel strategy.

2.1. Angiogenesis. Growth and progression of tumour cells requires an increased supply of both oxygen and nutrients. For this the formation of a new vascular network is required to meet these demands [1]. Under regular physiological conditions, angiogenesis in cells results in a structurally well organized and highly efficient network of vasculature. However, this is not the case in tumours, where angiogenesis leads to a chaotic network of blood vessels. This is a distinctive component of the cancer microenvironment. This vasculature may be characterized by disorganization, dilation, branching, shunts, and varied diameters which results in inconsistent blood flow and further alters the microenvironment by creating hypoxic areas and regions high in acidity [1, 2]. Angiogenesis is a crucial component in the metastasis of cancer cells, and if it can be controlled, has the capability of halting the spread of cancer to other tissues. Additionally, the chaotic nature of the blood vessel and its network in the tumour microenvironment poses a problem for current cancer therapies in that it makes it difficult to administer drugs uniformly and in effective concentrations in all areas of the tumour. These are two problems which are trying to be overcome by instead treating the tumour environment and controlling angiogenesis as opposed to killing the cancer cells directly.

The key mediator of angiogenesis is the glycoprotein vascular endothelial growth factor (VEGF), which stimulates blood vessel growth by acting on endothelial cells [3]. Additionally, VEGF can afford viability of immature blood vessels by preventing apoptosis, and evidence has shown it can also make vasculature more permeable causing leaky cell walls resulting in an increase of interstitial pressure [4–6]. Mediation by the VEGF proteins is a critical component in creating the chaotic vasculature of the tumour microenvironment, and as such is a primary research target for new therapeutic strategies.

2.2. Hypoxia and HIF-1. Acute and chronic lack of blood supply resulting in the absence of oxygen and known as hypoxia is also a definitive characteristic of the tumour micro milieu and is caused by inefficient blood supply to cancerous cells. The hypoxic environment poses a challenge to traditional cancer treatment options in that this oxygen-depleted environment makes the solid tumour cells less susceptible to killing with chemotherapy and radiation, less genetically stable (thus more difficult to predict its response to treatment), and resistant to apoptosis [7]. Since these cells are devoid of oxygen, carbohydrate metabolism occurs via glycolysis resulting in the production of lactic acid and decreasing the pH of the tumour environment. Critical in the control of tumour hypoxia are the hypoxia-inducible factors (HIFs), a family of transcription factors which serve in the regulation of mammalian response to the absence of oxygen. One of the targets of HIF-1 is VEGF, which is switched on in a hypoxic environment. VEGF then stimulates angiogenesis creating poor vasculature within the tumours, causing further hypoxia creating a positive feedback loop where both factors are stimulated. It is important to note that VEGF is not the only target of HIF-1, and in fact, more than 60 direct target genes have been identified, many playing crucial roles in cell survival, metabolism, and metastasis of cancer cells [8]. The crucial role that HIF transcription factors have in creating the tumour microenvironment makes these proteins potentially important cancer treatment targets.

2.3. Apoptosis and Necrosis. The proinflammatory cytokine tumour necrosis factor alpha (TNF-α) plays a unique role in the tumour environment where it permits cellular communication and is capable of promoting both growth and necrosis. TNF-α acts in conjunction with many other molecules within the cell to perform these tasks. For instance, tumour necrosis is carried out first by endothelial cell apoptosis caused by deactivation of the integrin $a_v b_3$ and disruption of the interface with the extracellular matrix (ECM), followed by T-cell activation to remove remaining tumour cells [9, 10]. In a contradictory role to this, in low chronic doses, TNF-α promotes tumour growth, invasion, and metastasis. One way it accomplishes this is by the remodelling of tissue by inducing matrix metalloproteins (MPPs) [11]. TNF-α also activates nuclear factor-κb (NF-κb), a transcription factor, which stimulates the proliferation of tumour cells and regulates antiapoptotic genes, and thus can protect the cancerous cells from the apoptotic cascade induced by TNF-α [12]. NF-κb has also hindered some forms of cancer treatment involving cytokines, chemotherapeutics, and radiation which have all been shown to activate NF-κb, helping to prevent apoptosis [13]. Both TNF-α and NF-κb can both be targets for future cancer therapies; TNF-α can be triggered to induce apoptosis, and NF-κb can be inhibited to prevent tumour growth and spread, as well as aid in making solid tumour cells more susceptible to apoptosis by treatment with other therapeutic cancer agents.

2.4. Tumour Structure and Stromal and Interstitial Pressure. Tumour structure greatly contributes to the microenvironment of cancer. The membrane structure, in particular, is an essential part of tumour invasion and metastasis. The extracellular matrix (ECM) is a key component to the tumour structure, and it comprises molecules essential to cell signalling [14]. Structural components of the ECM include fibrous elements, link proteins (fibronectin for example), and space-filling molecules, whereas the signalling components comprise the cytokines, growth factors, ions, and peptides [14]. Fibronectins, one of the most abundant structural proteins of the ECM, have additional functions in tumour cells aside from their structural roles and aid in regulation of adhesion, migration, differentiation, and proliferation [15]. Fibronectins can also serve in signalling as solid-phase ligands, further adding to the complexity of the tumour micro milieu [16]. Another set of structural components of the ECM that duals as signalling molecules are collagens. In solid tumours, collagen has sometimes shown to be upregulated, providing a rigid matrix aiding in cell growth. The primary role of collagen, however, is in anchoring cells to the membrane and aiding in regulation [17]. As such, both fibronectin and collagen are key regu-

lators of tumour invasion, metastasis, growth, proliferation, and signalling.

Additional regulators of structural elements of the tumour cell are essential in metastasis. For example, the steps involved in the spread of cancer between tissues include the degradation of basement membrane and ECM, modulation of cell adhesion molecules, followed by migration to adjacent tissue [18]. During degradation of the basement membrane and ECM, matrix metalloproteinases (MMPs) play a vital role. MMPs are a family of enzymes capable of degrading all parts of the ECM, as well as other nonmatrix substrates which also contribute to the tumour microenvironment and progression [19–21]. MMPs also play a crucial role in angiogenesis, where they degrade the cellular matrix surrounding endothelial cells making them a target for antiangiogenesis [18, 22]. Research has shown them to be involved in cell growth (both inhibitory and stimulatory), proliferation, and apoptotic prevention as well [14].

The high interstitial fluid pressure compared to that of normal cells is yet another key defining element of the tumour milieu. This is the result of the chaotic vasculature of solid tumours as previously described, due to high vessel permeability and cell density outside the vessels, low lymphatic drainage, and poor perfusion [23]. This causes problems for the administration of cancer therapeutics, which may not successfully be able to reach all tissues in sufficient quantities due to transcapillary transport [24].

2.5. Cancer-Related Inflammation. Inflammation is present in the microenvironment of some solid tumours prior to metastasis, and in others, an oncogenic change induces an inflammatory response which aids in the development of tumours [25]. This inflammation assists in proliferation, survival, angiogenesis, and metastasis, while simultaneously facilitating in avoiding the adaptive immune system, and disrupting signals from hormones and chemotherapeutics [25]. Therefore, molecules which coordinate these inflammatory responses are important constituents of the tumour micro milieu. Cytokines and chemokines are two classes of these regulatory components.

Chemokine is a family of cytokines which play a vital role in the directing leukocyte migration to sites of infection and inflammation in the body. In cancer, aberrant chemokine and cytokine receptor production facilitates tumour growth by mediating migration of leukocytes to tumour cells and stimulating the release of growth factors, such as tumour transforming growth factor (TGF), and promoting angiogenesis [26]. Cytokines are components of the immune system which stimulate the generation of antitumour specific responses. Additionally, however, cytokines may influence carcinogenesis and metastasis by modifying the tumour phenotype [27]. A primary example is interleukin-2 (IL2), a pluripotent cytokine which augments innate immune responses such as activation of natural killer and lymphokine-activated killer cells, and neoplastic cell killing by MHC-restricted T-cell responses [28].

3. The Impact of Tumour Micro Milieu on Current Cancer Therapy

Thus far, the development of successful cancer therapies has been hampered by various aspects of the tumour micro milieu. Primarily, the obstacles of angiogenesis, hypoxia, and heterogeneous cell arrangement must somehow be overcome in order to develop viable treatment strategies for solid tumours. Along with increased efforts to understand the tumour microenvironment, alternative cancer treatment strategies have, however, emerged which seek to utilize this environment to an advantage.

3.1. Selective Tumour Colonization. As previously discussed, the unique microenvironment of solid tumour cells consists of a complex, disarrayed set of blood vessels due to tumour angiogenesis, which leads to hypoxic areas, varying pH, and inconsistent blood flow. This poses a problem to current cancer treatment strategies in that therapeutics cannot be evenly distributed to all tumour tissue in effective concentrations, hindering some conventional chemotherapeutics. Additionally, many anticancer drugs target rapidly dividing cells and ignore the stromal cells and other cells that make up a tumour and can often result in tumour regrowth. Hypoxia in tumour cells may allow for inhibited cell cycle progression and proliferation, thus rendering these types of treatments ineffective [29]. In ionizing radiation, DNA damage occurs, which under aerobic conditions becomes lethal due to fixation by oxygen. In the anaerobic environment of solid tumours, however, the DNA can be restored to its original condition and may remain unharmed [30, 31]. The hypoxia of the tumour is undesirable for treatment in that it contributes to a higher degree of malignancy in the cancer cells, whilst aiding in cell development and angiogenesis [18]. Severe hypoxia in tumours, not surprisingly, has therefore been linked to poor prognosis [32]. Other treatment strategies have also been rendered less effective due to this hypoxic environment. The initially very promising area of utilizing adenoviral vectors to treat cancer was thwarted by this tumour hypoxia, where it induced halting of G1 cells responsible for viral replication, resulting in less effective treatment [33, 34]. The hypoxic milieu negatively influenced treatment via retroviral vectors due to the phosphorylation of eIF2α leading to inhibition of translation [35].

Bacterial-based cancer therapy using *Clostridium* spores offers a selective advantage in overcoming the obstacles of hypoxia and necrosis. *Clostridium* species, being strictly anaerobic will only colonize in areas devoid of oxygen, and when systematically injected, spores germinate and multiply in the hypoxic/necrotic areas of solid tumours [36]. *Clostridium*, although anaerobic, possesses the ability to sporulate, allowing them to remain dormant in environments where oxygen is present. However, when growth conditions are suitable (i.e., in the hypoxic/necrotic milieu of solid tumours), the *Clostridium* spores germinate and begin to colonize these areas. This aspect of *Clostridium* growth is being exploited for use in a number of various novel cancer treatment strategies currently being developed which utilize

Table 1: Summary of current methods being researched in *Clostridium*-based cancer therapies.

Method	Premise	Target/drugs	Clostridium species being used	Reference
Clostridium directed enzyme prodrug therapy (CDEPT)	*Clostridium* is genetically engineered to express an enzyme which cleaves a prodrug into its cytotoxic form.	CD/F-FU	*C. sporogenes*	[39]
			C. beijerinckii	[49]
			C. acetobutylicum	[41]
		NTR/CB1954	*C. beijerinckii*	[37]
			C. sporogenes	[40]
		NTR/PR-104		
Administration of cytokines/cytotoxic agents	*Clostridium* is used to deliver agents (cytokines) to either act directly cytotoxic to cells or enhance immune system response to tumour cells.	murine TNFα	*C. acetobutylicum*	[41, 50, 51]
		IL-2	*C. acetobutylicum*	[42]
Clostridium directed antibody therapy (CDAT)	*Clostridium* is modified to produce highly specific antibodies against tumour antigens.	VHH against HIFα	*C. novyi-NT*	[45]
Combined bacteriolytic therapy (COBALT)	*Clostridium* which demonstrate direct antitumour effects are administered in conjunction with other known cancer therapies to increase oncolysis.	*Clostridium*/ mitomycin C and cytotaxin *Clostridium*/vinorelbine or docetaxel	*C. novyi-NT*	[47]
			C. novyi-NT	[52]
Release of liposomal encapsulated drugs	Species of *Clostridium* which secrete lipid-degrading enzymes are used for the targeted release of liposome-encapsulated drugs at the tumour site.	*Clostridium*/Doxil	*C. novyi-NT*	[48]

Clostridium as a vector to deliver therapeutics directly to the solid tumour site (Table 1). Clostridial vectors can be safely administered as spores, and their efficacy in delivering and secreting therapeutic proteins has been demonstrated in a number of preclinical trials.

3.2. Clostridium-Directed Enzyme Prodrug Therapy. One novel treatment strategy, known as suicide gene therapy or gene-directed enzyme prodrug therapy, utilizes an enzyme which cleaves or modifies a prodrug such that cleavage results in the active, oncolytic form of the toxin. Using *Clostridium* species as a vector, known as *Clostridium* directed enzyme prodrug therapy (CDEPT), the bacterium can be genetically modified to express these proteins. When spores are administered systematically, the *Clostridium* selectively grows and colonizes in the tumour cells where it expresses the prodrug cleaving enzyme. When the therapeutic is then administered, it is only cleaved into its active component in the localized tumour environment, resulting in tumour cell specificity as opposed to the nonspecific targeting exhibited by most other current treatment options, such as radiation. Although other vector systems exist (primarily viral vectors), bacterial vectors, *Clostridia* in particular, convey benefits such as lower toxicity, higher safety, and nonexistence no restraints on the gene size to be delivered. Additionally, bacteria can be rendered inactive rather quickly by the administration of antibiotics further increasing the safety of these approaches.

Several enzyme/prodrug combinations are currently available. The cytosine deaminase (CD) and 5-fluorocytosine (5FC) system was one of the first systems to be cloned into *Clostridium*. CD converts the prodrug 5FC into 5-fluorouracil (5FU), a cytotoxic compound. Another common combination is the nitroreductase (NT R) enzyme, which converts CB1954 to a DNA cross-linking agent [37, 38]. Although these enzymes have been successfully cloned into a number of *Clostridium* strains, *C. sporogenes* has shown the highest potential thus far. Injection of recombinant CD expressing *C. sporogenes* NCIMB10696 spores into tumour-bearing mice was successful in tumour-specific expression of CD [39]. Moreover, when this was combined with its prodrug 5FC, significant tumour growth delay was achieved. Promising results were also obtained with the NTR enzyme/Pr-104 prodrug combination as well. NTR was successfully transformed into and expressed by *C. sporogenes*, and *in vivo* studies where spores were injected into tumour-bearing mice showed significant tumour reduction [40].

3.3. Clostridium to Enhance the Immune System and Tumour Cell Recognition. Another novel treatment being developed in cancer therapy is genetic engineering of bacteria that express an enzyme possessing direct cytotoxic actions, as opposed to having prodrug cleaving actions. As previously discussed, TNF-α, a cytokine, can act as a tumour regressor in high doses where it functions as a vasculotoxic agent and as such is being researched as one of these cytotoxic enzymes. Over a decade ago, Theys et al. [41] genetically engineered *C. acetobutylicum* DSM792 to express and secrete murine TNF-α. Although TNF was secreted and biologically active,

colonization levels of recombinant *C. acetobutylicum* were low, and sufficient amounts of TNF to combat the tumour cells were not secreted, and therefore no therapeutic benefits were observed [41]. More recently, an attempt was made at increasing TNF-α expression and secretion levels in *C. acetobutylicum* by adjusting transcription, translation, and secretion processes; however, no significant advances were made and TNF-α expression remained constant [42]. For this protein to be excreted in levels high enough to act as an antitumour agent, ways must be developed which would either allow increased TNF-α production at the tumour site or utilized the synergistic effect of interleukin-2 and TNF-α [42, 43].

Interleukin-2 (IL-2) is a cytokine which enhances the immune system's natural anticancer functions [42]. Administration of cytokines such as IL-2 to the tumour environment may stimulate the immune system to discern and assail the solid tumour cells; however caution must be taken as high systemic levels of IL-2 cause toxicity. This was the basis for the genetic modification of *C. acetobutylicum* DSM792 by Barbe et al. [42] in 2005 to express increased levels of IL-2. By introducing rat IL-2 into *Clostridium*, solid tumours were specifically targeted and sufficient levels of IL2 were produced and excreted to decrease tumours in mice, while avoiding the effects of systemic toxicity. It may be possible to make treatment of this sort more effective by combining with other cytokines, enzymes, or chemotherapy. Additionally, by combining IL-2-recombinant *Clostridium* with a vascular targeting agent, it is hypothesized that colonization of the bacteria will increase while also increasing the release of tumour antigens from cells that have become necrotic, thus increasing the antitumour response by immune cells [42].

3.4. Clostridium-Directed Antibody Therapy (CDAT). Another area in which this is being utilized is *Clostridium*-directed antibody therapy (CDAT) where *Clostridium* is modified to produce high specificity antibodies against tumour antigens. Recently, CDAT was used to target HIF1α cells using the variable domain of the heavy-chain subclass of antibodies (VHH). A VHH against HIF1α, which when expressed in mammalian cells binds and inhibits HIF activity [44], was introduced into *C. novyi-NT* by heterologous gene transfer. The VHH, when isolated from the *Clostridia*, retained its binding capacity and specificity for the target, and overall the study demonstrated successful conjugation, expression, and functionality of these antibodies [45]. Further research into codon usage and promoters of the VHH antibody gene must be performed, however, to increase expression levels in *Clostridia* in order to successfully utilize this method in future cancer therapies. The potential of this would be in that by targeting HIF1α, hypoxia in the solid tumour could be controlled, and subsequently, factors contributing to metastasis and invasion could be eliminated, thus impeding the spread of the cancerous cells. This type of treatment would be useful as a combined modality treatment, where Clostridia spores are administered and express VHH upon germination. Once VHH minimizes tumour hypoxia by targeting HIF1α, a second treatment such as chemotherapy or radiation may be applied, which would be more effective as a result of the decreased hypoxia.

Our laboratory has recently created a hybrid toxin that could be expressed and delivered using the clostridial system. This toxin utilises the high affinity of receptor binding fragment of *Clostridium perfringens enterotoxin* (CPE). CPE naturally binds to CLDN-4 through the C-terminal 30 amino acid. Taking advantage of the fact that CLDN-4 is overexpressed on a range of cancer cells, we thus constructed a cDNA comprising C-CPE and a fragment of exotoxin A(ETA') (C-CPE-ETA'). The recombinant C-CPE-ETA' fusion protein was shown to retain the specificity of binding to CLDN-4 and initiate rapid penetration into cytosol in five different CLDN-4-positive cancer cells (MCF7, A431, SW480, PC3, and DU145) but not to CLDN-4-negative cells (HELA, HUVEC). C-CPE-ETA' was strongly cytotoxic towards CLDN-4-positive cancer cell, as opposed to cells lacking CLDN-4 expression. Moreover, we have also demonstrated that the recombinant fusion protein had significant anticancer ability in CLDN-4-positive cancer models *in vivo*. Subcutaneously implanted MCF7 and SW480 xenograft tumours were significantly decreased or abolished after three repeated injection of the hybrid toxin [46].

3.5. Combined Bacteriolytic Therapy (COBALT). COBALT is a proposed method of cancer treatment which has been relatively successful thus far. In this type of therapy, a bacterium is engineered to exhibit antitumour properties, such as proteolytic enzymes, which is then administered in conjunction with other known cancer therapies to work synergistically and improve oncolysis. A new strain of *Clostridium*, *C. novyi-NT*, which expresses proteolytic proteins was recently developed which showed significant antitumour activity in mice. To identify and manufacture this strain, 26 strains of bacteria were tested for tumour colonisation efficiency, and *C. novyi* was particularly promising. However, this contained a lethal toxin, which was subsequently genetically removed, and the newly engineered strain was designated *C. novyi-NT* [47]. Systemic administration of *C. novyi-NT* spores destroys adjacent cancer cells while simultaneously prompting inflammatory action by the recruitment of cytokines, attracting neutrophils, monocytes, and lymphocytes which attack cancer cells [38]. In an attempt to increase the antitumour action of the bacteria, spores of this strain were administered alongside of known anticancer drugs. Very promising results were obtained when bacterial *C. novyi-NT* spores were administered in conjunction with microtubule-interacting chemotherapeutic agents such as vinorelbine and docetaxel, and in 2006 a phase 1 clinical trial was commenced. Unfortunately, the first study was terminated due to design problems, but a second attempt at a phase one clinical trial to test safety has recently started and is still in the stages of recruiting participants [http://www.clinicaltrials.gov/ct2/results?term=c.novyi-NT]

3.6. Liposome-Mediated Preferential Release of Drugs at Solid Tumour Site. Genome analysis of *C-novyi-NT* has demonstrated that some of its oncolytic capabilities are due to the

presence of several lipid degrading enzymes, which are highly expressed when *C. novyi-NT* is colonised in tumours [31]. In 2006, Cheong et al. [48], exploited this property of *C. novyi-NT* to release liposomal drugs within the tumour. It was hypothesised that since this species lyses red blood cells, its ability to disrupt membranes might be able to increase the release of liposome-encapsulated drugs directly at the site of the tumour. A form of doxorubicin incorporated within a liposome called Doxil was utilised, and promising results were obtained. Systemic injection of *C. novyi-NT* spores in conjunction with Doxil completely eliminated tumours in two models. Further analysis of protein fractions of the culture medium, as well as mass spectroscopy analysis, revealed that lipase was indeed the protein product secreted by *C. novyi-NT* which increased the effectiveness of the liposome-encapsulated drug. The significance of this study is that further research can be performed using other liposome encapsulated drugs with specific targeting and release within the tumour microenvironment [31, 48].

Although theoretically very promising, there are still some obstacles that must be overcome in order for *Clostridium*-directed therapies to be developed as a viable treatment option for cancer patients. Clostridium species which are easily transformable, such as *C. acetobutylicum*, do not typically have high colonisation efficiencies, whereas species which typically colonise well in tumour cells, such as *C. sporogenes*, *C. oncolyticum*, and *C. novyi-NT* are not easily transformed. However, recent advancements in genetic engineering technologies have allowed for improvements in this area. Using a new method of conjugative transfer from *E. coli*, Theys et al. were able to successfully transform plasmid vectors at higher frequencies into these strains of *Clostridium*, opening many new doors in *Clostridium*-directed cancer therapies [49]. Keeping this in mind, there are still many areas which need to be improved before *Clostridium* can be successfully implemented in current cancer therapies, but research efforts continue and advances are being made regularly in this field.

4. Conclusions and Future Perspectives

Clostridium-based cancer therapy is a promising approach for the treatment of solid tumours. Recently, understanding the solid tumour microenvironment and its influence on cancer therapy has profoundly changed our thinking about cancer therapy. We have realised that the unique solid tumour micro milieu has been one of the greatest hindrances thus far in successful cancer treatment. The utilisation of anaerobic *Clostridium* species allows for a targeted and curative treatment by destroying tumour microenvironment first, creating opportunity for combinational therapies. In addition, numerous strategies involving the administration of *Clostridium* spores to selectively deliver cancer therapeutics directly to the site of the solid tumour are currently being developed, and in many cases, promising oncolytic capabilities have been demonstrated. However, successful implementation of this mode of therapy in clinical trials relies on developmenting and manufacturing a *Clostridium* species to have both high colonisation efficiency and expression and excretion of sufficient high levels of the therapeutic proteins. Improving genetic engineering methods to genetically modify bacteria and modulation of gene expression to yield maximum protein secretion are areas which may enhance this field. *Clostridium*-based cancer therapies are one of the most novel and promising methods of cancer treatment currently being researched. The ability of these *Clostridium* based modalities to selectively target the microenvironment has provided a firm foundation for which to build towards efficient, safe and effective cancer treatments for the future and to improve the prognosis and treatment of so many individual patients suffering from solid tumours, relieving the burdens of patients, their families and the healthcare systems.

Acknowledgments

The authors would like to acknowledge and thank other members of the Wei Laboratory for their comments, as well as provision of reference and resources. They also want to thank the grant supports to M. Q. Wei from the Dr. Jian Zhou smart state fellowship, Queensland government, and the National Health and Medical Research Council and Cancer Council, Queensland Australia.

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Hindawi Publishing Corporation
Journal of Toxicology
Volume 2012, Article ID 159726, 9 pages
doi:10.1155/2012/159726

Review Article

Kinetic and Reaction Pathway Analysis in the Application of Botulinum Toxin A for Wound Healing

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Received 24 May 2011; Accepted 5 September 2011

Academic Editor: Martha L. Hale

A relatively new approach in the treatment of specific wounds in animal models and in patients with type A botulinum toxin is the focus of this paper. The indications or conditions include traumatic wounds (experimental and clinical), surgical (incision) wounds, and wounds such as fissures and ulcers that are signs/symptoms of disease or other processes. An objective was to conduct systematic literature searches and take note of the reactions involved in the healing process and identify corresponding pharmacokinetic data. From several case reports, we developed a qualitative model of how botulinum toxin disrupts the vicious cycle of muscle spasm, pain, inflammation, decreased blood flow, and ischemia. We transformed this model into a minimal kinetic scheme for healing chronic wounds. The model helped us to estimate the rate of decline of this toxin's therapeutic effect by calculating the rate of recurrence of clinical symptoms after a wound-healing treatment with this neurotoxin.

1. Introduction

As characterized by Hanchanale and coworkers [1], the perception of botulinum toxin A has been transformed "from poison to a healing agent." Much of our present knowledge about this toxin comes from the toxicological literature. Information concerning botulinum neurotoxin serotypes, molecular structures, substrate specificities, mechanisms of zinc-dependent peptide hydrolysis, ion channel formation, and other detailed topics has been extensively reviewed [2–4]. Of the seven immunologically distinct serotypes (A–F) from *Clostridium botulinum* and several other species, type A is the best characterized and is among the most potent of all toxins. The neurotoxin is initially expressed as a single polypeptide of nearly 1300 amino acid residues (MW ~150 kDa). Crude toxin extracts (MW ranges from ~300 to 900 kDa) contain several nontoxic ancillary proteins that form a complex with the neurotoxin. When ingested, these additional proteins are thought to protect the neurotoxin against austere environments such as those found in the certain regions of the gastrointestinal tract [5]. The neurotoxin is posttranslationally modified to form two chains that are covalently bridged with a disulfide bond. The light (L) chain (MW ~50 kDa) has zinc-dependent proteolytic activity, while the heavy (H) chain contains the translocation and binding domains. Subsequent to binding to specific receptors (SV2) [6] at peripheral cholinergic nerve terminals, the receptor-toxin complex is internalized into a membrane-bound compartment that undergoes a drop in pH. This acidification initiates a series of interrelated reactions. Conformational changes occur that allow the insertion of the H-chain into this compartment's membrane. As a result, the disulfide bond that links the L and H chains is reduced, an ion channel is formed, and the presumed proteolytic active moiety, the L-chain, is translocated into the neuroplasm. When the type A toxin

substrate, SNAP-25, is selectively cleaved, synaptic vesicle-mediated neurotransmission is blocked that could eventually lead to fatal paralysis.

Since the 1980s, the therapeutic potential of this toxin has been exploited. Extraocular muscles have been injected with the partially purified neurotoxin as an adjunct or alternative to surgical correction in treating strabismus [7, 8]. The chemodenervation effects of this most poisonous of poisons [9] have been used to relax hyperkinetic striated muscle groups to diminish the effects of dystonia and related diseases [10]. Currently, BOTOX has been approved by the U.S. Food and Drug Administration (FDA) for the following indications: strabismus, blepharospasm, cervical dystonia, upper limb spasticity, maxillary hyperhidrosis, chronic migraine [11], and urinary incontinence [12]. These indications along with the temporary enhancement in the appearance (cosmesis) with BOTOX COSMETIC of moderate to severe wrinkles in adults [13, 14] introduces the theme of muscle immobilization in terms of a desired therapeutic outcome. Immobilization has been characterized as a fundamental principle of wound healing [15]. Advantage has also been taken of this toxin's chemoimmobilization property to improve the healing of wounds.

In contrast to the relatively vast amounts of information regarding this toxin's structure and mechanism of action, the newer, off-label uses for botulinum toxin have been less extensively reviewed. To gain further insight regarding the scope of these efforts, we have gathered and examined biomedical research articles by conducting systematic searches of the relevant literature in PubMed, in a manner similar to that of Steele and Madoff [16]. We have examined studies that range from descriptive observations to randomized controlled clinical trials to obtain more information about the components and processes involved in wound healing and the related time courses of action of botulinum toxin A.

The processes observed clinically on the wound healing effects of the type A toxin are at an early stage of our understanding. This proposal is substantiated by evidence-based reviews that critically evaluate this toxin's effects with different indications [17, 18]. We previously noted [19, 20] that only a few clinical studies have focused on kinetic analyses. Constructing even partial models for the clinically observed effects by this toxin remains a challenge. To advance our understanding, we have selected some of those clinical studies that have examined the timing of this toxin's effects.

2. Methods

2.1. Literature Searches. botXminer, the botulinum reference tool of clostridial neurotoxin citations in Entrez-PubMed/MEDLINE [20], was initially used to search in article titles, abstracts, and MeSH headings for the words "wound" and "heal" or "healing." A more extensive list of 29 wound-related keywords was then generated: anal, angiogenesis, collagen, cytokine, fibroblast, fibroblastic, fibrosis, fissure, flap, glycosaminoglycan, heal, healing, hemorrhoid, hemorrhoidectomy, hypertrophic, incision, inflammation, inflammatory, keloid, lesion, repair, scar, scarring, sphincterotomy, surgical, tendon, tensile, ulcer, and wound. This controlled vocabulary was used in the batch mode [21] to search botXminer for additional related citations. Another set of filter terms were used to find time-course-related information about this toxin. This set included 26 terms: clearance, day, decay, decline, delay, diffusion, duration, follow up, frequency, hour, hr, interval, kinetic, latency, minute, month, onset, period, persistence, recurrence, repeat, resistance, sec, time, week, and year.

Within the two lists in the batch matrix search, $(a_1, a_2, \ldots, a_m)$ and $(b_1, b_2, \ldots, b_n)$, the terms undergo the OR operation ($\bigcup$) which can be represented in a general form of (a_1 OR a_2 OR, etc.). In addition, the lists are combined with the AND operation ($\bigcap$) [21] which results in a batch query that can be represented by

$$\left(a_1 \bigcup a_2 \bigcup \cdots a_m\right) \bigcap \left(b_1 \bigcup b_2 \bigcup \cdots b_n\right). \quad (1)$$

Summaries from the two sets of terms were returned by botXminer in the form of tables, histograms, and lists. Lists of citations were subsequently manually examined. Additional keywords and phrases within the more than 70 downloaded text files were automatically searched with file search assistant (v. 3.1, 2009, AKS-Labs, Raleigh, NC, USA).

2.2. Analysis of Kinetic Data. Data from the clinical literature that were analyzed were fitted to an exponential function

$$y = y_0 + (a)\exp\left(-k_{\text{decay}}t\right), \quad (2)$$

where y is the cumulative number of patients who are free of symptoms at time = t, y_0 is the cumulative number of patients who are symptom-free at $t = \infty$, a is a preexponential constant, and k_{decay} is the rate constant for the decay of this response. SigmaPlot (v. 11.0, 2008, Systat Software, Inc. Chicago, Ill, USA) was used to conduct a least-squared fit for the values of y_0, a, and k_{decay}. The 95% confidence intervals for y values were also calculated. This equation is commonly used for simulating the decay rate of a reactant from a single model compartment.

2.3. Nomenclature. The FDA has approved generic, nonproprietary names for commercial formulations of botulinum toxin [22–24]. For botulinum toxin type A, BOTOX (Allergan, Calif, USA) is onabotulinumtoxinA, DYSPORT (Ipsen Biopharm Limited Co., UK) is abobotulinumtoxinA, and Xeomin (Merz Pharma GmbH & Co KGaA., Germany) is incobotulinumtoxinA [25]. For botulinum toxin type B, MYOBLOC/NeuroBloc (Solstice Neuroscience, Inc., Pa, USA; Eisai Ltd., UK) is now rimabotulinum toxin B [26]. The changes in nomenclature emphasize the different potencies and the noninterchangeable unit dosages of these distinct brand name products. As reviewed by Alberto [24], these distinctions in names emphasize the differences in manufacturing and formulation techniques that may contribute to differences in the pharmacokinetics, efficacy, safety, and antigenicity among these products.

In the present paper, partially purified toxin with nontoxic or accessory proteins is referred to as botulinum toxin type A to distinguish it from botulinum neurotoxin A (BoNT/A), the pure holotoxin.

2.4. Sources of Error and Uncertainty. The lists of selective keywords are not intended to be exhaustive but serve as a starting point for the present work. Additional terms, such as proctology and coloproctology [27], can be used in more comprehensive studies.

Software development involves verification and validation. Verification confirms that, for example, the equations being coded are producing the correct calculations. Software can be validated when it can model the results that best fit existing data, and is subsequently reinforced by further data obtained experimentally. For kinetically related clinical problems, the underlying processes that need to be included are still uncertain. Furthermore, mathematical models are not designed to replace validation by basic research experiments or clinical observations. Rather, models are meant to enhance validation procedures by providing stimuli for new ideas, hypotheses, and perspectives on the problems being examined.

3. Results and Discussion

3.1. Literature Search for Botulinum Toxin A and Wound Healing. A preliminary search of botXminer, using the query "wound AND (heal OR healing)" for years 1980–2010, returned over 150 citations about half of which were true positives due to the large number of references related to tetanus (false positives). From the list of true positive citations, additional keywords were identified.

A variety of indications were found, in which botulinum toxin A has been used for wound healing and related conditions. These examples included experimental cutaneous scars in animal models [15, 28] and in clinical studies: chronic anal fissures [29, 30], cleft lip surgical repair [31], traumatic head lacerations or elective excisions of forehead masses [32], focal fold granuloma [33], hypertrophic scarring [34], pressure ulcers [35, 36], Raynaud's phenomenon (vasospastic ischemia of the digits, digital ischemia, including chronic ulcers) [37], and self-mutilation injuries in Lesch-Nyhan syndrome [38]. Another healing application of botulinum toxin A, referred to as protective ptosis, is used against persistent corneal ulcers, burns, and other ophthalmic-related problems [39, 40].

Conducting a matrix batch search in which "tetanus" was filtered out, using 26 time-related terms along with the 29 wound healing-related terms, yielded 671 unique citations. From this filtered output, we concentrated on references dealing with wound conditions and indications in which botulinum has been used for therapeutic purposes in which some mechanistic, dosage, and/or kinetic information was also available (Methods, Figure 1).

3.2. Botulinum Toxin A and the Components of the Healing Process. The normal wound-healing process has been described as being comprised of four overlapping phases: haemostasis, inflammation, tissue proliferation, and remodeling [41]. If any of these processes are disrupted, healing is impeded leading to a chronic wound state. The interference by botulinum toxin in reducing muscle movement has helped to define healing phases further. For example, a vicious cycle involving inflammation, pain, and muscle spasm was first noted to be the underlying cause for the development of chronic anal fissures [29, 42]. Subsequently, low blood flow and ischemia were added to this cycle [43–45]. Additional components of the healing process have been identified for other conditions as presented in this section.

Scar formation is a hallmark of wound healing, and it usually causes significant physical, psychological, and cosmetic problems. Hypertrophic scarring is a common, refractory dermal disease that is manifested by the abnormal appearance of wound healing which can be the result of different types of injuries [34, 46, 47]. In the study by Xiao et al. [46], 19 patients were treated once a month over 3 months with 1.8–35 U botulinum toxin A (Hengli/CBTX-A, Lanzou Biochemical Co., China [48]). Improvements in wound healing were based on subjective grading by patients and plastic surgeons. Scores were assigned before treatment for the associated erythema, pliability (lesion softening), and the severity of itching. After a 6-month followup of the 19 patients participating in that study, 15 gave an overall assessment of their lesion improvement as "good," and seven others rated their improvement as "excellent." Some critical comments provided by the authors included the lack of control subjects, the study was not double blinded, and a small patient population size. Finally, there was only a relatively short follow-up time of 6 months so that no determination of the total time course of toxin action could be established.

A number of quantitative parameters may be accessed to evaluate the effectiveness of wound healing by botulinum toxin A. Increased metabolic activity and inflammation during the healing process induce muscle contractions around the edges of the skin wound [32, 46]. The major role of botulinum toxin A in this healing process is to prevent the repeated, small contractions that produce "microtraumas" near the hypertrophic scar and thereby decrease the tensile force (muscle activity) during scar formation. Traditional surgical techniques that align incisions along Langer's lines do not prevent repeated contractions [15]. The development of fibrosis also involves the deposition of extracellular collagen and glycosaminoglycans that can cause the scar to hypertrophy, invert, and become hyperpigmented resulting in poor color matching of this tissue with the neighboring skin [15, 28]. Other parameters for wound healing include size of wound, amount of and infiltration of inflammatory cells, blood vessel proliferation, and wound thickness [28].

Additional cellular and molecular mechanisms of healing by the formation of scar tissue (traumatic cicatrisation) are beginning to be elucidated [46]. Transforming growth factor $\beta 1$ (TGF- $\beta 1$) is a fibrotic cytokine that stimulates cellular growth, differentiation, and adherence and leads to the collagen deposition. This cytokine initiates these processes by extracellularly binding to a coupled pair of

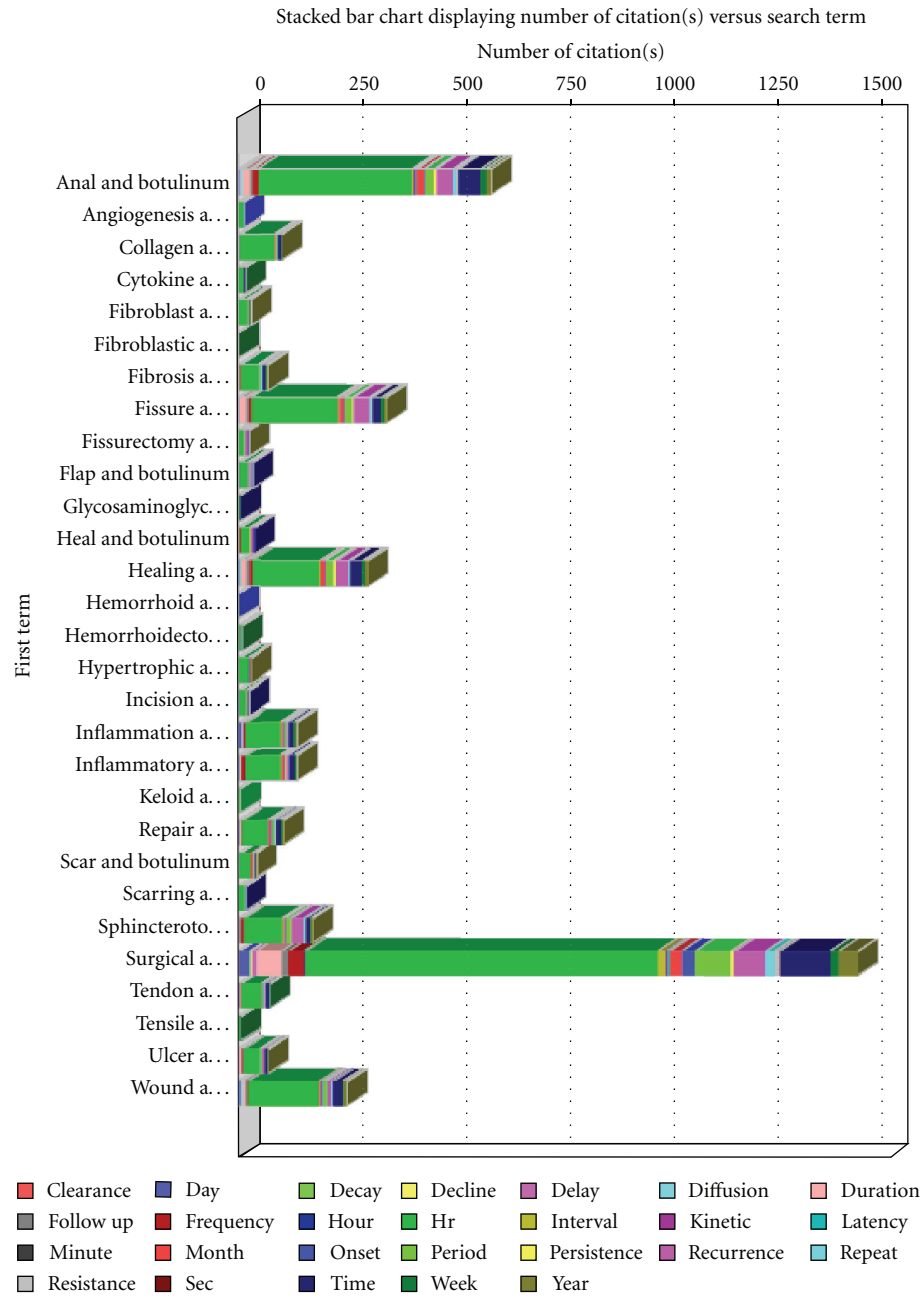

Figure 1: Example output from a botXminer database search. The numbers of botulinum toxin citations are shown for each of the combined 29 wound-healing (first term) and 26 time-related terms (legend) that were associated with 671 unique citations. The wound-related keywords associated with "botulinum" are associated with histograms that represent the different numbers of articles found in these multiple searches. The color-coded, time-related keywords used in these searches are simultaneously displayed in these histograms. Each "a..." in the left-hand labels signifies "and botulinum."

serine-threonine kinases. On binding, one receptor recruits and phosphorylates the other. This signaling pathway eventually stimulates transcription of the collagen gene and the formation of hypertrophic scars. Because human fibroblasts derived from hypertrophic scars overexpress and secrete TGF-β1, another wound-healing effect of botulinum toxin A has been speculated to be inhibiting the secretion of TGF-β1 [47]. Similarly, suramin, an antifibrotic polysulfonated naphthylurea compound, has been reported to promote wound healing by antagonizing TFG-β1 in muscle-derived fibroblasts [49].

The circulatory system is also affected by the apparent ability of botulinum toxin to enhance wound healing. Using a rat model for wounds, Yoo's group [50] observed that pretreatment with botulinum toxin A increased dorsal skin flap survival and concluded that this process was caused by increased perfusion. Because this toxin inhibits secretion of norepinephrine from sympathetic vasodilator

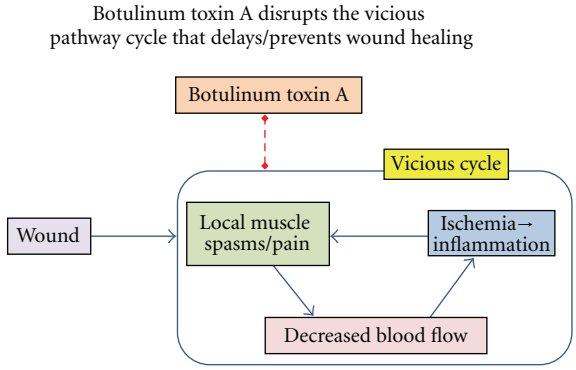

Figure 2: Botulinum toxin is depicted to disrupt the vicious pathway cycle that delays or prevents the healing of moderate wounds. This cycle involving spasm, pain, diminished blood flow, ischemia, and inflammation is blocked by the inhibition of chemical transmission at neuromuscular junctions by botulinum toxin A [29, 42, 43]. Dashed line is inhibitory action.

and vasoconstrictor neurons [51, 52], the effect of botulinum toxin A may involve increased perfusion by decreasing sympathetic vasoconstriction in the skin flaps, thus promoting skin flap survival.

3.3. Kinetic Data for Onset and Duration of Healing Effects Produced by Botulinum Toxin A. The protective effects of botulinum toxin-induced ptosis have been used for the conditions of recalcitrant corneal ulcers and other surface disorders as an alternative to the surgical practice of partially sewing the eyelids together (tarsorrhaphy) [39]. This secondary healing effect is produced when botulinum toxin A is injected into eye muscles, typically the levator palpebrae superioris (LPS) muscles [53]. This therapeutic application has been the subject of studies that have also generated kinetic data [39]. From an open-label, multicentered study with 16 ophthalmic patients who received 5 U BOTOX in the LPS muscle, the time to "suitable" ptosis was 4.0 ± 0.5 days (mean ± SE, range: 2–8 days), and the duration of this ptosis was 46.0 ± 12 days (1–206 days) [39]. A similar number of patients who received a single, lower dose (2.5 U) had a comparable mean time to ptosis (3.6 days) and a shorter, mean duration (16 days). Diplopia was the only adverse effect experienced by five patients. Although statistical analyses with more patients are required to make more definitive conclusions, these trends are not unexpected but are, nevertheless, remarkable because a twofold change of a low dose apparently resulted in appreciable differences in duration.

3.4. Kinetic Analysis of Recurrence of Symptoms. Chronic anal fissure (CAF) is a painful condition caused by spasms of the internal sphincter smooth muscles. The traditional surgical approach has been sphincterotomy that can result in the adverse effect of incontinence [30]. Traditional nonsurgical approaches that have been used include sitz bath, topical anaesthesia, nitroglycerin, isosorbide dinitrate, nifedipine, Diltiazem, L-arginine gel, hyperbaric oxygen, and botulinum toxin A [43]. The first use of botulinum toxin as a medical

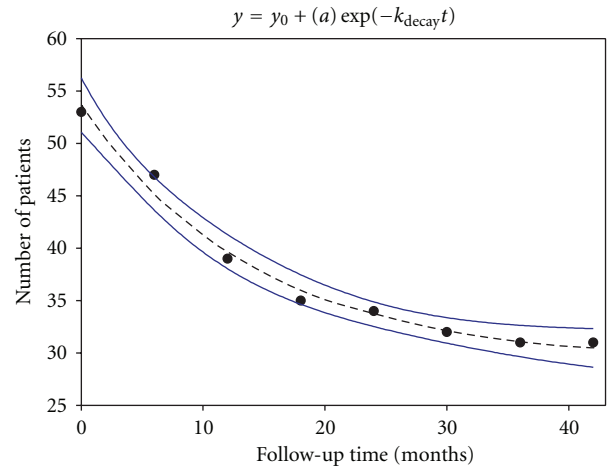

Figure 3: Time course of recurrence of CAF symptoms in some patients (22 of 53) in a 42-month follow-up study [55]. Initially ($t = 0$, i.e., 6 months after injection), all 53 patients were symptom-free for 6 months after injection of 10–20 U BOTOX. The fitted values from (2) (see text) are $y_0 = 29.2$, $a = 24.4$, and $k_{\text{decay}} = 0.85$ years^{-1}. The y-axis represents the number of symptom-free patients. Dashed line is fitted data; solid lines are 95% C.I.

alternative for CAF was conducted by Jost and Schimrigk [29]. For this indication, we chose what has been described as one of the longest follow-up studies using botulinum toxin (type A) in the treatment of CAF [54].

We analyzed data for the time course of recurrence of CAF symptoms in patients in a 42-month follow-up study [55, Table 1]. As illustrated in Figure 3, at the beginning of the followup (at $t = 0$), all 53 patients were symptom-free for 6 months after one to two injections of 10–20 U BOTOX. Subsequently, 22 of 53 patients showed a recurrence of symptoms. Most patients (31 of 53) did not undergo recurrence indicating that they remained healed during this follow-up period. From the nonlinear fit of these data to (2), the fitted values ± SEMs are $y_0 = 29.2 \pm 1.2$, $a = 24.4 \pm 1.3$. The k_{decay} (decay rate constant) is $7.08 \times 10^{-2} \pm 1.07 \times 10^{-2}$ month^{-1}, 1.64×10^{-6} min^{-1} (see below), or 0.85 years^{-1} (see Table 1). The value of $1/k_{\text{decay}}$ or τ is 14.1 months, and the corresponding value of $t_{1/2} (= \tau \ln 2)$ is 9.8 months or 294 days.

The long-term success rate was only 31/53 (58%) or a 42% rate of recurrence [30]. From Arroyo's group [54], recurrence rate of 12% occurred initially, and a rate of 53% at the 3-year follow-up point. Bilateral fissurectomies of the internal anal sphincter combined with toxin injections [56] have been reported to have a high success rate although the follow-up time in that study was limited to 1 year. Also, it was not determined whether surgery itself produced a similar rate of healing.

An interpretation of the single exponential decay curve during the 42-month followup (Figure 3) is that it reflects a zero-order elimination or inactivation step of the persistent, intraneuronally located toxin [57]. This step is described by the rate constant of decay, k_{decay}, in Figure 4 and k_e in a previous publication [58]. The table summarizes the processes displayed in Figure 4 and highlights the recurrence of symptoms that may be used to gauge the slow elimination

Table 1: Summary of processes involved in wound healing as described in text. Recurrence of symptoms results from long-term observations [55] was analyzed in the present paper and is highlighted in bold.

Parameter	Relative rates	Rate constant symbol	Units	Value
Onset of initial wounding	Very fast	k_1	msec^{-1} or sec^{-1}	N.A.[a]
Development of chronic wound	Moderate	k_2	days^{-1} or weeks^{-1}	N.A.
Healing of acute wound	Slow	k_3	weeks^{-1} or months^{-1}	N.A.
Healing of chronic wound	Very slow	k_4	years^{-1}	N.A.
Recurrence of symptoms [55], [Figure 3]	**Very slow**	$\mathbf{k_{decay}}$	$\mathbf{years^{-1}}$	**0.85**
4-step reaction [19]				
Diffusion toxin to receptors	Moderate	k_5	min^{-1}	0.001
Binding of toxin	Fast	k_B	min^{-1}	0.058
Translocation of toxin	Fast	k_T	min^{-1}	0.141
Toxic reaction (lysis)	Fast	k_L	min^{-1}	0.013

[a]N.A., because data are not presently available, order of magnitude estimates are given.

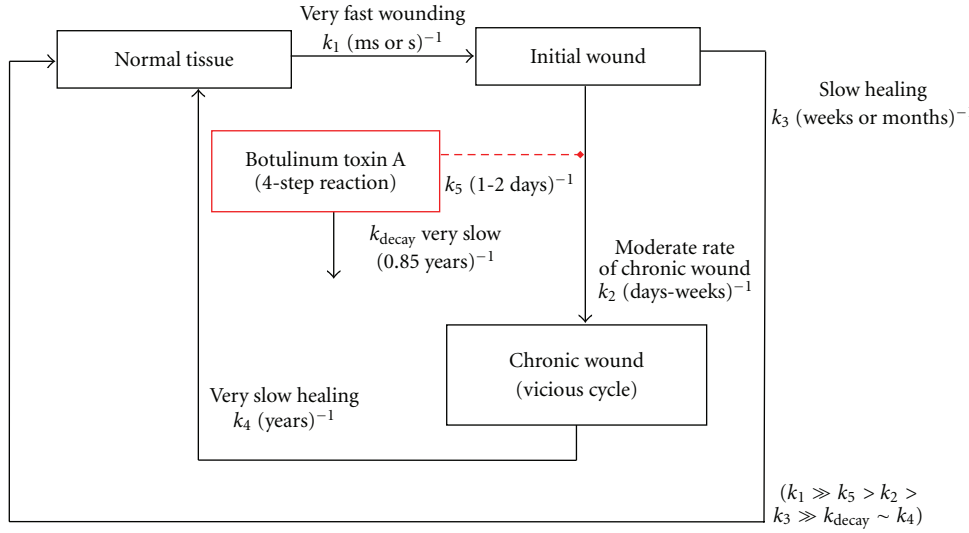

Figure 4: A minimal kinetic model for enhanced wound healing with botulinum toxin. Wounding of the normal tissue state is assumed to occur most rapidly leading to an initial wound state of the tissue. Healing from a significant wound is typically a slow process taking weeks or months. In the meantime, without treatment, this system enters, at a moderate rate, into a vicious cycle state (Figure 2) from which its escape represents a very slow healing process. With botulinum toxin treatment, this cycle is blocked thus allowing the tissue to return to the normal state by the slow healing route. The four-step reaction of toxin binding, translocation, internalization, and toxicity is described elsewhere [19]. A competing reaction is depicted as the slow decay or the inactivation rate of this toxin (k_{decay}) that leads to a recurrence of symptoms.

or inactivation of the type A botulinum toxin. Notably, this rate may be comparable to the slow rates of healing of some chronic wounds.

Alternatively, the long-lived toxic effect may be related to the rate of restoration of intact SNAP-25 intracellular levels or a combination of a slow degradation and a persistent inhibitory action of the SNAP-25 BoNT/A cleavage product [59]. The value of $t_{1/2}$ of 294 days is comparable to the recurrence times of 444 ± 132 days (mean ± S.D.; range 270–718 days [60]) for achalasia patients after receiving a single injection of 80 U BOTOX. On the other hand, our calculated decay rate constant of $1.64 \times 10^{-6}\,\text{min}^{-1}$ is about 1000 times slower than the estimated rate constant for decay of efficacy ($1.1 \times 10^{-3}\,\text{min}^{-1}$) in a single dystonic patient [58, 61, 62]. This difference may be due to several factors, among which are the different patient populations, the muscles being injected, the conditions being treated, and the assessment methods.

4. Future Directions

While the above studies show encouraging trends in support of using botulinum toxin A in wound-healing paradigms, additional studies are necessary. Overall, more prospective clinical studies of these treatments with botulinum toxin A are needed. Evidence from blinded, randomized, placebo-controlled, multicentered studies will help determine if these

toxin treatments have significant benefit and if the minimal adverse reactions can be sustained.

Future trials should also use larger populations of more homogeneous (standardized) patients and control subjects, plan to examine long-term outcomes, and conduct cost-benefit analyses [63]. Although randomized controlled trials are considered the gold standard of clinical research [64], assessing them using criteria for standardizing phase III trials remains a substantial challenge [65]. Moreover, there is also a need for additional controlled studies to clearly establish an advantage of botulinum products over other methods.

Retrospective meta-analysis studies are also needed for all of these new treatments. An outcome of one of these analyses was the low probability that type A botulinum toxin or calcium channel blockers were found to be more effective in treating CAF than nitroglycerin ointment in 182 patients [30]. Another example is Shao et al.'s analysis [44] which determined that for 279 CAF patients the traditional surgical procedure of lateral internal sphincterotomy (LIS) was more effective than BOTOX in healing chronic anal fissure. While LIS produced a higher rate of minor anal incontinence, botulinum toxin was associated with a higher rate of recurrent disease. For those patients who had a high risk of incontinence, local injection of the toxin was considered appropriate.

Computational modeling and simulation studies at different levels of granularity (i.e., detail) should also be beneficial. Starting with existing minimal kinetic models [19, 66], dose-dependent kinetic models could be developed to predict the time course (onset, duration, and recurrence of symptoms) and the extent of botulinum toxin A's effectiveness. Kinetic models could help to identify what research gaps exist and which ones can be experimentally or clinically resolved. One gap that could be experimentally verified is to determine if the intracellular diffusion of botulinum toxin A [67] is influenced by other coinjected materials, for example, epinephrine and local anesthetics (lidocaine, Xylocaine), compounds that have been considered in controlling local diffusion and predicting the extent of this toxin's paralytic effect [15, 32].

As more realistic physiological-pharmacological models are developed, more free parameters and more sources of error, assumptions, and caveats will need to be evaluated. Potentially confounding factors associated with wounds include cell viability, alkali conditions [41], the generation of reactive oxygen species, and inflammation that is related to low blood flow and ischemia [45]. Changes in the ion flow through transmembrane channels and in metabolism could also have roles in wound healing. Another confounding factor is the well-known, persistent muscle weakness (up to 5 years) that results from protracted patient immobilization due to critical illness polyneuropathy or myopathy [68, 69]. This persisting weakness and residual neurologic deficits are likely due to denervation, combined with catabolic muscle wasting and potential myopathic changes [68]. It remains for future studies to differentiate these physiological deficits from botulinum toxin-induced effects in either a botulism patient or with therapeutic treatments with this toxin.

The tissues involved in the healing process in cytoskeletal architectures and in membrane structures associated with various organs also need to be considered. Neurophysiological abnormalities, such as transient denervation-induced muscle fibrillations and presynaptic alterations producing muscle fasciculations, may also need to be considered. Other complicating factors in the healing process could also involve the biomechanical dynamic properties of soft tissue (e.g., stiffness) in response to stress and strain, and tissue anisotropy (directionality of nonhomogeneous material).

5. Summary/Conclusions

This succinct review examines the soft tissue wound-healing properties of botulinum toxin. When viewed from the perspective of treating neurologic and other disorders, it is noteworthy that the efficacy of this toxin is predicted to be transient as the toxin's effect wanes, while for wound healing, a permanent resolution is expected. Further, with respect to wound healing, the concept is described that for some lesions, botulinum toxin interferes with the vicious cycle of muscle spasm, pain, inflammation, decreased blood flow, and ischemia [43]. A reaction pathway scheme is outlined to illustrate botulinum toxin's involvement in stopping the vicious cycle. A minimal kinetic scheme for healing chronic wounds is also presented that includes different macroscopic states of soft tissue conditions (normal, initial wound, and chronic wound), and quantitative estimates for the relevant rate constants are provided. A definitive validation of the results, that is, the minimal kinetic model for predicting the beneficial effects of type A toxin, awaits additional clinical data. Perhaps the most useful outcome is that our kinetic model is capable of identifying a measurable gap (decay rate of toxin's effect) in our attempt to comprehend how complex, interacting biological systems respond to environmental stressors.

Disclaimers

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. The content of this publication does not necessarily reflect the views or policies of the U.S. Army, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. This paper has been approved for public release.

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Hindawi Publishing Corporation
Journal of Toxicology
Volume 2012, Article ID 760142, 9 pages
doi:10.1155/2012/760142

Research Article

Retargeting *Clostridium difficile* Toxin B to Neuronal Cells as a Potential Vehicle for Cytosolic Delivery of Therapeutic Biomolecules to Treat Botulism

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Received 16 May 2011; Accepted 13 July 2011

Academic Editor: S. Ashraf Ahmed

Botulinum neurotoxins (BoNTs) deliver a protease to neurons which can cause a flaccid paralysis called botulism. Development of botulism antidotes will require neuronal delivery of agents that inhibit or destroy the BoNT protease. Here, we investigated the potential of engineering *Clostridium difficile* toxin B (TcdB) as a neuronal delivery vehicle by testing two recombinant TcdB chimeras. For AGT-TcdB chimera, an alkyltransferase (AGT) was appended to the N-terminal glucosyltransferase (GT) of TcdB. Recombinant AGT-TcdB had alkyltransferase activity, and the chimera was nearly as toxic to Vero cells as wild-type TcdB, suggesting efficient cytosolic delivery of the AGT/GT fusion. For AGT-TcdB-BoNT/A-Hc, the receptor-binding domain (RBD) of TcdB was replaced by the equivalent RBD from BoNT/A (BoNT/A-Hc). AGT-TcdB-BoNT/A-Hc was >25-fold more toxic to neuronal cells and >25-fold less toxic to Vero cells than AGT-TcdB. Thus, TcdB can be engineered for cytosolic delivery of biomolecules and improved targeting of neuronal cells.

1. Introduction

Clostridial toxins in nature are remarkably efficient cell cytosol delivery vehicles with highly evolved cell-specific delivery features that may be ideal for therapeutic applications. Specifically these toxins (1) gain entry to animals; (2) survive in blood; (3) bind to target cells expressing a specific receptor; (4) penetrate the target cells; (5) deliver an enzymatically active cargo to the cytosol. *C. difficile* toxins A and B (TcdA and TcdB) contain a receptor-binding domain (RBD) that binds to receptors that are broadly expressed on cells and then enters by endocytosis. Once in the endosome, the toxins employ a translocation domain (TD) to deliver a glucosyltransferase (GT) to the cytosol which inactivates Rho GTPases and leads to cell death [1]. The toxins also contain a cysteine protease (CPD), located between GT and TD, that cleaves the GT enzymatic "cargo" from the "delivery vehicle" at the endosomal membrane and liberates it into the cytosol [2–4].

C. difficile bacteria generally reside in the gut where the released toxins intoxicate intestinal epithelial cells and cause the disruption of tight junctions of epithelium and its barrier function. It is likely that in severe cases of the infection, the toxins penetrate into the submucosa and disseminate systemically [5]. We recently identified *C. difficile* toxins in the blood of the experimentally infected animals [6, 7], suggesting that the toxins may be reasonably stable in serum.

Recent developments have enabled the application of TcdA and TcdB as therapeutic delivery vehicles. The *Bacillus megaterium* (*B. megaterium*) expression system has been shown to permit high-level expression of functional recombinant TcdB (5–10 mg/L culture) [8]. Secondly, the toxicity of these agents is virtually eliminated by introducing two

point mutations within the GT domain that should have no effect on endosomal uptake and translocation to the cytosol (Haiying Wang and Hanping Feng, unpublished data). Finally, the limits of the GT, TD, and RBD domains have recently been carefully defined in the literature [3], facilitating efforts to replace one or more domains with similar domains from other toxins. We recently showed that it was possible to replace the RBD from TcdB with the RBD from TcdA and retain most or all of the toxin activity (Haiying Wang and Hanping Feng, unpublished data).

One drawback to the use of TcdA or TcdB as cytosolic delivery vehicles is the lack of cell specificity. This is in contrast to botulinum neurotoxins (BoNTs) which display a marked specificity for neuronal cells. BoNTs are CDC Category A biodefense threat agents that cause paralysis by entering the presynaptic terminal of motor neurons and inhibiting neurotransmitter release. All seven BoNT serotypes bind to a neuronal receptor through a receptor-binding domain. The toxins then undergo endocytosis, delivery of the BoNT protease cargo to the cytosol, and subsequent cleavage of SNARE proteins [9–11]. Reversal of neuronal intoxication must involve either the inhibition and/or elimination of the protease. We and others have reported development of biomolecules that potently inhibit BoNT protease [12–14] or promote its degradation [15, 16]. In this study, we demonstrate that biomolecules fused to the amino terminus of TcdB can be successfully delivered to the cytosol of cells and that replacement of the TcdB RBD with the equivalent RBD from BoNT serotype A (BoNT/A) leads to a chimeric toxin with enhanced specificity for neurons. These results indicate that it may be possible to develop therapeutic agents based on TcdB in which biomolecules are delivered to BoNT-intoxicated neurons that inhibit and/or destroy the toxin protease. Such a treatment would promote accelerated neuronal recovery from intoxication and thus could serve as the first antidotes for treatment of botulism.

2. Materials and Methods

2.1. Bacterial and Mammalian Cell Cultures. Bacterial cultures of *Escherichia coli* (TOP10 cells; Invitrogen, Carlsbad, CA) and *B. megaterium* (MS941 strain; kindly provided by Dr. Rebekka Biedendieck, Germany) were grown at 37°C in Luria-Bertani (LB) medium, containing ampicillin (Amp) and tetracycline (Tet), respectively.

Mammalian cell lines were obtained from ATCC (Manassas, VA) and cultured as monolayers in 100 mm cell culture dishes at 37°C and 5% CO_2. Cells were reseeded twice a week after harvest using 0.05% trypsin to suspend cells. The murine neuroblastoma cell line, Neuro2A, and the human neuroblastoma line, M17, were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 50 μg/mL streptomycin sulfate. Vero cells (kidney epithelial cells from African green monkey) were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 50 ug/mL streptomycin sulfate.

2.2. Cloning of TcdB Constructs. Full-length TcdB was expressed in *B. megaterium* as described previously [8]. To generate AGT-TcdB, the DNA encoding an alkylguanine-DNA alkyltransferase (AGT) flanked by 5′-BsiWI and 3′-BsrGI sites was synthesized (Geneart, Germany). AGT is a commercially available tag for producing AGT-fusion proteins. AGT catalyzes its own covalent binding to the substrate benzylguanine (BG) derivatives. The BG derivatives can be labeled with probes such as biotin or fluorescein to permit detection and/or cell localization of AGT-fusion proteins [17]. The AGT was appended in frame to TcdB by digestion of AGT with BsiWI and BsrGI and ligation into pHis1525/TcdB digested with BsrGI as represented in Figure 1. To generate AGT-aTcdB-ΔGT construction, a unique BamHI site (position 542) was created between coding sequences of GT and CPD by overlapping PCR. Then the AGT-tag coding DNA precisely replaced the GT in frame with the CPD, by ligation into pHis1525/TcdB digested with 5′-BsrGI and 3′-BamHI.

To generate AGT-TcdB-BoNT/A-Hc, an AgeI site was installed between the TD and RBD of TcdB. The entire RBD of TcdB, consisting of the C-terminally combined repetitive oligopeptides (CROPs, residues 1852–2366), was then replaced by the heavy chain C-terminus of BoNT/A (BoNT/A-Hc, residues 861–1296). BoNT/A-Hc coding DNA [18] was amplified by PCR from BoNT/A coding DNA and flanked by 5′-AgeI and 5′-XmaI restriction sites using primers; sense: 5′-cgaccggtggtggaggcggttcaggcggaggtggctctggcggtggcggttcccgcctgctgtcaactttcac-3′ and antisense: 5′-cggcccgggttagtgatggtgatggtgatggagaggacgttcaccccaac-3′. A flexible spacer $(GGGGS)_3$ was encoded in the forward primer to separate TcdB and BoNT/A-Hc in the chimera. The reverse primer encoded a His_6 sequence at the carboxyl coding end. All plasmid constructions were confirmed by DNA sequencing and transformed into *B. megaterium* for protein expression as described previously [8]. All DNA cloning and plasmid construction were performed at Tufts University and approved by the Institutional Biosafety Committees in agreement with NIH Recombinant DNA technology guidelines.

2.3. Characterization of Recombinant TcdB Chimeric Proteins. Expression and purification of His-tagged TcdB proteins was performed essentially as described previously [8] with a few modifications.

For Western blots, AGT-TcdB and AGT-TcdB-BoNT/A-Hc were separated on a 4–20% gradient polyacrylamide gel by SDS-PAGE. AGT-tag fused to TcdB was detected using a rabbit polyclonal serum anti-AGT (New England Biolabs, Boston) at a dilution of 1 : 1000. Detection of full-length TcdB was performed using an alpaca polyclonal anti-TcdB serum, generated in our laboratory and diluted $1 : 10^6$. The BoNT/A-Hc domain in AGT-TcdB-BoNT/A-Hc chimeric protein was detected by a mouse anti-BoNT/A-Hc monoclonal antibody (A11G12.4B- kindly provided by Dr. Jean Mukherjee, Tufts University) diluted at 1 : 25,000. Detection was performed using Amersham ECL Western Blotting Detection Reagents for chemiluminescence (GE Healthcare, UK).

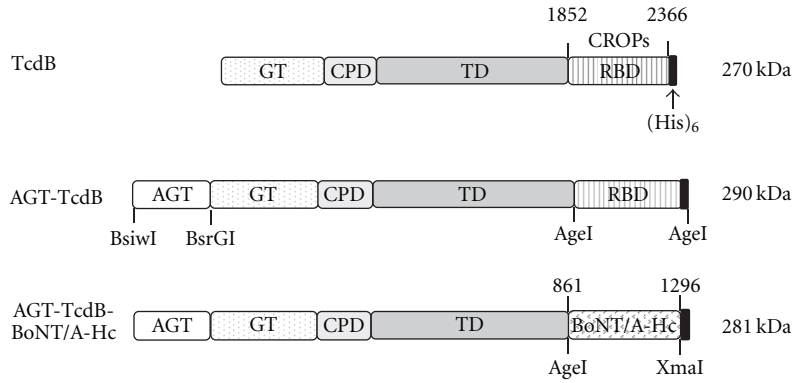

Figure 1: Engineered recombinant TcdB proteins. Native TcdB contains a glucosyltransferase domain (GT), a cysteine-protease domain (CPD), a translocation domain (TD), and a receptor-binding domain (RBD) as shown. The AGT-tag coding DNA was appended to the amino terminus in frame with the full-size TcdB coding DNA to create the AGT-TcdB expression vector. The TcdB RBD was replaced in frame with the full-size BoNT/A heavy chain carboxyl terminus (BoNT/A-Hc, amino acids 861–1296), containing the receptor-binding domain for BoNT/A, to generate AGT-TcdB-BoNT/A-Hc. The sizes of the boxes are approximately proportional to the sizes of the protein domains. Restriction sites used in preparing the constructions are indicated.

2.4. AGT-Tag Labeling with Biotin. AGT fused to TcdB was labeled *in vitro* with biotin in the absence of DTT, according to the manufacturer's instructions (New England Biolabs). Briefly, 2 μM AGT-TcdB was mixed with 3 mM BG-biotin (AGT substrate labeled with biotin) in a 25 μL reaction and incubated overnight at 4°C. Biotin-labeled AGT-TcdB was analyzed by Western blot using streptavidin conjugated to horseradish peroxidase (HRP). DTT was not added in the labeling reaction as recommended, because it has been reported that DTT mimics intracellular activation of the toxin [19].

2.5. Cytotoxicity Assay. Cell lines at semiconfluence were treated with purified TcdB, AGT-TcdB, or AGT-TcdB-BoNT/A-Hc in 5x serial dilutions starting from 100 ng/mL. Cells were cultured for a period of 24 h, and morphological changes were monitored every hour by light microscopy. Cell toxicity was quantified as the percentage of rounded cells per total cells.

3. Results

3.1. Construction and Expression of AGT-TcdB and AGT-TcdB-BoNT/A-Hc. To test the potential of TcdB-based vectors for delivery of biomolecules to the cytosol of neuronal cells, DNAs were created that encode two chimeric forms of TcdB fused to a C-terminal His_6-tag (see diagrammatic representations in Figure 1). In the AGT-TcdB construct, an alkylguanine-DNA alkyltransferase, referred to as AGT, was appended to the TcdB N-terminus in frame with the GT coding DNA. Another construct was also prepared in which the GT was replaced by AGT, but this failed to yield meaningful amounts of full-size protein apparently because of proteolysis (data not shown) and was not further pursued. For the second chimeric toxin, AGT-TcdB-BoNT/A-Hc, the putative receptor-binding domain (RBD) from AGT-TcdB was replaced in frame with the well-defined BoNT/A receptor-binding domain [20, 21] designated BoNT/A-Hc. The recombinant TcdB chimeric proteins were expressed in *B. megaterium* and purified by nickel affinity as previously described for wild-type TcdB [8].

3.2. Expressed AGT-TcdB Retains AGT Enzymatic Activity. Recombinant AGT-TcdB was expressed, and the purified protein had the expected molecular weight. Western blots with polyclonal anti-TcdB serum recognized both the parental TcdB and AGT-TcdB, while AGT antiserum recognized only the AGT-TcdB (Figure 2). To confirm the proper folding and function of the AGT fusion partner, the enzymatic activity of the AGT alkyltransferase was tested. AGT-TcdB was incubated with BG-biotin which catalyzes the covalent linkage of biotin to AGT. The efficiency of *in vitro* AGT-protein labeling is generally ~95% according to the manufacturer (New England Biolabs). Western blotting with streptavidin demonstrated that AGT-TcdB became biotinylated following incubation with BG-biotin (Figure 2), thus demonstrating that the AGT domain in recombinant AGT-TcdB fusion retained enzymatic activity.

3.3. TcdB Delivers Glucosyltransferase Fusion Protein Cargo to the Cell Cytosol. Cytosolic delivery of the GT domain is a requirement for cytotoxicity [22, 23]. Since each of the TcdB chimeric proteins (Figure 1) contain a fully functional GT domain, delivery of the AGT/GT fusion cargo into the cell cytosol could thus be assessed by measuring cell toxicity. In a dose-response toxicity assay in Vero cells, AGT-TcdB was found to retain a potency nearly equal to wild-type TcdB (Figure 3(a)). Cells exposed to the lowest toxic dose of AGT-TcdB were somewhat slower to become rounded than when exposed to TcdB indicating that the presence of the AGT partner may cause a small delay in toxin entry (Figure 3(b)). Even so, the half maximal effective concentration (EC_{50}) is virtually identical for both toxin forms. Enzymatic labeling of AGT-TcdB with BG-biotin did not cause any significant

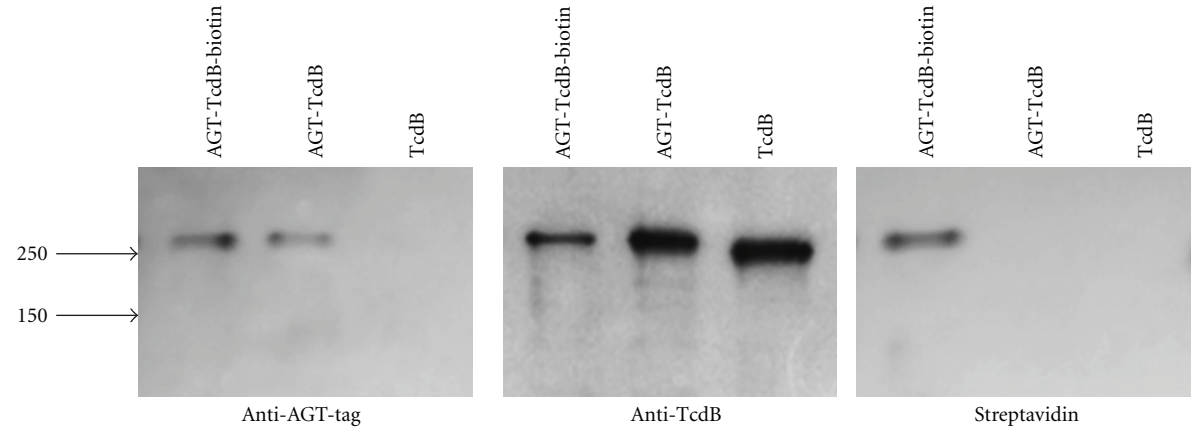

Figure 2: AGT-tag expressed at the N-terminus of TcdB retains enzymatic activity. Recombinant TcdB and AGT-TcdB were expressed and purified. 250 ng aliquots of purified TcdB and AGT-TcdB (± autobiotinylation) were analyzed by Western blots using anti-AGT-tag sera (left) and by anti-TcdB sera (middle). The positions of molecular weight markers are indicated with arrows. Each of the protein preparations was subjected to enzymatic reactions with BG-biotin and analyzed for bound biotin by Western blot with streptavidin (right). The data shown are representative of 2 independent experiments.

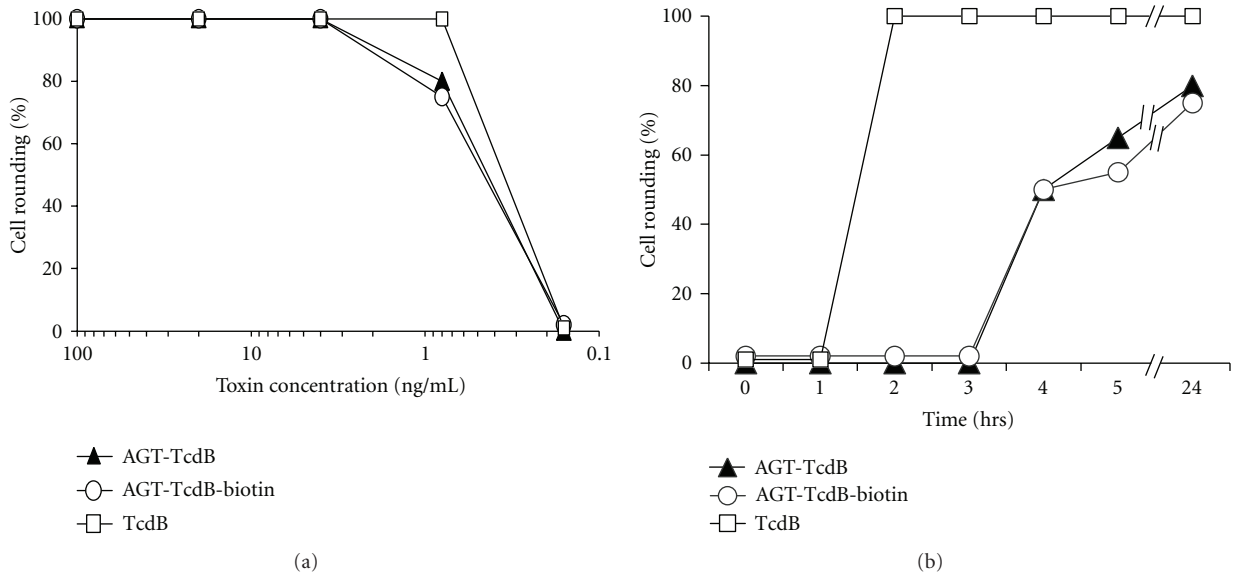

Figure 3: TcdB with an N-terminal AGT-tag retains cytotoxic potency. (a) AGT-TcdB potency as a function of protein concentration. Vero cells were treated with serial dilutions of TcdB and AGT-TcdB ± autobiotinylation. The dilution series started at 100 ng/mL and continued with fivefold serial dilutions. The % cell rounding was assessed after 24 hr for each concentration tested. (b) AGT-TcdB potency as a function of time after exposure. Vero cells were exposed to 0.8 ng/mL of TcdB or AGT-TcdB, and the % cell rounding was assessed hourly for five hours and then after 24 hr. The data shown in both (a) and (b) are representative of 3 independent experiments.

decrease in toxin potency (Figures 3(a) and 3(b)). We conclude that the AGT-GT fusion protein with fully functional glucosyltransferase activity is delivered to the cytosol by TcdB with an efficacy nearly that of GT alone, thus demonstrating the potential of TcdB-based vectors to function as cytosolic delivery vehicles.

3.4. Replacing the TcdB Receptor-Binding Domain with the Equivalent Domain from BoNT/A Increases Neuronal Cell Toxicity. Recombinant AGT-TcdB-BoNT/A-Hc protein was expressed from an expression vector in which the TcdB RBD coding region from AGT-TcdB was replaced by DNA encoding the BoNT/A RBD, the carboxyl 50 kDa portion of the BoNT/A heavy chain (Figure 1). The goal was to engineer TcdB for improved entry into neuronal cells and reduced entry into nonneuronal cells. The AGT-TcdB-BoNT/A-Hc preparation, purified only by nickel affinity, contained a protein of the predicted molecular weight for the full-size protein (~280 KDa) as well as some lower-molecular-weight species that likely result from both protein degradation and protein contamination (Figure 4(a)). Nevertheless, Western blot analysis confirmed that the 280 kDa AGT-TcdB-BoNT/A-Hc protein was full size as it stained with both anti-AGT and anti-BoNT/A-Hc antibodies (Figure 4(b)).

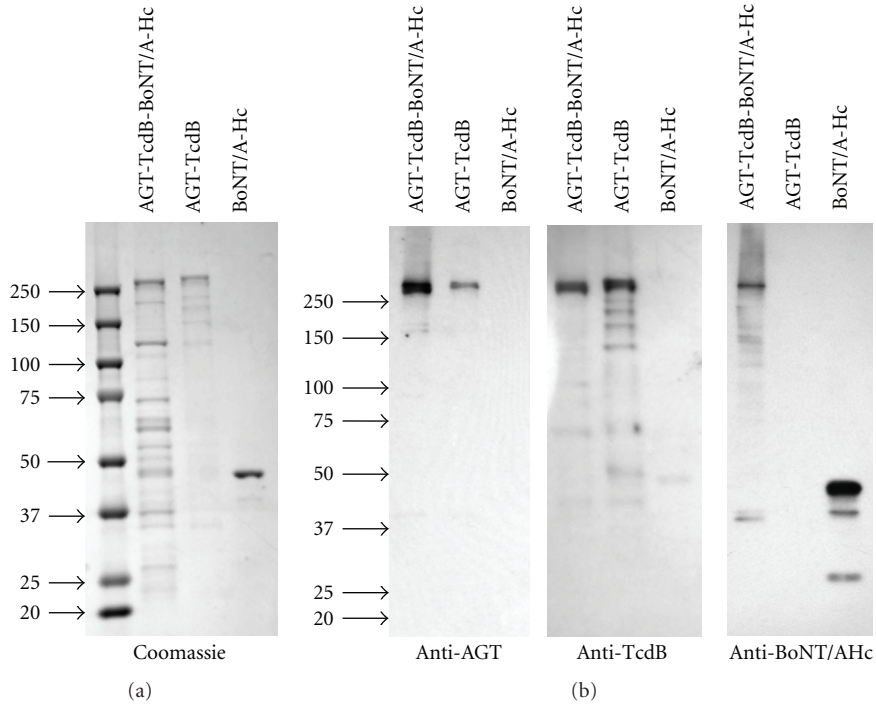

Figure 4: Recombinant expression of TcdB with a BoNT/A RBD. Recombinant AGT-TcdB, AGT-TcdB-BoNT/A-Hc, and BoNT/A-Hc were expressed and purified. (a) Each preparation containing 250 ng of protein was analyzed by SDS-PAGE and protein stain. (b) The preparations were also analyzed by Western blots using anti-AGT serum, anti-TcdB serum, or anti-BoNT/A-Hc mAb as indicated. The position of molecular weight markers is indicated with arrows.

Next, we tested the cytotoxicity of AGT-TcdB-BoNT/A-Hc with AGT-TcdB on two neuronal cells lines, Neuro2A and M17, and on Vero cells, a nonneuronal cell line highly sensitive to TcdB [24, 25]. The presence of the BoNT/A-Hc domain dramatically improved the potency of the AGT-TcdB for both neuronal cell lines (Figure 5(a)). The EC_{50} of AGT-TcdB-BoNT/A-Hc was approximately 25-fold lower than AGT-TcdB when assessed 24 h following toxin exposure. In contrast, the EC_{50} of AGT-TcdB-BoNT/A-Hc for Vero cells was approximately 25-fold higher than AGT-TcdB. The finding of AGT-TcdB-BoNT/A-Hc toxicity in Vero cells (Figure 5(a)) corroborates previous reports that TcdB retains some toxicity in the absence of the putative RBD [23, 25, 26]. The rounded phenotype of the neuronal cells exposed to the two toxin forms was indistinguishable, as exemplified in representative images of Neuro2A cells (Figure 5(b)). Neuro2A cells exposed for 24 h to 0.16 ng/mL AGT-TcdB-BoNT/A-Hc were 100% rounded, while exposure to this dose of AGT-TcdB caused no observable changes compared to untreated cells. Even at 4 ng/mL, AGT-TcdB induced only 50% rounding of Neuro2A cells (Figure 5(b)).

It is noteworthy that cell rounding was also observed to occur more rapidly in the two neuroblastoma cell lines following exposure to AGT-TcdB-BoNT/A-Hc compared with exposure to AGT-TcdB. A time course of intoxication of Neuro2A cells exposed to one of these two toxin preparations at three different concentrations clearly demonstrates that it takes about 125-fold more AGT-TcdB to achieve ≥80% cell rounding in 5 hrs than with AGT-TcdB-BoNT/A-Hc (Figure 6). These results suggest that TcdB enters and intoxicates neuronal cells significantly more rapidly and efficiently when the toxin contains the BoNT/A RBD in place of the native TcdB RBD.

4. Discussion

BoNT-mediated paralysis is caused by inhibition of neurotransmission in poisoned neuronal cells. This blockage is induced following delivery of the endopeptidase domain (light chain) to neurons which then inactivates one or more cytosolic proteins specifically required for neurotransmitter release. While biomolecules that inhibit or eliminate the BoNT light chain have been developed [16], the *in vivo* delivery of these therapeutic agents to the cell cytosol of intoxicated neurons to promote their recovery remains a challenge. One viable option for delivery vehicles is to reengineer clostridial toxins, which are already well evolved for delivery of their enzymatic cargo to cell cytosol, such that the toxicity is removed, while the ability to enter cells and deliver cargo remains intact.

Here, we demonstrate that wild type TcdB can be engineered as a delivery system for selective targeting of neuronal cells. *C. difficile* toxins (TcdA and TcdB) have a major advantage over other clostridial toxins as cytosolic delivery vehicles for therapeutic biomolecules. These toxins naturally carry their own protease (cysteine-protease domain) that enzymatically cleaves and releases their cargo into the cytosol, eliminating the need to engineer a mechanism that

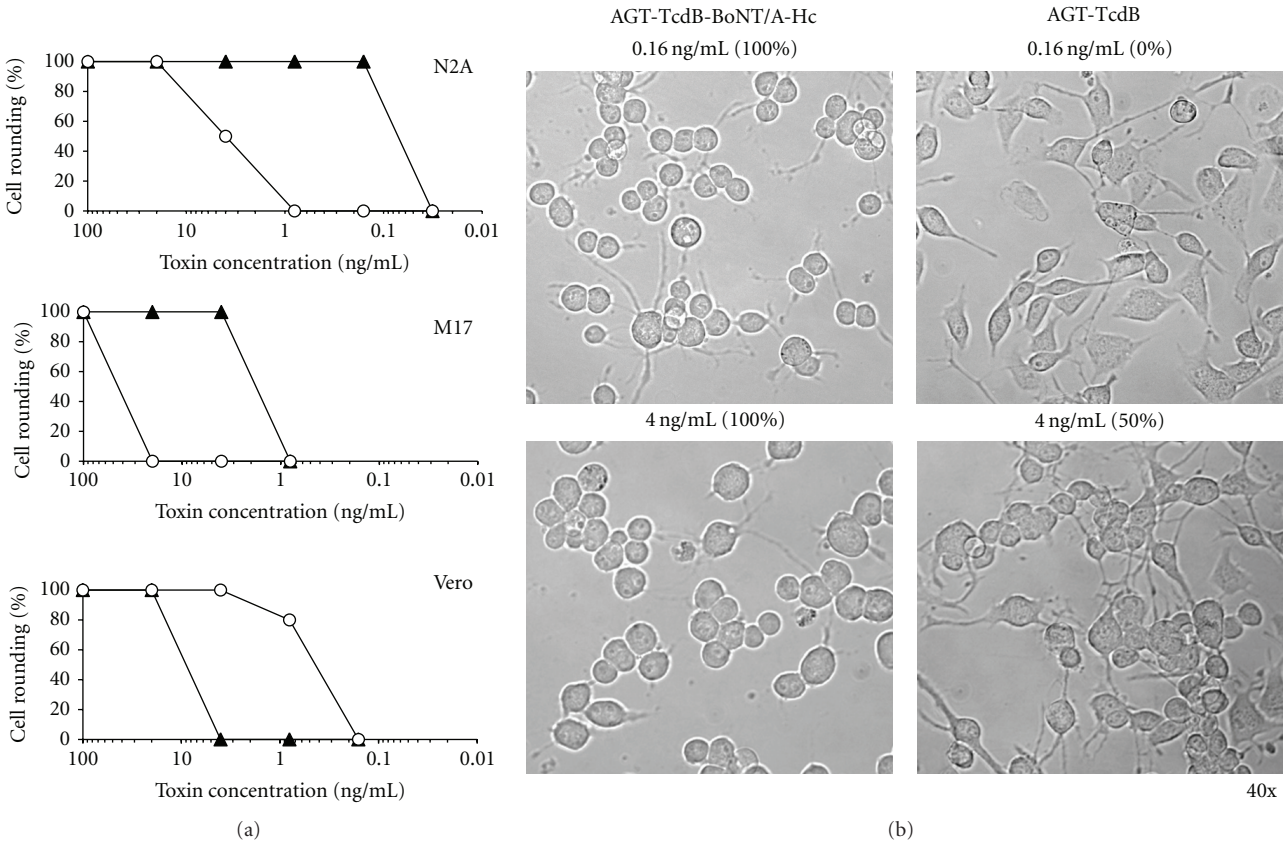

Figure 5: A TcdB chimera with the BoNT/A receptor-binding domain has increased specificity for neuronal cells. (a) Toxin potency of AGT-TcdB and AGT-TcdB-BoNT/A-Hc on two neuronal cell lines and Vero cells. Potency was assessed by serial dilutions of purified recombinant AGT-TcdB-BoNT/A-Hc (▲) and AGT-TcdB (◦). The potency was determined on Neuro2A and M17 neuroblastoma cells and Vero cells. Fivefold serial dilutions of the proteins were added to medium, and cells were monitored for toxicity by assessing the % cell rounding after 24 hr. Data shown are representative of 4 independent experiments. (b) Microscopic images of Neuro2A cells exposed to AGT-TcdB or AGT-TcdB-BoNT/A-Hc. Representative images are shown of Neuro2A cells exposed for 24 hr to 0.16 ng/mL or 4 ng/mL of either AGT-TcdB-BoNT/A-Hc or AGT-TcdB, respectively.

permits this release. For example, it has been reported that the GT domain of TcdA can be removed and replaced with luciferase to generate a functional delivery vehicle capable of translocating luciferase to the cytosol of target cells [27]. Previous studies have shown that BoNTs can also be adapted as delivery vehicles, but unlike Tcds, BoNTs do not have an autocatalytic domain to release the light chain into the cell cytoplasm. Thus, in constructs in which the toxic light chain was removed, it was necessary to insert a linker that promotes a disulfide bond between the cargo and the translocation domain to make possible cargo release into the cytosol following disulfide bridge reduction in the endosome [28, 29]. Such an approach may be less efficient or ineffective for some therapeutic cargo.

In this work, we have engineered expression vectors for two chimeric TcdB proteins in which a functional GT domain remains in place. The strategy was to test the TcdB ability to deliver cargo to the cytosol by measuring the cytotoxic potency of the two chimeric proteins in comparison to wild-type TcdB. This eliminates the need for microscopic or fractionation methods to distinguish between endosomal and cytosolic cargo. In AGT-TcdB, an alkylguanine-DNA alkyltransferase, referred to as AGT, was appended to the GT domain of wild-type TcdB. Our results show that AGT was at least partially functional, since it enzymatically labeled AGT-TcdB with biotin. AGT-TcdB was capable of intoxicating Vero cells nearly as efficiently as wild type TcdB. Thus, we infer that AGT and GT domain were delivered together to the cell cytosol, and that adding a cargo to the N-terminus of the toxin did not interfere substantially with GT domain translocation and activity. Earlier work had shown that gluthatione-S-transferase (GST) could also be appended to the wild-type TcdB and detected in the cytosol of intoxicated cells, but the fusion toxin was not fully active, and there was no data as to the efficiency of delivery or that the GST fusion protein retained its enzymatic activity [22].

Next, we tested whether TcdB could be engineered for selective neuronal toxicity by exchanging the TcdB receptor-binding domain (RBD) on AGT-TcdB with the equivalent RBD from the neuronal-specific BoNT/A toxin, generating AGT-TcdB-BoNT/A-Hc. Our results showed clearly that

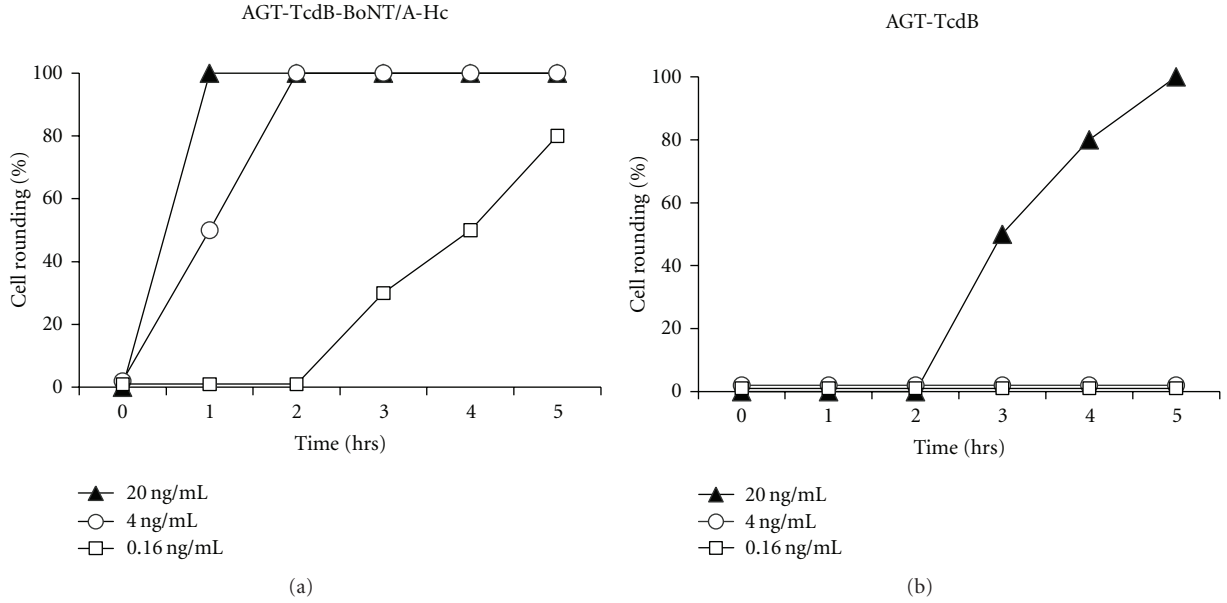

Figure 6: A TcdB chimera with the BoNT/A receptor-binding domain more rapidly elicits Neuro2A cell rounding. Neuro2A cells were exposed to three different concentrations (20 ng/mL, 4 ng/mL, and 0.16 ng/mL) of AGT-TcdB-BoNT/A-Hc or AGT-TcdB and the % cell rounding was assessed hourly for 5 hr. Data shown are representative of 2 independent experiments.

neuronal cells were at least 25-fold more sensitive to the toxic effects of AGT-TcdB-BoNT/A-Hc than AGT-TcdB, and signs of intoxication appeared more rapidly. The results imply that it may be possible to engineer TcdB with specificity for almost any cell type by replacing the RBD with a RBD that binds to an appropriate receptor expressed on the target cell population. It is interesting to speculate that TcdB-BoNT/A-Hc chimeras may also be capable of transcytosis through endothelial cells in the same manner as native BoNT can accomplish because the BoNT/A-Hc domain has been shown to be sufficient for retention of this BoNT function [30]. This feature may permit delivery of therapeutic agents via oral or respiratory routes.

Although AGT-TcdB-BoNT/A-Hc showed enhanced toxicity for neuronal cells, this chimera retained some toxicity for nonneuronal cells. Previous work has also reported evidence that TcdB retains some toxicity in the absence of the putative RBD and suggested that TcdB internalization into host cells may be mediated by additional receptor-binding regions that are outside of the CROP domain of the toxin [25, 26]. Indeed, a recent report [23] has shown that deletion of the C-terminal region (residues 1500–1851) of putative translocation domain (TD) reduces cell toxicity without greatly affecting GT translocation and function. The authors conclude that this portion of the putative TD likely contains additional RBD properties. The results thus indicate that deletion or replacement of residues contributing to receptor binding within the C-terminus of TcdB putative TD will likely reduce or eliminate non-neuronal cell intoxication by TcdB-BoNT/A-Hc without loss of its capability to deliver cargo to neuronal cells.

In addition to engineering TcdB with AGT fused to the amino terminus of the GT domain, we also prepared a TcdB protein in which AGT replaced the GT domain. Expression of this fusion protein apparently produced an unstable fusion protein, resulting in very low yields of purified full-size protein. One possibility is that the removal of GT resulted in an altered conformation of the protein that resulted in activation of the cysteine protease domain which then led to autoproteolysis. The results suggest it may be important to retain the GT domain in order to maintain stability of the chimeric protein. In this case, it will be necessary to eliminate the GT enzymatic activity to render the glucosyltransferase inactive, so that the therapeutic delivery agent does not retain toxicity. Towards this goal, we recently produced an atoxic and safe TcdB, by introducing two point mutations in the GT domain (Haiying Wang and Hanping Feng, unpublished data). We expect that TcdB-based vehicles with its cargo fused to the atoxic GT domain will have better stability and perhaps lead to a product having more native conformation, thus resulting in a more efficient endosomal uptake and cargo translocation to the cytosol than TcdB vehicles lacking the GT domain. Thus, in future studies, we are opting to append foreign coding DNA to a mutated TcdB-BoNT/A-Hc rather than to replace the GT domain.

In summary, this study strongly suggests it will be possible to engineer TcdB as a cytosolic delivery vehicle of therapeutic cargo to neuronal cells, and perhaps other cell types, by redirecting its cellular binding specificities. As an example, we recently developed a small biomolecule that specifically inhibits BoNT/A protease and promotes its rapid degradation [16], and a mutated TcdB containing the

BoNT/A-Hc may permit specific delivery of this therapeutic cargo to neurons as an antidote for botulism.

Acknowledgments

The authors thank Weijia Nie and Amelie Debrock for technical assistance, Dr. Jong-Beak Park for purified recombinant BoNT/A-Hc, Dr. Jean Mukherjee for the anti-BoNT/A-Hc mAb, and Dr. Rebekka Biedendieck for the MS941 strain. They also thank Dr. Saul Tzipori for his early and continuing support of this project and for helpful discussions. This project was funded with federal funds from the NIAID, NIH, DHHS, under Contract no. N01-AI-30050, and awards R21AI088489 and R01AI088748. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

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Hindawi Publishing Corporation
Journal of Toxicology
Volume 2012, Article ID 981626, 9 pages
doi:10.1155/2012/981626

Review Article

Use of *Clostridium perfringens* Enterotoxin and the Enterotoxin Receptor-Binding Domain (C-CPE) for Cancer Treatment: Opportunities and Challenges

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Received 15 May 2011; Accepted 6 June 2011

Academic Editor: Martha L. Hale

Clostridium perfringens enterotoxin (CPE) causes the symptoms associated with several common gastrointestinal diseases. CPE is a 35 kDa polypeptide consisting of three structured domains, that is, C-terminal domain I (responsible for receptor binding), domain II (responsible for oligomerization and membrane insertion), and domain III (which may participate in physical changes when the CPE protein inserts into membranes). Native CPE binds to claudin receptors, which are components of the tight junction. The bound toxin then assembles into a hexameric prepore on the membrane surface, prior to the insertion of this oligomer into membranes to form an active pore. The toxin is especially lethal for cells expressing large amounts of claudin-3 or -4, which includes many cancer cells. Initial studies suggest that native CPE has potential usefulness for treating several cancers where claudin CPE receptors are overexpressed. However, some challenges with immunogenicity, toxicity, and (possibly) the development of resistance may need to be overcome. An alternative approach now being explored is to utilize C-CPE, which corresponds approximately to receptor binding domain I, to enhance paracellular permeability and delivery of chemotherapeutic agents against cancer cells. Alternatively, C-CPE fusion proteins may prove superior to use of native CPE for cancer treatment. Finally, C-CPE may have application for other medical treatments, including vaccination or increasing drug absorption. The coming years should witness increasing exploitation of this otherwise formidable toxin.

1. Introduction to *Clostridium perfringens* Enterotoxin (CPE)

Clostridium perfringens is a major pathogen of humans and livestock [1]. This Gram-positive anaerobe causes both histotoxic infections, such as gas gangrene (clostridial myonecrosis), and enteric infections, such as human food poisoning. As typical amongst pathogenic clostridial spp., the virulence of *C. perfringens* is largely attributable to its ability to produce an arsenal of potent protein toxins. Production of four of these toxins (alpha, beta, epsilon, and iota) is used to classify *C. perfringens* strains into one of five types (A–E). Less than 5% of all *C. perfringens* type A isolates produce another toxin named *C. perfringens* enterotoxin (CPE) that is biomedically important, although not used in the toxin typing classification system [2]. After a brief introduction to this unique toxin, promising efforts to utilize CPE, or its derivatives, for cancer therapy will be described

1.1. The Role of CPE in Natural Disease. CPE-producing *C. perfringens* type A strains cause the second most common bacterial food poisoning in the USA, as well as many cases of human nonfoodborne gastrointestinal diseases, such as antibiotic-associated diarrhea [2]. The food poisoning develops when foods contaminated with large numbers of CPE-producing type A strains are ingested; the ingested bacteria then briefly grow in the small intestine before committing to sporulation. It is during this *in vivo* sporulation that

the enterotoxin is produced. Molecular Koch's postulates analyses have demonstrated that production of CPE is essential for CPE-positive type A human food poisoning or nonfoodborne gastrointestinal disease isolates to cause gastrointestinal effects in animal models [3].

Most, but not all, *C. perfringens* type A food poisoning strains carry their enterotoxin gene (*cpe*) on the chromosome [2]. In contrast, the *cpe* gene of type A nonfoodborne human disease strains is located on large (~70–75 kb) plasmids [4]. Amongst *cpe*-positive type A strains there are two major families of *cpe* plasmids [5]. These enterotoxin plasmids can be conjugative [6], presumably due to the presence of a Tn*916*-like region named *tcp* that has been experimentally shown to mediate transfer of other *C. perfringens* conjugative plasmids [7].

Expression of the *cpe* gene is regulated by sporulation-associated alternative sigma factors [8, 9]. Specifically, one alternative sigma factor (SigF) controls expression of two other alternative sigma factors (SigK and SigE), which then direct transcription of *cpe* mRNA from several SigK- or SigE-dependent promoters located upstream of the *cpe* ORF. Exceptionally large amounts of CPE can be produced during sporulation; for example, CPE can represent 20% of total protein in some sporulating CPE-positive *C. perfringens* type A strains [2].

Once produced, CPE is not immediately secreted [2]. Instead it accumulates in the cytoplasm of the mother cell until the completion of sporulation. When the mother cell then lyses to release the mature spore, CPE is released into the intestinal lumen, where it binds and acts as described in the following section.

The *in vivo* outcome of natural CPE action during gastrointestinal disease is desquamation of the intestinal epithelium, intestinal necrosis, and the accumulation of luminal fluid [1]. These effects account for the natural gastrointestinal symptoms of CPE-associated disease, which most commonly include diarrhea and abdominal cramps. Typically, people are sickened with *C. perfringens* type A food poisoning for 12–24 hours and then recover. However, this illness can be fatal in the elderly or in people suffering from medication-induced constipation [10].

1.2. The Cellular Action of CPE. As shown in Figure 1, the current model of CPE action begins with binding of this toxin to claudin receptors (described in detail below). This binding results in formation of a small (~90 kDa) SDS-sensitive complex. Besides CPE, the small complex also contains [11] both claudin receptors and claudins incapable of binding CPE (i.e., nonreceptor claudins). Presumably the presence of nonreceptor claudins in small complex is attributable to claudin: claudin interactions. Six small complexes are then thought to oligomerize into an SDS-resistant large complex named CPE hexamer-1, or CH-1. This hypothesis is based upon results of heteromer gel shift analyses, which identified the presence of six CPE molecules in each CH-1 complex [11]. CH-1 is ~450 kDa in size and contains, in addition to six CPE molecules, both receptor and nonreceptor claudins [11]. CH-1 initially assembles as a prepore on the membrane surface; however, at 37°C this prepore then rapidly inserts into membranes to form an active pore [12].

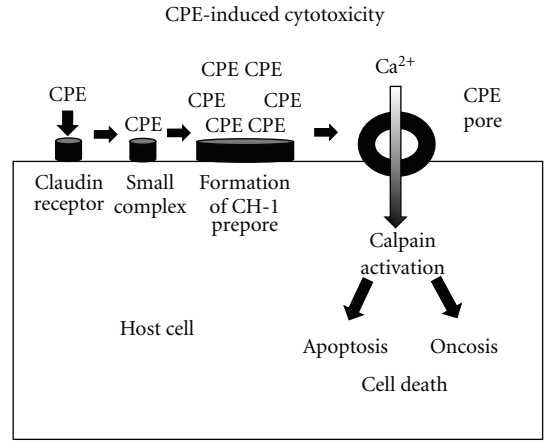


Figure 1: Model for CPE-induced cytotoxicity. CPE first binds to claudin receptors to form an ~90 kDa small complex. Six small complexes are then thought to oligomerize on the membrane surface to form a CH-1 prepore. The prepore then inserts into membranes to form the active pore. This results in entry of calcium into cells, which activates calpain. When a high CPE dose is used, there is substantial entry of calcium into cells, causing a strong calpain activation; this results in cell death by oncosis. Lower CPE doses cause more limited calcium influx and thus a milder calpain activation; these cells die by classical caspase 3-mediated apoptosis.

Formation of the CH-1 pore results in calcium influx, which (via calpain activation) leads to cell death [13, 14]. At moderate CPE doses, where modest CPE pore formation allows only limited calcium influx, CPE-treated cells die from a classical caspase 3-mediated apoptosis. At higher CPE doses, where large amounts of CPE pore formation results in a massive calcium influx, cells die from oncosis.

CPE pore formation also leads to morphologic damage that exposes the basolateral surface of cells. This allows formation of a second bigger (~650 kDa) large complex named CH-2 [15]. In addition to six copies of CPE and both receptor and nonreceptor claudins, CH-2 also contains another tight junction protein named occludin [11, 15]. Formation of CH-2 leads to internalization of occludin into the cytoplasm [11]; claudins are also internalized inside native CPE-treated cells although it is not clear if this is due to CH-1 formation, CH-2 formation, or to formation of both complexes. These effects likely help to explain the observed ability of native CPE to disrupt tight junctions [16].

2. The CPE Structure/Function Relationship

2.1. Cytotoxicity Domains of CPE. CPE consists of a 319 amino acid polypeptide (Mr 35,317) with a unique primary sequence [2]. The CPE structure/function relationship has been extensively analyzed by combined genetic, biochemical, and structural biology approaches (Figure 2). As this review was being prepared, the structure of the native CPE has just been reported [18]. This structure revealed that CPE is

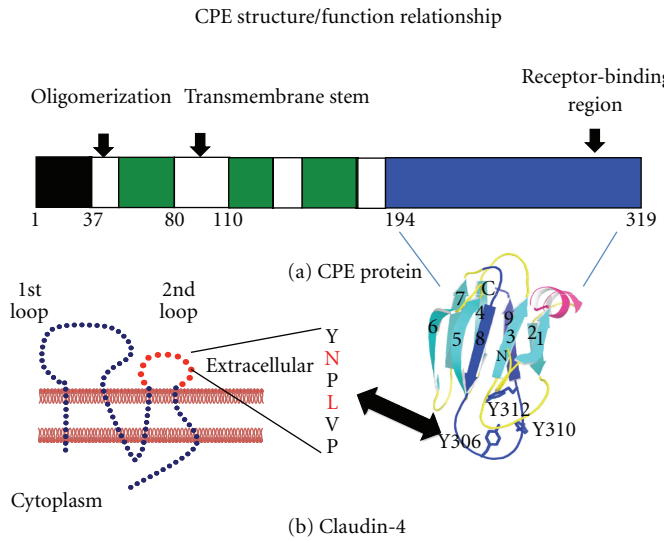


Figure 2: CPE structure versus function relationship. Panel A shows the functional regions of CPE, including the unstructured N-terminal sequences comprising amino acids 1–37 (black box), the domain II sequences mediating oligomerization and membrane insertion (white boxes), the domain III sequences that may participate when CPE inserts into membranes (green boxes), and the domain I sequences that mediate CPE receptor binding (blue box). Shown below the drawing is the structure of domain I (used with permission from [17]), including three tyrosine residues that interact with claudin receptors. Panel B shows the predicted structure of claudins. The amino acids in the turn region of extracellular loop 2 are shown to the right of claudin, with the two residues (N and, to a lesser extent, L) important for CPE binding highlighted in red.

a three-domain protein, reminiscent of several other pore-forming toxins.

The N-terminal 37 amino acids of native CPE lack a definable structure and are not necessary for toxicity [2]. In fact, removing these amino acids by chymotrypsin, as may occur in the intestines during disease, produces a 2–3-fold more potent toxin [2]. This proteolytic activation occurs because removal of the N-terminal CPE sequences exposes the CPE region between amino acids 47 to 51, which reside in domain II, to promote CH-1 formation. Thus, this CPE region apparently functions as a latch that facilitates CPE oligomerization. Site-directed mutagenesis studies showed that particularly important domain II residues for CH-1 formation are (i) the Asp at CPE residue 48 and (ii) the Ile at CPE residue 51 [19].

CPE domain II also contains a region spanning from residues 81 to 106 that appears to be a transmembrane stem [12]. Removal of these residues produces a CPE variant that can form CH-1 but is unable to kill cells or form pores. This effect is consistent with CPE residues 81 to 106 mediating the insertion of this transmembrane stem into membranes. Membrane insertion of all six transmembrane stems, from the six CPE proteins present in CH-1, results in β-barrel pore formation.

Domain III may undergo structural changes during the prepore to pore transition that facilitate insertion of the CPE transmembrane stem into lipid bilayers, facilitating pore formation [18].

2.2. Receptor-Binding Domain of CPE. Over 20 years ago it was shown that a recombinant CPE fragment corresponding to the C-terminal half of the native toxin retains full receptor-binding activity [2]. However, (as expected from the preceding section) this C-terminal CPE fragment named C-CPE was nontoxic since it lacks the N-terminal regions necessary for CH-1 formation and insertion of CH-1 into membranes to form a pore. Deletion mutagenesis and synthetic peptide approaches later localized [2] most CPE receptor-binding activity to the 30 C-terminal amino acids (Figure 2). More recently, site-directed mutagenesis studies demonstrated that three Tyr residues, located at positions 306, 310, and 312 of the native toxin protein are important for receptor binding [21].

C-CPE (residues 194 to 319 of the native toxin) approximately corresponds to domain I of the native CPE protein [17, 18]. Domain I consists of a nine beta strand sandwich that shares structural similarity with the receptor binding domains of some other bacterial toxins, including the large Cry family of *Bacillus thuringiensis* toxins [17]. By correlating this C-CPE structure with the previous binding-activity mapping studies described above, it is apparent that the receptor-binding site of CPE resides on a large loop located between beta strands 8 and 9.

3. Claudins as CPE Receptors

3.1. Introduction to the Claudins. Mammalian tight junctions act as both fences and gates, that is, they represent important barriers for an epithelium and also regulate paracellular permeability [22]. Studies conducted over the past 15 years have determined that the tight junction is comprised of several proteins, the most important of which are the claudins [22]. The claudins are a 24-member family of

~20–25 kDa proteins that are predicted to consist of four transmembrane domains, two extracellular loops (ECL-1 and ECL-2), and a cytoplasmic tail that can mediate signaling cascades. Claudins polymerize into strands that comprise much of the tight junction. The distribution of individual claudins varies amongst different tissues. As described in detail later, claudins are also overexpressed in many cancers.

3.2. Evidence that CPE Binds to Claudin Receptors

3.2.1. Fibroblast Transfectant Studies. In 1997, Katahira et al. reported that when fibroblasts, which are naturally CPE-resistant, were transfected to express an ~22 kDa Vero cell protein, they gained CPE sensitivity [23]. The Vero cell protein expressed by these transfectants had properties of a CPE receptor since the fibroblast transfectants acquired the ability to bind significant levels of the toxin and to form high molecular weight complexes that are now recognized as CH-1. This Vero cell CPE receptor protein was later identified as claudin-4. It was also determined [24] that, at physiologic concentrations, the enterotoxin can bind to transfected fibroblasts expressing claudin-3, -4, -6, -8, or -14. However, no CPE binding was detected to transfectants expressing claudins-1, -2, -5, or -10.

3.2.2. Studies with Naturally CPE-Sensitive Enterocyte-Like Cells. A more recent study by Robertson et al. demonstrated that CPE also interacts with claudins in naturally CPE-sensitive Caco-2 cells, which are a human enterocyte-like cell line [11]. Using coimmunoprecipitation and electroelution approaches, this study showed both the CPE small complex and CH-1 large complex formed in Caco-2 cells can contain, in addition to CPE, receptor claudins-3 and -4, along with the nonreceptor claudin-1. In addition to CPE, receptor claudins, and nonreceptor claudins, the CH-2 complex formed in Caco-2 cells also contains occludin [15]. The stoichiometry of claudins in CH-1 and CH-2, or occludin in CH-2, has not yet been determined.

3.3. Mapping of the CPE Binding Site in Claudin Receptors. The structure of a claudin has not been solved at the time when this review is being prepared. However, as mentioned earlier (and depicted in Figure 2), claudins are predicted to possess two extracellular loops named ECL-1 and ECL-2. An early study using claudin chimeras consisting of the N-terminal half of CPE receptor claudin-4 fused with the C-terminal half of CPE nonreceptor claudin-1 suggested that CPE interacts with the putative ECL-2 region [24]. This hypothesis was rigorously confirmed by a recent study [25] using more specific chimeric claudins, which showed that substituting only the predicted ECL-2 sequence of claudin-4 into a claudin-1 backbone is sufficient to produce fibroblast transfectants that are very sensitive to CPE. The reverse was also true, that is, transfectants expressing a chimeric claudin, where only the claudin-1 ECL-2 had been specifically substituted into the claudin-4 backbone, were CPE-insensitive.

Several recent studies (reviewed in [26]) have focused on understanding the specific ECL-2 residues of claudin receptors that mediate CPE binding. These studies have utilized a variety of approaches including arrays of immobilized ECL-2 synthetic peptides, solubilized claudins, or transfectants expressing claudin mutant. Results from these studies suggested that ECL-2 possesses a helix-turn-helix motif that interacts with CPE. An Asn residue in the middle of this turn appears to be important for CPE binding; some evidence suggests that a Leu residue located two residues from this Asn may also participate in the binding of this toxin.

4. CPE and Cancer Treatment

4.1. Introduction. Since approximately 85% of malignant tumors are derived from epithelial cells, an epithelium-targeted therapeutic strategy has been the focus of cancer translational research. As mentioned earlier, claudins are the major components of paracellular tight junctions (TJs), distribute at the most apical junctions between epithelial cells, and play an essential role in the control of paracellular transport. Furthermore, Claudin-3 and -4 have been identified as the specific receptors for CPE, which is of potential therapeutic significance since these two claudins are abundantly expressed in ovarian, breast, uterine, and pancreatic cancers [27]. While CPE can trigger lysis of epithelial cells by binding to claudin-3 and claudin-4, with resultant initiation of massive permeability changes, osmotic cell ballooning, and cytolysis within 5–15 min (see previous sections of this review), cells lacking expression of the CPE receptors are completely unaffected by this enterotoxin [19]. These observations have raised the possibility that CPE may be an innovative claudin-targeted therapy for malignant tumors. In fact, efforts have been made to use CPE in the treatment of claudin-overexpressing cancers during the past few years.

4.2. CPE Treatment and Ovarian Cancer. Ovarian cancer remains the most lethal gynecologic malignancy in the United States. Approximately 90% of patients with advanced-stage ovarian cancer develop recurrence and inevitably die from the development of chemotherapy resistance. Therefore, the discovery of novel and effective therapy is of immediate clinical importance. Previous studies have demonstrated that claudin-3 and -4 were among the six most differentially upregulated genes in ovarian cancer cells, but their expression is undetectable in normal ovaries. Of particular note, chemotherapy-resistant/recurrent ovarian cancers express claudin-3 and -4 at significantly higher levels than chemotherapy-sensitive cancers [28].

These overexpressed claudins may represent promising targets for the use of CPE as a tumor-targeting therapy against this aggressive disease. Indeed, Santin and colleagues successfully used CPE to treat an animal model of chemotherapy-resistant human ovarian cancer [29]. They found that all ovarian cancer cells, regardless of their resistance to chemotherapeutic agents, showed a dose-dependent cytotoxic response and died rapidly after 24 hours of exposure to CPE. Furthermore, in this animal model employing

chemotherapy-resistant human ovarian cancer xenografts, multiple intraperitoneal (i.p.) sublethal doses of CPE ranging from 5 to 8.5 μg/mL significantly inhibited tumor growth and extended the survival of mice harboring a large tumor burden of chemotherapy-resistant ovarian cancer. The application was well tolerated throughout the treatment period. Therefore, CPE-based therapy may have potential as a novel treatment for chemotherapy-resistant ovarian cancer.

4.3. CPE Treatment and Breast Cancer. Breast cancer is one of the most common malignancies worldwide. Despite tamoxifen and aromatase inhibitor having significantly improved long-term survival of patients diagnosed at the early stages, advanced breast cancer and metastatic breast cancer are still incurable diseases. Claudin-3 and claudin-4 are overexpressed in most primary breast carcinomas and breast cancer brain metastases but undetectable in normal breast epithelial cells. In 2004, Kominsky et al. reported [30] for the first time that intratumoral CPE treatment of T47D human breast cancer cell xenografts resulted in significant tumor suppression and necrosis in SCID mice without any side effects. However, i.p. administration of the same dose of CPE was toxic and had no effect on tumor volume. CPE also damaged breast cancer cell lines in a claudin-dependent manner but did not affect cell lines lacking claudin-3 and -4 [30].

In later studies [20], those investigators found that intracranial administration of CPE retarded tumor growth and increased survival in two murine models of breast cancer brain metastasis without any apparent systematic or CNS toxicity (Figure 3). These two studies suggest that the local administration of CPE may be useful in the treatment of breast cancer.

Overexpression of claudin-3 and -4 has been found in uterine serous papillary carcinoma (USPC) and correlates with a more aggressive phenotype and a worse prognosis. A study by Santin et al. has demonstrated that CPE effectively and specifically triggers cytolysis of primary and metastatic USPC cell lines in a dose-dependent manner whereas normal cells lacking claudin-3 and -4 are unaffected by CPE treatment [31]. In particular, multiple intratumoral injections of CPE in large subcutaneous USPC xenografts led to tumor necrosis and even tumor disappearance in 100% of animals. Similarly, i.p. injection of sublethal doses of CPE significantly suppressed tumor growth and extended survival of animals harboring chemotherapy-resistant intra-abdominal USPC. The local/regional administrations of CPE were well tolerated without observable adverse events in animals.

4.4. CPE Treatment of Pancreatic and Prostate Cancers. CPE has also been used to treat pancreatic and prostate cancers in nude mice or *in vitro* by different research groups. Michl et al. reported that intratumoral injections of CPE in pancreatic cancer xenografts resulted in apparent tumor suppression and necrosis in mice, and that CPE treatment also caused an acute dose-dependent cytotoxic effect in pancreatic cancer cells *in vitro* [32]. In prostate cancer, Long et al. showed that the prostate cancer metastatic cells from the bone marrow were sensitive to CPE-mediated cytolysis *in vitro* [33]. These two preclinical studies suggest that CPE may have potential as a novel therapy for primary and metastatic malignancies expressing claudin-3 and -4.

4.5. Challenges of Using Native CPE for Cancer Therapy. Although the specificity and rapidity of CPE-mediated cytolysis may increase anticancer efficacy and reduce opportunity for the development of drug resistance, its clinical application encounters some challenges. Since claudin-3 and -4 are expressed in some normal tissues such as prostate, lung, and the gastrointestinal tract, systemic toxicity is an important concern for CPE therapy. In this context, many investigators have chosen the local administration of native CPE to treat cancers. This approach, however, does not appear to be a good option for some metastatic cancers such as lung metastasis or multiple metastases. Moreover, some adverse events have sometimes been observed even following the local administration of CPE during several studies [30, 34]. Furthermore, CPE is recognized as a virulence factor responsible for the pathophysiological responses associated with a common food poisoning, proinflammatory cytokine response and other human diseases [34]. Another potential problem would include possible immune responses against CPE. Since CPE-associated foodborne illness is so common, many people already have serum antibodies against this toxin. It is unclear whether these serum CPE antibodies include neutralizing antibodies but CPE does contain at least one neutralizing epitope, present in the CPE binding domain [2]. Other challenges include determining optimal dosage and regimen, development of drug resistance, and long-term safety concerns. Therefore, further detailed studies will be required to resolve these issues.

5. C-CPE and Cancer Treatment

Although the clinical application of CPE is limited by its potentially significant toxic side effects, the C-terminal binding domain of CPE (C-CPE) overcomes this drawback of CPE and has recently emerged as a promising cancer therapeutic agent due to its unique properties. For example, C-CPE can disrupt the paracellular TJ barrier by binding to claudin-3 and -4 in the epithelia and thus improve drug delivery in a noncytotoxic fashion. It also has a smaller molecular size that might provide less immunogenicity than CPE.

The paracellular TJs are the primary barrier to the transport of solutes from the apical surface to the core of cells. Agent uptake via the paracellular pathway in the epithe-lia is considered an attractive route for the absorption of chemotherapies. Given that claudin-3 and -4 are overexpressed in several cancers and are major components of cell TJs, their downregulation by C-CPE may prove a novel strategy for enhancing conventional chemotherapy delivery into claudin-positive cancer cells [36].

Our group recently investigated the efficacy of a combination therapy using a chemotherapeutic agent with C-CPE as

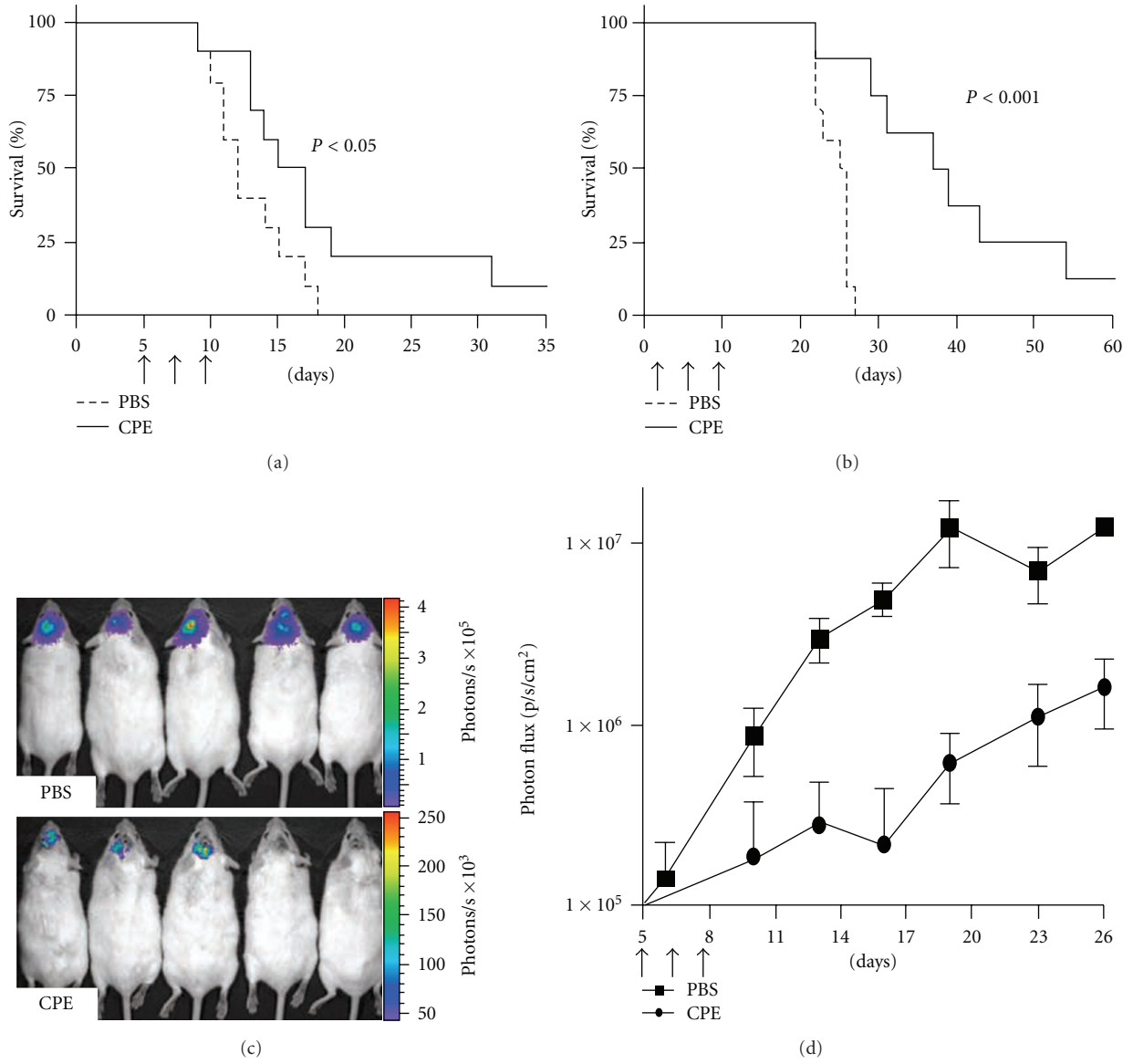
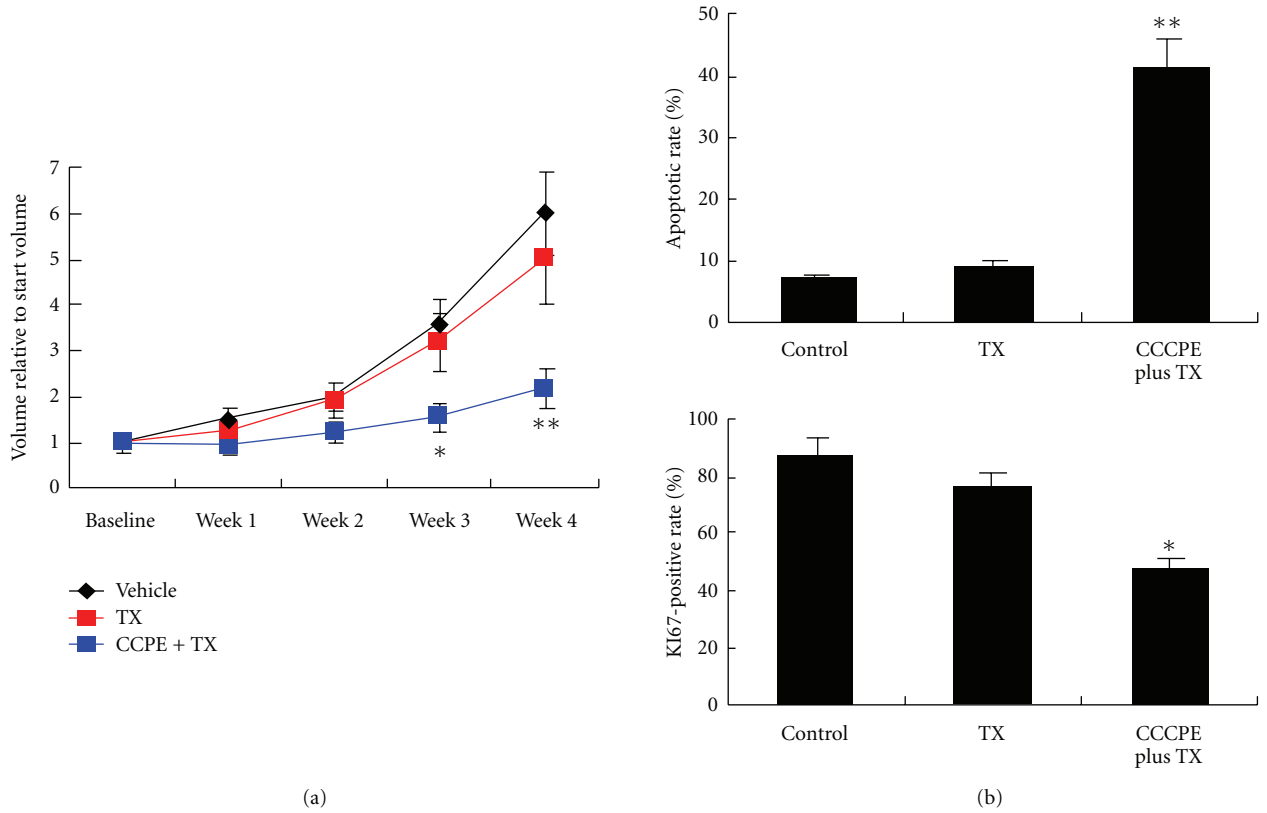


Figure 3: Efficacy of CPE in the treatment of breast cancer brain metastasis. (a, b) Brain tumors were established in mice using the human breast cancer cell line MDA-MB-468 and the murine breast cancer cell line NT2.5-luc. Tumors were treated by intracranial administration of 0.5 μg CPE versus PBS on days 5, 7, and 9. (c, d) For the NT2.5-luc brain tumor model, noninvasive bioluminescent imaging was done twice per week beginning on day 4. Bioluminescent images from five representative mice are shown for each experimental group at day 19 (c). Photon flux was measured over the indicated time course as an indication of tumor growth (d). Differences in survival between experimental groups were analyzed using the log-rank test. Reproduced with permission from [20].

compared to the single-agent chemotherapeutic agent [35]. Using three-dimensional and monolayer culture models and a xenograft mouse model of human EOC cells, we found that C-CPE enhanced the chemosensitivities of EOC cell lines to Taxol or Carboplatin at low concentrations in a claudin-dependent fashion. Moreover, repeated i.p. administration of C-CPE in combination with Taxol significantly suppressed large tumor burdens by about 59% compared with control or Taxol alone and showed no apparent toxic drawback of CPE as encountered in previous studies (Figure 4). Our study suggests that, at relatively low concentrations, C-CPE may enhance the sensitivity of EOC cells and other claudin-sensitive tumor cells to conventional chemotherapy and thus alleviate systemic side effects of the agents.

Indeed, C-CPE could be useful not only as an anticancer drug enhancer but also as a carrier to deliver a variety of toxins leading to a spectrum of new anticancer drugs of high selectivity. TNF-α has been demonstrated to be an attractive antitumor agent in a variety of animal models but clinical application has been limited due to its failure to concentrate at the site of tumors and the development of severe side effects. To solve the problem, Yuan et al. engineered a C-CPE-TNF fusion toxin that was >6.7-fold more cytotoxic



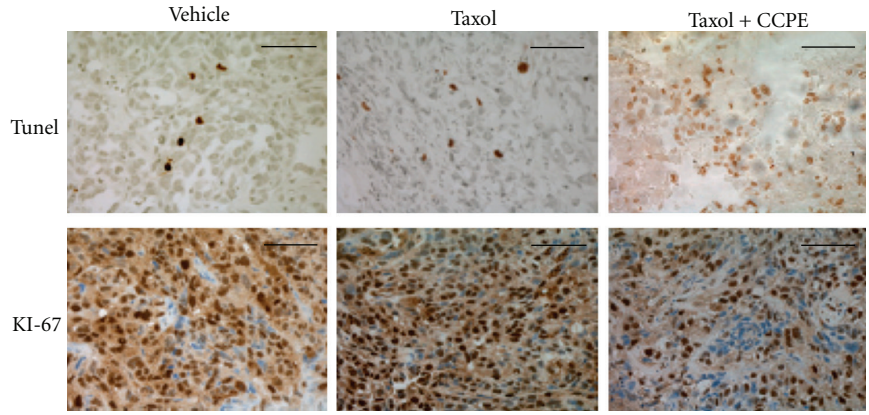


Figure 4: Combination therapy of C-CPE and taxol attenuates EOC xenograft growth *in vivo*. Female SCID mice were inoculated s.c. with 5×10^6 SKOV-3 cells. Four weeks later, the mice harboring large tumor burden were intraperitoneally administered with taxol alone (20 mg/kg), taxol combined with C-CPE (0.1 mg/kg), or vehicle (PBS) twice a week for 4 weeks. (a) Growth curves of tumors were presented as the mean volume normalized to the start volume. *The combination of C-CPE and taxol led to a significant tumor suppression compared with vehicle or taxol alone ($P < 0.05$). (b) After 4 weeks of treatment, immunostaining of Ki67 and TUNEL was performed to evaluate cell proliferation and apoptosis in EOC xenografts. $^*P < 0.05$; $^{**}P < 0.001$. Reproduced with permission from [35].

than free TNF to ovarian cancer cells expressing claudin-3 and -4; whereas the TNF component in the fusion was 5-fold less potent than free TNF, suggesting that C-CPE-TNF fusion may prevent or decrease the systemic side effects caused by TNF [37]. Besides ovarian cancer, Saeki et al. fused C-CPE to the protein synthesis inhibitory factor (PSIF) derived from *Pseudomonas aeruginosa* exotoxin A for breast cancer that expresses high levels of claudin-3 and -4 [38]. The C-CPE-PSIF fusion selectively damaged claudin-expressing breast cancer cell lines, and repeated intratumoral injections significantly suppressed tumor size by 36% without causing apparent side effects in mice [38]. These two studies indicate that C-CPE may be a useful carrier for delivering toxins to claudin-sensitive malignancies

with higher anticancer potency and less systemic side effects.

The advantages of this claudin-targeted C-CPE-based cancer therapy are significant: tumor-targeted therapy, less immune response, and an improved therapeutic potency not achieved with previous treatments. There remain challenges to be overcome, however, before we can see any major medical advances in treating cancer with C-CPE: (1) determining safe dosages and schedules, (2) choosing optimal administration routes, (3) avoiding potential immunogenicity, and (4) improving effectiveness for preventing and/or treating cancer metastases. Therefore, further studies and clinical trials are required to determine whether C-CPE could be developed as a claudin-targeted novel therapeutic agent for the treatment of cancer.

6. Use of C-CPE for Other Medical Applications

In addition to cancer treatment, C-CPE has been utilized to treat other medical conditions. For example, with the intention of developing a potent mucosal vaccination approach, a nasal vaccine of C-CPE-fused antigen has been prepared and applied in mice without mucosal injury and side effects [39]. In addition, the ability of C-CPE to enhance paracellular permeability has been exploited by using this protein to increase drug absorption from the intestines [40]. One potential issue regarding the use of C-CPE for increasing intestinal drug absorption could be possible gastrointestinal side effects such as diarrhea, due to increased paracellular permeability. However, C-CPE does not increase luminal fluid levels in rabbit ileal loops, possibly arguing against this concern [41].

Acknowledgment

A second independent manuscript solving the structure of CPE has just been accepted for publication (see D.C. Briggs et al., Journal of Molecular Biology, 2011, In press).

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Hindawi Publishing Corporation
Journal of Toxicology
Volume 2012, Article ID 802893, 12 pages
doi:10.1155/2012/802893

Review Article

Therapeutic Use of Botulinum Toxin in Neurorehabilitation

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Received 5 April 2011; Revised 28 June 2011; Accepted 13 July 2011

Academic Editor: S. Ashraf Ahmed

The botulinum toxins (BTX), type A and type B by blocking vesicle acetylcholine release at neuro-muscular and neurosecretory junctions can result efficacious therapeutic agents for the treatment of numerous disorders in patients requiring neurorehabilitative intervention. Its use for the reduction of focal spasticity following stroke, brain injury, and cerebral palsy is provided. Although the reduction of spasticity is widely demonstrated with BTX type A injection, its impact on the improvement of dexterity and functional outcome remains controversial. The use of BTX for the rehabilitation of children with obstetrical brachial plexus palsy and in treating sialorrhea which can complicate the course of some severe neurological diseases such as amyotrophic lateral sclerosis and Parkinson's disease is also addressed. Adverse events and neutralizing antibodies formation after repeated BTX injections can occur. Since impaired neurological persons can have complex disabling feature, BTX treatment should be viewed as adjunct measure to other rehabilitative strategies that are based on the individual's residual ability and competence and targeted to achieve the best functional recovery. BTX therapy has high cost and transient effect, but its benefits outweigh these disadvantages. Future studies must clarify if this agent alone or adjunctive to other rehabilitative procedures works best on functional outcome.

1. Introduction

Botulinum toxins are some of the most potent poisons present in nature produced by the anaerobic bacterium *Clostridium Botulinum*. Historically, these toxins were predominantly associated with a food-borne toxicosis producing a neurological life-threatening disease called "botulism", characterized by a severe generalized muscular paralysis and cholinergic autonomic blockade. Currently, botulinum toxins have become established as efficacious therapeutic agents for the treatment of numerous medical disorders. Seven types of toxins have been harvested from clostridium, designated A through G, but only type A (BTX-A) and B (BTX-B) are commercially available and used in clinical practice. In 1989, the Food and Drug Administration approved BTX-A for the treatment of strabismus; since then, the growing use of this drug in several neurological disturbances has made it one of the most important advancements in the therapeutics of movement disorders such as muscular dystonia and dyskinesia. At the same time, botulinum toxin (BTX) either alone or adjunct to other measures has emerged as a new important therapeutic strategy for clinicians, treating a wide range of disturbances including gastroenterological and urological diseases as well as dermatological and cosmetic applications.

In the last decade, one of the main indications of BTX is the treatment of disorders characterized by muscular hyperactivity and excessive or inappropriate muscle contraction. Neurorehabilitative medicine treats these patients and ameliorates severe neurological impairments that have scarcely available therapeutic interventions. This paper presents both the consolidated applications of BTX in spasticity as well as in other disturbances in which it has been shown to be a useful therapeutic tool. Since in clinical practice, BTX-B is less used than BTX-A, and few researches studies have been published regarding its use, most of data presented pertain to BTX-A treatment.

2. Structure and Type of BTX

The active BTX molecule is formed by two chains weighing ~150.000 daltons, in which a heavy chain is linked by a disulfide bond to a light chain [1]. The heavy chain is

responsible for neuron-specific binding and internalization. Once internalized and within a vesicle, the light chain across the vesicle membrane is released into the neuronal cytoplasm, where it binds to a specific target protein involved in the docking and fusion of acetylcholine-containing vesicles to the internal portion of the cell membrane. These target proteins are collectively referred to as the SNARE complex. The BTX-A cleaves a protein termed SNAP-25, whereas BTX-B binds a different protein designed VAMP, also known as synaptobrevin [2]. Both are responsible for vesicle acetylcholine release. The derangement of this process at the neuromuscular junctions cause clinical effects consisting in muscle weakness and paralysis. BTX-A and BTX-B are commercially available and used in clinical practice. To date, three formulation of BTX-A are commercialized and are marketed as Botox (Allergan, Inc., Irvine, Calif, USA), Dysport (Ipsen Ltd., Berkshire, UK) and Xeomin (Merz, Frankfurt, Germany), respectively. The preparations are manufactured by different processes, have different formulations and potencies, which are determined by different biological assays based on their clinical use. BTX-B is marketed by Solstice Neuroscience (Malvern, Pa, USA) as MyoBloc in the United States and NeuroBloc (Elan Pharmaceuticals, San Diego Calif, USA) in Europe. It is important to note that the potency of a single unit varies greatly among the commercial types. Although the potency of 1 U of Botox is roughly equal to 1 U of Xeomin, 3 U of Dysport, and 40 to 50 U of MyoBloc, it is very important to recognize that a simple ratio of dosing equivalencies cannot be applied [3]. BTX-B is commercially packaged in vials of 10 mL containing 5.000 MU and 10.000 MU of neurotoxin. For muscle injections, botulinum toxins are diluted with 0.9% sodium chloride solution at variable volumes depending on the dose that the clinician plans to inject. BTX doses are generally adjusted according to factors such as severity of the hyperactive muscle, number of muscles involved, age, and previous response to BTX therapy. The duration of BTX effect is variable depending from several factors including type of neurotoxin, dose, site of injections, and clinical applications. In disease botulism, neurotoxin A produces longer paralysis than botulinum neurotoxin B consistent with human observations [4]. Likewise, BTX-A has been shown to have a longer duration of effect in cervical dystonia compared with BTX-B [5]. Botulinum types A and B have a similar duration of clinical action in treating drooling due to neurological diseases [6]. In poststroke spasticity, the duration of action was not specifically addressed by the available studies although some trials suggested that efficacy of BTX-A may be appreciated 6 weeks after injection and for up to 9–12 weeks [7]. BTX-B has a tendency to produce more autonomic side effects than BTX-A [8] and can have a more enduring action than BTX-A in specific clinical applications such as hyperidrosis and sialorrhea [9, 10].

3. Spasticity

In a neurorehabilitation setting, BTX is predominantly used for the treatment of spasticity and to prevent muscular contractures. Spasticity is defined as a velocity-dependent increased resistance to passive limb movement in people with upper motor neuron syndrome [11]. Clinically, it is an involuntary motor disorder, characterized by hypertonic muscle tone with increased excitability of the muscle stretch reflex and increased tendon reflexes. Muscle weakness and limb paresis are associated to spasticity and contribute to the loss of motor dexterity and ability. Spasticity, if left untreated can hamper functional outcome by promoting persistent abnormal posture that, in turn, produces muscular-tendon contractures and bone deformity. Secondary complications arising from spasticity include impaired movement, hygiene, self-care, poor self-esteem, body image, pain, and pressure ulcers. Furthermore, patients with severe spasticity can develop poor social participation and quality of life (QOL) [12]. Because of these clinical concerns and related high social costs [13], several therapeutic strategies have been proposed for the treatment of spasticity including surgical, medical, and rehabilitative procedures. However, spasticity is not always harmful and patients with a combination of muscle weakness and hypertonic muscles may rely on the increased tone to maintain their posture and aid standing or walking. It is important to point out that BTX use is indicated when the spasticity is focal or segmental and if it interferes with active or passive functioning. Treatment of muscle hyperactivity may be considered when the condition is disabling. The primary aim of the treatment of spastic muscles is to maintain length and allow normal positioning of the limbs to prevent secondary soft-tissue shortening. Generally, BTX treatment is carried out as an adjunct to other rehabilitative strategies that are based on an individualized, multidisciplinary programmes and targeted to achieve patient goals. Treatment plans must consider a tradeoff between reduction of spastic hypertonia and preservation of residual motor function [14]. Although there is no consensus as to when BTX therapy should be initiated, or how long it should last, BTX-A injection is considered the hallmark or first line of medical treatment for focal/segmental spasticity [15]. Conversely, BTX-B has been predominantly used as an alternative agent for patients who developed resistance to BTX-A [16]. BTX-B has been used for the treatment of adult and child spasticity, but its effectiveness is unclear [17]. Data in the literature are insufficient to recommend it for the treatment of children with spasticity [18]. It is known that spasticity can follow several neurological diseases such as stroke, acquired brain injury, multiple sclerosis, cerebral palsy, and spinal cord injury. BTX use for the treatment of spasticity in some of those will be addressed.

3.1. Poststroke Spasticity. Spasticity is a frequent motor disorder in adult patients with stroke and its incidence is variable ranging from 17% to 43% [19–21]. A bulk of trials have demonstrated the efficacy of BTX-A in reducing poststroke spasticity [22–24]. Improvement of hypertonic muscles has been reported both in upper [12, 25–28] and lower limbs [29–31] after BTX-A injections. Thus far, administration modalities, and standard muscle doses of BTX-A (either Botox or Dysport) have been proposed for the reduction of limb spasticity in adult patients with stroke.

However, there is no clear evidence from the literature to guide optimal timing of interventions (e.g., early versus late), frequency of interventions, dilutions, injection sites, or doses. Current clinical recommendations for muscle-specific dosing in spasticity remain largely based on expert opinion, clinical experience, as well as the formulation of botulinum toxin being used. A mean BTX-A global dose ranging from 90 to 360 MU and from 350 to 1500 MU per intramuscular injection has been reported for upper spastic limbs when using Botox or Dysport, respectively. A BTX-A dose ranging from 100 to 400 MU and from 400 to 1500 MU for the two toxins, respectively, has been used in treating lower spastic limbs [7, 32]. According to the European Consensus on the use of BTX-A in adult spasticity, maximum doses should not exceed 1500 MU Dysport and 600 MU Botox per injection session [33]. The magnitude of response is dose dependent [34, 35] even if the dosage is largely titrated by the practitioner based on individual patient response.

BTX-B has also been used in treating poststroke spasticity, but its efficacy in reducing spasticity has been questioned and, thus far remains unclear [17]. A recent systematic review of BTX use in adult poststroke spasticity concluded that available data on BTX-B were insufficient to assess its effect on spasticity and that further controlled trials using BTX-B were necessary [7].

Although the reduction of spasticity is widely demonstrated with BTX-A treatment, its impact on the improvement of dexterity and functional outcome remains controversial. Some functional improvements may be seen after BTX injections, but global functional assessment methods do not consistently reflect these changes. Numerous studies have reported to attain prespecified goals [26, 36], active movement [37, 38], and gait [39]. Conversely, other studies did not find any benefit on functional gain in patients with post-stroke spasticity after BTX-A treatment [12, 40–44]. Fridman et al. [45] reported kinematic parameters improvement of spastic upper arm in post-stroke patients after BTX-A injections. They speculated that the improvement in velocity and time required to perform some motor tasks could be translated to countless situations in a patient's life, which is difficult to determine with objective functional scales. Likewise, Bensmail et al. [44] described improvement of kinematic parameters in upper spastic arm after BTX-A treatment but without significant changes in clinical outcomes. However, clinical practice shows that some patients benefit with improved motor function, but that predicting factors still have to be identified. Several reasons have been suggested to explain this contrasting finding. It is possible that spasticity does not contribute to limitation of function and that the underlying weakness is the only significant cause of activity limitation [46]. Many recovering patients with stroke experience significant reductions in functioning, QOL, and family relationships. Improvements in QOL, caregiver burden, and patient functioning are key measures of success in any rehabilitation program. Furthermore, these patients can develop shoulder pain that interfere with the rehabilitative process and has been associated with poorer outcomes and prolonged hospital stays [47]. BTX-A treatment of poststroke spasticity has been demonstrated to produce improvement of patients' quality of life [12, 25] and pain relief [48].

3.2. Spastic Cerebral Palsy. Cerebral palsy (CP) is the most common nonprogressive cause of motor disturbance and disability in children. Even with improvements in medical technology and clinical practice, the overall rate of CP remains high, with 2 to 3 per 1000 live births [49]. CP is the main cause of spasticity in children. Therapeutic management may include splinting/casting, passive stretching, facilitation of posture and movement, spasticity-reducing medication, and surgery. Many clinicians frequently face the dilemma of whether and how to medically treat spasticity in children with CP. When pharmacologic intervention is deemed appropriate, treatment decisions must first be based on accurate assessment using valid and reliable clinical instruments, and, even more importantly, measurable, achievable, and realistic treatment goals should be delineated. Successful use of BTX in children with CP was first reported in 1993 by Koman et al. [50]. Since then, there has been a growing interest of the therapeutic effects of BTX-A, and many trials have investigated its effectiveness for treatment of spasticity in children and adults with CP [18, 51–55]. Likewise in poststroke spasticity, BTX-A has been effective in the reduction of spasticity of both upper [52, 56] and lower extremities [54, 57].

In the late 1990s, pediatric doses of BTX-A in the treatment of children with spastic CP ranged from 12 to 16 U/kg and from 15 to 25 U/Kg of body weight for Botox [58] and Dysport [59], respectively. These dosages have increased over time [60]. Higher BTX-A doses of 15 to 22 U/kg and of 20 to 30 U/kg have also been used without serious adverse events for Botox [61] and Dysport [62, 63], respectively. Maximum doses of BTX-A should not exceed 300 U Botox and 900 U Dysport per injection session, respectively. Children typically receive higher doses per kilogram of body weight than adults and can develop more adverse events. BTX-A treatment is effective and safe, maintaining long-lasting effects after repeated injections. A recent review of relevant literature concerning the treatment of spasticity in children with CP recommended that the use of BTX-A should be offered as an effective and generally safe treatment to reduce localized/segmental spasticity in upper and lower extremities. The same review did not find sufficient data to support or refute the use of BTX-B [18]. In this rehabilitation area, BTX-A is generally used as an adjunct to physiotherapy or other rehabilitative interventions such as casting or orthotics to obtain reduction of spasticity and functional improvement. A review by Ryll et al. [54] reported that BTX-A injections combined with usual care or physiotherapy can have a positive effect on walking in children with CP. Trials comparing BTX-A with usual care or physiotherapy showed evidence that functional outcomes improved at different follow-up times of 2 to 6 weeks [64, 65], 12 weeks [66], and 24 weeks [64, 66] when BTX-A injections were combined with usual care or physiotherapy compared to usual care or physiotherapy alone. Similarly, another recent systematic review found a high level of evidence supporting the use of BTX-A as an adjunct to

managing upper limbs in children with spastic CP [56]. However, several issues remain unsolved including timing, duration of BTX-A action, and its effectiveness in the long term. Furthermore, when BTX-A is used as adjunct to other measures, the type of physiotherapy that is best indicated, application, timing, and type of casting to obtain better results remain unclear.

3.3. Brain Injury. About 75% of patients with physical disability following severe brain injury (BI) will develop spasticity requiring specific treatment. Also, patients with focal spasticity due to BI can benefit from BTX treatment. Unlike poststroke spasticity, scarce data about the use of BTX in treating spasticity following traumatic BI have been reported. Generally, these studies used BTX-A and enrolled small or heterogeneous samples of patients including subjects with stroke and traumatic BI [67–70]. BTX-A injection was used as an adjunct to physiotherapy [71] or casting [67] strategies. However, all studies demonstrated the efficacy of BTX-A in reducing spasticity either in adults [23, 67–70] or children [71, 72].

4. BTX as Adjunct Therapy for Management of Spasticity

Treatment of spasticity incorporating BTX is usually part of an integrated multidisciplinary rehabilitation program. BTX is rarely a sole treatment, and it is generally used combined with physiotherapy or casting, particularly in children with spastic CP. Physiotherapy procedures associated with BTX-A treatment can be variable including stretching posture, constraint induced therapy, occupational therapy, and electrical stimulation [73–76]. A previously mentioned systematic review reported that a combination of BTX-A and occupational therapy for the treatment of spastic arm in children with CP is more effective than occupational therapy alone in reducing impairment, improving activity level outcomes, and goal achievement [56]. Furthermore, the authors found a high level of evidence to support the use of BTX-A as an adjunct to managing upper limb spasticity in these children.

4.1. BTX and Casting/Orthotics. BTX is also used to prevent muscular contraction and to facilitate cast application or orthotics. Serial casting is a method used for reducing contractures due to spasticity and can be applied both to upper and lower limbs. BTX can facilitate this process by producing temporary weakness and relaxation of the targeted muscles, allowing them to be stretched more easily, thus reducing the neurogenic and biomechanical components of spasticity. Casting is the application of fiberglass and/or plaster to the spastic upper or lower limb to immobilize a joint and has been proposed for the treatment of spasticity following stroke, acquired brain injury and CP, in particular. This strategy has been recommended as a treatment option in the management of equinus in children with CP for many decades. Early researches reported that BTX is as effective as serial casting in improving dynamic function for children suffering with cerebral palsy [77–79]. Hence, there has been a growing interest of the therapeutic effects of BTX-A as adjunct to casting [80–82]. However, the effect of BTX-A combined with casting on the reduction of spasticity remain controversial and unclear [53, 80–82]. A systematic review of the effects of the casting on equinus of children with CP did not find any differences between groups comparing BTX-A plus casting or BTX-A alone versus casting [81].

Furthermore, similar questions and doubts about the improvement of functional outcome were raised when BTX and casting was used for the treatment of spastic children with CP. Studies comparing BTX-A injections plus casting or BTX-A injections alone versus casting showed strong evidence for no effects on the functional outcomes in the application of BTX-A injections, casting alone, or a combination of both treatments [54] in children with CP. On the other hand, Yaşar et al. [82] recently observed that in chronic stroke patients, casting might be an appropriate intervention following BTX-A injection to prevent equinovarus deformity and to improve the quality of walking. Likewise, casting and application of orthotics might potentiate the effect of BTX treatment. Lai et al. [83] reported that in poststroke spastic patients, dynamic splinting after BTX injection increased the range of active elbow extension and suggested that it might be a useful adjunct procedure for optimizing BTX effects.

5. BTX Use without Spasticity

Neurological diseases can produce variable and complex impairments requiring tailored rehabilitation strategies. In a neurorehabilitation setting, clinicians have to approach numerous motor and nonmotor disorders other than spasticity. BTX use in specific neurological diseases and disorders with complex neurological dysfunction will be provided.

6. BTX and Focal Hand Dystonia

Focal hand dystonia (FHD) is a motor disturbance characterized by a task specific muscle spasms, in which learned or repetitive motor tasks (such as writing or playing a musical instrument) trigger muscle spasms and interfere with practiced motor execution, whereas other actions remain normal. FHD include a variety of disorders affecting many different skilled functions. Writer's cramp and musician's dystonia are the most common forms of idiopathic FHD [84]. Writer's cramp is characterized by involuntary, repetitive, or sustained contractions of finger, hand, or arm muscles that occur during writing and produce abnormal postures or movements that interfere with normal handwriting. Although the prevalence is relatively low, varying from 3 to 7/100 000, [85, 86], it may be responsible for considerable morbidity in terms of working impairment, pain, embarrassment, low self-esteem, and poor social interaction. Musician's dystonia is a task-specific movement disorder that manifests itself as a loss of voluntary motor control in extensively trained movements. Approximately 1% of all professional musicians develop musician's dystonia, and in many cases, the disorder terminates the careers of affected

musicians [87]. Therapeutic strategy proposed for the treatment of FHD including muscle relaxation techniques, physical and occupational therapy, and medical and surgical therapies have all been disappointing. Several researches have demonstrated that BTX injections into selected hand and forearm muscles provides the most effective relief in patients with these task-specific occupational dystonias [88–90]. Injection of BTX into the muscles responsible for the abnormal postures can be very effective and is often considered the first choice. A muscle dose ranging from 5 to 40 MU of BTX-A (Botox) and from 15 to 150 MU of BTX-A (Dysport) has been injected for writer's cramp [91]. There has also been a specific study showing utility for musician's cramps [92]. Patients can continue to respond to injections for many years. Lungu et al. recently reported that BTX-A treatment was safe and effective after more than a decade of treatment [93] in 20 patients with FHD. Of these, the musicians were more likely to wait longer between injections. The dose of BTX is based on the size of the muscle affected, the intensity of the spasm, and the number of muscles involved [94]. Likewise, in other motor disorders, BTX can correct abnormal hand posture and relieve discomfort. However, the restoration of normal hand function can be difficult to achieve. Not only are some of the underlying dystonic defects, such as loss of speed and coordination, not fully addressed by the botulinum toxin injection, but the weakness that accompanies injection can be an additional source of hand disability. In some patients, the tradeoff between disability due to weakness from injection and disability due to the dystonia itself is not acceptable. The cyclic improvement with BTX injection and the worsening of the dystonia when the benefit wears off, does not allow for consistent sustained performance which is especially problematic for professional musicians [95].

7. BTX and Obstetrical Brachial Plexus Injury

Obstetric brachial plexus injury (OBPI) can be a dramatic sequela of dystocia or complicated delivery. A recent study showed an incidence of 1.3 per 1000 live births in the United States [96]. A higher incidence, ranging from 3 to 4.6 per 1000 live births was found in Europe [97, 98]. Severe brachial plexus palsies can result in disabling due to impairment and imbalance of the muscular contraction in the paretic limb. In spite of physical therapy, some children continue to experience contractures and abnormal posture that hamper complete recovery. In the last decade, an increasing number of reports on the treatment of BTX-A for OBPI have been published [99–103]. BTX-A has been used to improve muscular imbalance of the internal rotator-adductor muscles of the shoulder, limited active elbow extension, and triceps cocontraction in combination with conservative treatment, including long-term physiotherapy, occupational therapy, and functional orthopaedic or plastic surgery. Furthermore, BTX-A as adjunct to serial casting has been successfully used in children with OBPI to improve muscular contracture, arm position, elbow extension, and dexterity in the paretic limb [99, 100]. However, a recent systematic review about the treatment indications of BTX-A in children with OBPI emphasized the need for randomized controlled trials to determine its benefits and efficacy in order to support the continued use of this intervention in managing muscle imbalance and muscle cocontraction in children with OBPI [104].

8. BTX in Sialorrhea

Since BTX also inhibits the release of presynaptic acetylcholine at the neurosecretory junctions of the salivary glands, it has been proposed as a possible efficacious pharmacological treatment for hypersalivation and sialorrhea, which can occur and complicate the course and management of some severe neurological diseases such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and CP. Indeed, numerous studies have demonstrated that BTX-A and BTX-B are effective and safe for the reduction of drooling in patients with ALS and PD [105–108]. The injected doses into salivary glands is variable depending on the disease, the BTX used, and the clinician's experience. The mean global doses injected into salivary glands ranged from 55 U to 200 U (Botox) [109, 110] and from 250 U to 450 U (Dysport) [111, 112] for BTX-A and from 2500 to 4000 U for BTX-B [106, 108, 113]. A recent research study comparing the two toxins in controlling sialorrhea of ALS and PD patients reported that either 250 U BTX-A (Dysport) or 2500 U BTX-B (Neurobloc) have similar effectiveness and safety [6].

In children with CP, drooling and sialorrhea have an incidence of 10% to 37% [114]. These symptoms can have a devastating effect on the family's social relationships and the patient's quality of life. Several studies have demonstrated that BTX-A can be used with success in controlling sialorrhea in children with CP [115, 116]. A mean dose of BTX-A (Botox) ranging from 2 to 22.5 U/kg of body weight per single gland has been injected [117–119]. BTX-B can also be a safe and effective therapy for the treatment of drooling in these children. A recent randomized trial comparing three doses of 1500 MU, 3000 MU, and 5000 MU BTX-B injection into the salivary glands with ultrasound guidance [10] reported that the 3000 MU injection of BTX-B significantly improved the frequency and severity of sialorrhea in those children. The lower dosage was ineffective, and the higher dosage produced no greater benefit and more side effects. It has been proposed that BTX-B is more effective and could have a more enduring effect than BTX-A on autonomic function [10, 106]. Indeed, autonomic side effects are sometimes observed far from the injection site (such as dry mouth) after treatment for axillary hyperhidrosis with BTX-B [120]. As previously mentioned, BTX-B has a tendency to produce more autonomic adverse events than BTX-A [5, 8, 121, 122], mainly due to the hypothesized affinity for postganglionic neurons containing M3 receptors (such as those responsible for salivation) [123]. However, the study of Guidubaldi et al. found that that BTX-B had a shorter latency than BTX-A and comparable duration [6]. The most relevant finding was that BTX-B had a significantly shorter latency than BTX-A (3 versus. 6 days). The different latencies

might be due to various characteristics of the two serotypes, perhaps diffusion and/or affinity for autonomic fibers.

9. BTX Use in Rare Rehabilitative Clinical Conditions

Neurologically disabled subjects can present with complex dysfunction and clinicians have to face unique and difficult to treat clinical conditions. BTX can be a useful therapeutic tool in some of these conditions. Anecdotal reports have been published concerning the use of BTX-A in specific rare conditions such as sustaining posture after surgery in patients with cervical disk herniation, secondary to dystonic cerebral palsy [124]. BTX-A has been used to hasten the healing of lower lip ulcers due to oromandibular dyskinesia in a subject in a vegetative state following a severe subarachnoid hemorrhage [125]. Likewise, BTX-A treatment was used to hasten the healing of a buttock pressure sore in a subject with severe spastic paraplegia following a traumatic spinal cord lesion. In this last case, several therapeutic agents were applied without success, since all efforts at healing the ulcer by topical medication were hampered by recurrent spasms involving the gluteal muscles and the ulcer region [126]. Gluteal injections of 660 U BTX-A (Dysport) reduced the movement disorder and improved buttock ulcer healing.

10. Adverse Events

Before performing BTX-A injections for therapeutic purposes, the expected risks and benefits for each patient must be carefully considered. Currently, dosages are largely titrated by the practitioner based on the previously mentioned criteria and the individual patient's response. Reported adverse events associated with BTX are infrequent and predominantly concern the BTX-A formulation. Local and remote effects following BTX injections have been described. The former consisted in a reaction at the injection site, including pain, rush, and edema, whereas remote effects are due to diffusion of toxin and cause variable effects characterized by autonomic, regional, or systemic muscular weakness. Most adverse events after BTX treatment arise through weakness of the muscles injected or those nearby, which become weak through local spread of the toxin. Allergic or possible immune-mediated mechanisms have been proposed to be the cause of symptoms such as general malaise, fever, and skin rush.

Observed adverse events include nausea, urinary incontinence, falls, seizures, fever, dry mouth, and dysphagia [127]. These disturbances have often been found in patients with preexisting comorbidities, for example, seizures in subjects with previous epileptic disorders. General malaise and "flu-like" symptoms have also been described [42]. Generally, they are mild to moderate and transient. A pooled analysis including 792 patients concluded that nausea was the most frequent minor adverse event in poststroke patients treated with BTX-A, affecting 2.2% of cases [127]. No serious adverse event was reported in a recent systematic review regarding BTX-A use in adults with poststroke spasticity [7]. Conversely, because children receive higher doses per kilogram than adults, they can develop more adverse events. A variable incidence of side effects ranging from 4% to 7% has been reported [40, 128, 129]. In a previously mentioned paper, 28.5% of CP children who were injected with 5000 MU of BTX-B for sialorrhea developed generalized weakness and severe dysphagia requiring hospitalization and nasogastric tube feeding [10]. A very infrequent systemic effect manifested was generalized weakness distant from the site of injection [130]. A recent review of cases described in the literature indicates that risk of developing systemic effects does not seem to be related to dose based on body weight [131]. It may be more likely that risk for this condition is related to the total injection dose and injection frequency. Doses greater than 600 units of Botox with follow-up injections occurring every 3 months may lead to an increased risk of developing severe adverse events. Repeated contralateral weakness and fatigue after high doses of BTX-A injection for poststroke spasticity have been also described [132].

11. Neutralizing Antibodies

BTX-A effects can be abolished by the development of neutralizing antibodies (NAbs). Antibody formation against BTX proteins is one of the reasons for therapy failure. Studies have demonstrated that antibodies-binding toxins, specifically in the region responsible for entry into neurons, neutralize or inactivate the toxin. In order to overcome therapy failure, injecting increased BTX doses with short injection intervals and using different BTX serotypes [133, 134] has been suggested. This phenomenon is reported with a variable incidence according to the treated disorder. In cerebral palsy, the incidence of NAbs has been reported in a range from 6% to 31% [128, 135, 136]. NAbs are rare in poststroke spasticity. In a sample of 235 poststroke patients with spasticity receiving a dose ranging from 100 to 400 MU of BTX-A, Yablon et al. [137] found <0.5% of NAbs. The development of NAbs are facilitated if repeated injections and high dosages of BTX are used independently from the treated disorder. However, NAbs are more frequent in patients with cervical dystonia compared to other hyperactive muscular disturbances. The development of NAbs has been also observed in subjects who underwent BTX injections for nonmotor disorders such as sialorrhea. Although no BTX-A resistance in the treatment of sialorrhea has yet been reported, this disappointing phenomenon has recently been described for BTX-B after repeated injection into the salivary glands [138].

12. Considerations

Muscle selection is a key feature for the efficacy of BTX treatment, and the infiltration modalities are a further source of heterogeneity. BTX-A injections are more efficacious if the muscles are targeted by needle EMG or ultrasound guidance. There is evidence from dystonia that EMG targeting increases accuracy and improves outcome [139].

However, when high doses are injected into sufficiently large muscles, as in spasticity, toxin diffusion compensates for this limitation. Salivary glands are generally injected by ultrasound guidance. A drawback for BTX therapy is its high cost and the transient nature of the toxin. In this respect, recent papers have reported that the clinical benefits of BTX-A treatment outweigh the apparent high costs of this intervention, showing it to be a cost-effective treatment [13, 42].

13. Conclusions

Botulinum toxin types A and B are valuable agents in the multiple therapeutic strategies that clinicians carry out in a neurorehabilitation setting. It is important to strive to attain the best clinical and functional benefit that improves the quality of care of patients undergoing rehabilitation. Since neurologically disabled subjects present complex dysfunction, prior to initiating BTX therapy, specific functional limitations, goals, and expected outcomes of treatment should be discussed with the patient and caregiver. Muscle selection and the order and priority of treatment should be tailored to the treatment of spasticity and muscular imbalance. BTX-A and BTX-B strategies should be viewed as adjunct measures based on the individual's residual ability, and competence and tailored rehabilitation programs are needed to achieve the best functional outcome. Although BTX-A treatment has been demonstrated safe and effective in managing several neurological disorders, many questions still remain unsolved. Future studies should address if this agent alone or as an adjunct to other rehabilitative procedures optimizes functional outcome.

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