Localized tetanus in immunized mice

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ABSTRACT

The capacity of tetanus toxin to enhance motor neuron excitability has suggested its potential use as a therapeutic. Widespread active vaccination against tetanus in all developed countries is considered the major obstacle to clinical use of the toxin. We wished to determine the response to localized intramuscular injection of tetanus toxin in both passively and actively immunized animals as an initial exploration into the possible use of tetanus toxin as a clinical therapeutic. Unvaccinated mice (n = 18) underwent intramuscular injection of tetanus toxin into the gastrocnemius muscle (0.2 ng, 1 ng, 5 ng). All animals in the lowest dose group developed only local tetanus of the injected limb of at least 2 weeks duration, while all animals in the higher dose groups also rapidly developed generalized tetanus and were euthanized. Another group of mice (n = 20) received anti-tetanus immunoglobulin (20–40 IU) at the time of toxin injection. These animals although dramatically resistant to the toxin developed predominantly local tetanus for over one month at doses of 2.5 µg and 5.0 µg. A third group of mice (n = 30) underwent active vaccination with tetanus toxoid to induce protective anti-tetanus immunity and then was challenged with high dose toxin injection (5 ng, 50 ng, 0.5 µg, 1.25 µg, 2.5 µg, or 5 µg). All animals developed local tetanus in the injected limb at a dose of at least 0.5 µg. The severity and duration of local tetanus was generally related to dose, but was more variable in the actively vaccinated group than in the naive or passively immunized animals. Response to the toxin over the first few days was predictive of both the duration and maximal severity of the motor response. Although vaccination dramatically increases resistance to tetanus toxin, by virtue of its extremely high potency, the toxin can produce prolonged localized tetanus even in vaccinated animals with relatively small amounts of protein. These results suggest the possible use of tetanus toxin to enhance local motor activity in a variety of neurologic conditions even in immunized humans. This study in uniformly vaccinated animals also illustrates the potential difficulties in determining an appropriate dose of toxin in a human population with variable degrees of immunity.

1. Introduction

Tetanus toxin has a unique mechanism of action that has direct impact for potential therapeutics (Bleck, 1991). Tetanus toxin, unlike the related and widely used therapeutic botulinum neurotoxin, is transported by motor neurons to the spinal cord (Schwab et al., 1979). In the spinal cord, tetanus toxin is then transferred to inhibitory presynaptic terminals surrounding those motor neurons. There the toxin destroys one of the SNARE proteins, vesicle associated membrane protein (VAMP, also known as synaptobrevin), resulting in inactivation of the inhibitory neurotransmission that normally suppresses motor neuron and muscle activity. This action results in enhanced excitability and activation of the affected motor neurons. Although clinical intoxication with tetanus toxin frequently results in widespread and continuous involuntary muscle contractions that characterize generalized tetanus, intramuscular injection of tetanus toxin can result in a localized state of muscle hyper excitability and contraction. Severe localized tetanus shows sustained contraction of the injected muscles, while injection of lower doses of toxin result in enhanced activation and contraction of the targeted muscles during attempts at voluntary movement both in experimental animals and humans with local tetanus (Struppier et al., 1963, Webster and Laurence, 1963). Such observations support the hypothesis that intramuscular injection of an appropriate dose of tetanus toxin could produce a state of hyper-excitability and over activity in a targeted population of motor neurons.
While a large number of agents including the botulinum neurotoxins are capable of reducing motor neuron or muscular activity, tetanus toxin is the only substance described that has the potential for selective enhancement of motor activity (Fishman, 2009, Goonetilleke and Harris, 2004, Benecke et al., 1977). Sanders has described potential uses of tetanus toxin for a wide range of disorders of inadequate muscle tone, ranging from sleep apnea to flaccid paralysis after stroke to even cosmetic use (Sanders, 2004). At this point the only published attempt at therapy is injection of tetanus toxin into pharyngeal muscles in a single bulldog resulting in improvement of sleep apnea without any observable adverse effects (Sasse et al., 2005). This observation raises the possibility that tetanus toxin could be dosed in a manner to produce enhancement of voluntary motor neuron activation without the widespread involuntary muscle activation typically seen in clinical tetanus. The hope is that dosing of tetanus toxin could be developed in an analogous manner to botulinum toxin, where the goal is localized reduction of muscle activation rather than flaccid paralysis.

Although one can envision the use of tetanus toxin to enhance inadequate muscle activity in a manner analogous to the widespread clinical use of the related clostridial toxin botulinum neurotoxin, there has been little consideration of the possible clinical utility of tetanus toxin. This disregard can be traced to the view that anti-tetanus antibodies, present in the vast majority of individuals in developed countries, would prevent the biological action of the toxin (Johnson, 1999). In contrast to the clinical use of botulinum toxin, the vast majority of individuals in developed countries have been vaccinated to prevent the occurrence of clinical tetanus. Vaccination with tetanus toxoid (formaldehyde denatured toxin) is well established to prevent clinical tetanus (Bleck, 1991). However, protection by vaccination from clinical tetanus is not absolute in humans or experimental animals. There have been several reports of clinical tetanus in vaccinated individuals even in the presence of what would be considered protective levels of anti-tetanus antibodies by ELISA (Berger et al., 1978, Passen and Andersen, 1986, Risk et al., 1981). Local effects of tetanus toxin on the obicularis oculi muscles have also been demonstrated in rabbits who received passive immunization with anti-toxin at the time of toxin injection (Fezza et al., 2000). This study however utilized a model of cephalic tetanus, an unusual anti-toxin at the time of toxin injection (Fezza et al., 2000). This observation raises the possibility that tetanus toxin could be dosed in a manner to produce enhancement of voluntary motor neuron activation without the widespread involuntary muscle activation typically seen in clinical tetanus. The hope is that dosing of tetanus toxin could be developed in an analogous manner to botulinum toxin, where the goal is localized reduction of muscle activation rather than flaccid paralysis.

2. Materials and methods

Injection of tetanus toxin (List Laboratories) was performed into the gastrocnemius muscle of adult male mice (C57/BL6, male, Jackson Labs). The toxin was reconstituted in phosphate buffer and injections were made with a microliter syringe (Hamilton 30G needle) with a volume of 5 μl while under isoflurane inhalation anesthesia per an approved protocol of the IACUC of the University of Maryland. All but the highest doses of toxin utilized a standard commercially available preparation that was intended to be reconstituted at a minimum dilution of 2.5 μg/25 μl. The manufacturer also kindly provided us with a second preparation of toxin designed for reconstitution at 10 μg/25 μl. This preparation is clearly soluble at a concentration of 5 μg/5 μl, and was utilized for the two highest doses in the study (2.5 μg/5 μl and 5 μg/5 μl). Animals were observed daily for any signs of distress.

Animals showing clear signs of generalized tetanus such as hyperextension posturing of the spine were euthanized (100 mg/kg pentobarbital). Clinical tetanus was evaluated using a motor behavior scale modified from Webster and Laurence, 1963 where 5 = generalized tetanus, 4 = sustained localized limb tetanus characterized by extension at the ankle and toes, 3 = intermittent spontaneous limb tetanus, 2 = limb tetanus consistently evoked on attempted limb movement and usually involving the entire limb, and 1 = limb tetanus that involved only part of limb (usually toe extension/spreading) or that was observed inconsistently with movement. Mice were videotaped during spontaneous walking and during attempts to grasp onto a wire platform when lifted by the tail. Tapes were scored by a rater blinded to any information about the toxin injection or vaccination status of the animal.

Passively immunized mice received human hyper immune anti-tetanus immunoglobulin (TIG, Talecris Biotherapeutics) by intraperitoneal (IP) injection at the time of toxin injection. Actively vaccinated mice received tetanus toxoid (Sanovis Aventis) by intraperitoneal injection with 1/50th of the recommended human dose in 0.1 ml of saline and underwent a second (booster) vaccination 30 days later (30 days prior to tetanus toxin injection). Sera for determination of anti-tetanus titers were obtained at the time of euthanasia. Anti-tetanus titers were performed by ELISA in a modification of a previously published protocol (Fairweather et al., 1987). Briefly, plates were coated with tetanus toxoid overnight and were then incubated with dilutions of sera from vaccinated or unvaccinated mice with a starting dilution of 1/50. Final titers were calculated by logarithmic plot of dilution versus optical density at a level of three times control (saline).

3. Results

3.1. Unvaccinated (naive) mice

Unvaccinated (naive) mice (n = 6 per dose group) were injected with one of three doses of tetanus toxin (0.2 ng, 1 ng, 5 ng). Motor responses are summarized in Fig. 1. All mice injected with the two higher doses developed generalized tetanus and died or were euthanized. Animals injected with the highest dose group developed severe localized tetanus and signs of generalized tetanus within 24 h of toxin injection, while animals receiving the mid-dose developed signs of generalized tetanus at a slightly longer duration after injection (2–4 days). All of the animals receiving the lowest dose developed localized tetanus of at least 2 weeks duration, with no animal in the low dose group developing generalized tetanus.

3.2. Passively immunized mice

Passively immunized mice (n = 5 per dose group) were injected with one of the following protocols: (1) 1.25 μg toxin (IM) and 20 IU TIG (IP), (2) 2.5 μg toxin with 20 IU TIG, (3) 5.0 μg toxin and 20 IU TIG and (4) 5.0 μg toxin and 40 IU TIG. All of the animals receiving the highest dose of toxin (5.0 μg) and the lower dose of TIG (20 IU) developed severe generalized tetanus within 24–48 h and were euthanized. The motor responses of the other three dose groups are shown in Fig. 2. All animals developed prolonged localized tetanus with some degree of generalization within the first 1–2 weeks. The dose response of animals within each group was highly consistent. Protection from the effects of toxin was substantial with animals surviving doses of toxin more than 2000 fold a uniformly lethal dose in naive animals. Some animals in the highest dose group of both toxin and anti-toxin were allowed to survive with generalized tetanus of an unusual appearance. These animals had severe but unilateral localized tetanus with curvature of the spine toward the injected side. Lack of involvement of the
contra lateral hind limb was not seen in any naïve animals with comparable severity of localized motor signs.

3.3. Actively vaccinated mice

Actively vaccinated mice (n = 5 per dose group with one anesthesia related death in the 1.25 μg group) were challenged with intramuscular injection of toxin of 5 ng, 50 ng, 0.5 μg, 1.25 μg, 2.5 μg, and 5.0 μg of toxin. No vaccinated animals showed any signs of localized or generalized tetanus at doses at doses lower than 0.5 μg. All five animals given a dose of 0.5 μg had mild transient localized tetanus and the motor responses of the highest three groups are summarized in Fig. 3. Actively vaccinated mice were also dramatically resistant to the toxin with only one animal in each of the two highest dose groups developing generalized tetanus. Unlike the unvaccinated and passively immunized animals, actively immunized mice had a more variable response to the same dose of toxin, although this variability was less apparent at the higher dose groups, with all animals showing moderate to severe localized tetanus of a prolonged duration. All animals had protective anti-tetanus antibody levels by ELISA (mean titer 1:20,000, range of titers 1:8000–1:46,000).

Different vials of toxin were used in the three groups of animals (naïve, passive immunization and active immunization) as well as two different batches between the two higher doses and other doses. A group of 5 naïve mice were injected intramuscularly with 5 ng of toxin from each new vial to confirm its level of activity, with all animals developing generalized tetanus within 24 h.

4. Discussion

We now report the first study evaluating the local response to tetanus toxin of animals that have been actively vaccinated against the toxin in a manner comparable to clinical practice in humans and compare them to both naïve animals and animals passively immunized with TIG. In our limited dose ranging study, unvaccinated (naïve) mice showed typical and consistent responses to the toxin ranging from local limb tetanus to generalized tetanus and death. In contrast, both passively and actively immunized animals showed dramatic protection from lethal effects of the toxin with only 2 out of 40 animals developing generalized tetanus at doses that ranged up to 5000 times a uniformly lethal dose in unvaccinated animals. Vaccinated mice were also much more resistant to the localized effects of the toxin, showing signs of local toxin action beginning at doses 500 times a uniformly lethal dose for unvaccinated mice.

These results support the concept that it is possible to produce prolonged localized tetanus which is likely on the basis of changes in motor neuron excitability even in a population that is either passively or actively immunized. This study also identified what is likely the major obstacle to any potential clinical use of the toxin: the variability in the relationship of dose to clinical response particularly in actively vaccinated animals. Our data illustrates variability in response even in a highly uniform population (inbred strain, uniform vaccination and toxin injection protocol). The target clinical population will have a much more variable degree of immunity to the toxin than these experimental animals, ranging from fully and recently immunized individuals to those with inadequate or remote vaccination. Overdosage with intramuscular tetanus toxin has the potential to result in generalized tetanus while underdosage would preclude any potential benefit. However, vaccination against tetanus toxin is one of the most well studied aspects of vaccination immunology, where previous literature provides us with many tools to help establish a clinically useful relationship between toxin dose, immune status of the individual and a physiologic effect of the toxin. These include not
only several established immunoassays, but functional bioassays such as the mouse protection assay.

The results of this study are highly consistent with, but extend the previous literature on the action of protein neurotoxins in immune animals and humans. Injections of hyperimmun serum against botulinum neurotoxin A into muscles adjacent to the site of toxin injection resulted in a more localized response to the toxin in human extraocular muscles (Scott, 1988). A study of the plant derived toxin ricin demonstrated that rats that received anti-ricin antibody at the time of toxin injection into the sciatic nerve, showed death of local motor neurons through a retrograde transport of toxin. These animals were, however, protected from systemic poisoning by a dose of ricin that was several fold greater than the lethal amount in naive animals (Wiley and Oeltmann, 1989).

The only previous study of tetanus toxin related to this issue used a model of cephalic tetanus where the toxin causes flaccid muscular weakness rather than typical spastic tetanus seen in limbs, and showed a localized muscle effect in spite of immunity (Fezza et al., 2000). Injection of tetanus anti-toxin in this study was again successful in reducing remote effects of the toxin while allowing local action in the injected muscle. Cephalic tetanus is a distinct syndrome from focal tetanus of a limb evoked in our current study (Jagoda et al., 1988). Cephalic tetanus in humans as well as animals frequently shows a flaccid paralysis which has evidence for blockade of synaptic activity at both the level of the brainstem and at the level of the neuromuscular junction (Garcia-Mullin and Daroff, 1973, Gonzalez-Forero et al., 2005). Focal limb tetanus has been much more intensively studied where neuromuscular junction blockade occurs only at relatively high doses and is not a pre-dominant part of the physiologic action of the toxin. We did not observe clinical signs of neuromuscular blockade such as a flaccid paralysis that have been previously described with high dose of IM tetanus toxin into limb muscles. Our observations are very consistent with the consensus view of presynaptic inhibitory terminals onto motor neurons in the spinal cord as the primary site of action of the toxin. All of these previous studies utilized a passive immunization paradigm in their assessment of immune effects on the toxin's action.

These previous studies along with our current report not only support the conclusion that local action of a toxin can occur in spite of immunity but also suggest mechanisms by which anti-toxin may actually enhance the safety of local use of a toxin. Toxin that is rapidly internalized into local synaptic terminals after intramuscular injection becomes inaccessible to antibodies, accounting in some part for the prolonged effects of protein toxins. Circulating antibodies, by neutralizing toxin that has not been locally internalized, may decrease the amount of active toxin that leaks from the injected muscle reducing unwanted effects on nearby and remote sites.

Widespread human vaccination with tetanus toxoid and resulting active immunity is in the setting that presents the true challenge to the possible clinical use of tetanus toxin. Published preclinical and clinical literature also support the conclusion that active vaccination and immunity against tetanus toxin, although highly effective in preventing clinical tetanus, is not absolute. Effective titers in actively vaccinated animals typically provide protection from at least 50–100 times a lethal dose of toxin (Chargelegue et al., 2005). There are several reports of clinical tetanus in individuals with not only a history of active vaccination, but also with anti-tetanus antibody titers usually associated with protection from the toxin (Berger et al., 1978, Passen and Andersen, 1986, Crane and Reder, 1992).

There are potential strategies to develop a more consistent dose response to tetanus toxin injection in spite of the variability in immunoresistance of the population. One strategy would be to determine the relationship between some laboratory measure of immunoresistance and the physiological effect of a specific dose of toxin. Although we measured anti-tetanus antibodies by ELISAs as part of this study, this assay was not designed to address this issue. ELISAs were performed on sera obtained at the time of euthanasia, well after toxin injection with its potential anti-tetanus booster effect. The appropriate setting to determine any correlation between anti-tetanus antibody titers and the response to the toxin would be from sera obtained immediately prior to toxin injection. In this preliminary study ELISAs were performed only to ensure that all animals were adequately vaccinated, while minimizing anesthesia and manipulation of study animals during life. Whether any correlation exists between anti-tetanus titers and physiological immunoresistance remains a question for later study with a greater number of animals in each dose group. In the current study ELISA was performed using tetanus toxoid as the antigen. Although this is a common practice in many human and animal studies for both safety and cost reasons, it may also confound any comment regarding antibody titer and clinical response. Antibodies directed against the binding domain of the toxin are most strongly correlated with immunity to the toxin. Another assay of anti-tetanus immunoresistance in this setting that may correlate with immunoresistance is a functional bioassay of protection called the mouse neutralization assay (MNA). The anti-tetanus titer determined in this assay by the extent to which the patient's sera can be diluted, then mixed with a standard amount of toxin, and protect the mouse from tetanus toxin induced death after injection of this mixture (Christiansen, 1981).

In general there is a correlation between anti-tetanus immunity by ELISA and by mouse neutralization assay (Melville-Smith et al., 1983, Hagenaaers et al., 1984). As noted earlier there have been several patients who developed clinical tetanus in spite of “protective” levels of anti-tetanus titers by ELISA or other in vitro assays (Berger et al., 1978, Passen and Andersen, 1986, Crane and Reder, 1992).

In spite of the variability in the dose response relationship of actively vaccinated animals, our observation of the time course of local and generalized tetanus provide us with very useful information to devise future protocols in actively vaccinated animals. Specifically, the response to the toxin within the first few days was highly predictive of the overall response-duration profile for individual mice regardless of their vaccination status. It may be possible to consistently induce long-lasting localized tetanus without generalized tetanus using a series of repeated injections with escalating doses of toxin, followed by an observation period such as 1–2 weeks between injections to guide any further dose escalation.

For most protein based therapeutics the degree of immunoresistance demonstrated in this study would practically preclude any further clinical use (Chance et al., 1976, Farrell and Giovannoni, 2007). Clostridial neurotoxins are however the most potent biologic toxins known, where effective doses of tetanus toxin in vaccinated animals are increased over naive animals from the fraction of a nanogram range to only in the single microgram range, a dose that is easily attainable with currently available preparations. Although a similar situation may occur with botulinum neurotoxin, it is designed for use in immunologically naive individuals, so that development of neutralizing antibodies associated with declining efficacy make continued treatment with currently available commercial preparations impractical.

The maximal duration of the local response observed even in immune mice was over one month, and is compatible with earlier literature in animals and humans where symptoms and signs of tetanus intoxication can last from a few weeks to many months (Risk et al., 1981, Struppler et al., 1963). In agreement with earlier experimental studies and human cases, localized tetanus of at least a moderate degree is well tolerated. Mice that developed only
localized tetanus showed no signs of distress, and demonstrated normal behavior except for the unusual posture of the injected limb.

These observations clearly support the need for continued preclinical investigation of this biologic toxin with a unique mechanism of action. While a large number of agents including the botulinum neurotoxins are capable of reducing motor neuron activity, tetanus toxin is the only substance described that has the potential for selective enhancement of motor activity (Brooks et al., 1957). Studies of the clinical use of botulinum toxin in the setting of post-stroke spasticity are particularly illustrative of both the utility of a targeted anti spasticity treatment, and the need for a complimentary agent that enhances rather than reduces motor activity. Anti-spasticity treatments such as botulinum neurotoxin result in reduction of muscle activity, tone, improvement in limb posture, but infrequently result in improvement in use of the limb (Simpson et al., 2008, Sheean, 2006). Several physiologic studies support the hypothesis that inability to voluntarily activate affected muscles rather than overactivity of spastic antagonist muscles is the major source of disability in the large number of patients with so-called upper motor neuron weakness, particularly after stroke (Fellows et al., 1994, Newham and Hsiao, 2001, Horstman et al., 2008).

Although muscles with inadequate activation can be readily identified in these patients, there is no current medical therapy to enhance their activation. Our working hypothesis is that affected muscles could be specifically injected with an appropriate dose of tetanus toxin, potentially enhancing voluntary contraction of the target muscles resulting in an increase in strength. The clinical and electrophysiological studies in human with localized tetanus of Strupppler and Adams suggest that intramuscular tetanus can amplify the activity of affected motor neurons. In patients with mid to moderate localized tetanus, spasms were evoked only with attempts at voluntary movement, with associated excessive activity on EMG (Strupppler et al., 1963). At this time there are no studies of localized tetanus in any setting of neurologic dysfunction. It remains to be determined whether tetanus toxin can reduce the activation threshold of target motor neurons with out producing extensive involuntary discharges characteristic of localized tetanus observed in this study. The goal of use of the toxin to enhance or amplify voluntary movement has implications for the large number of patients with motor disability due to conditions such as brain or spinal cord injury, stroke or multiple sclerosis.

Our current study combined with the previous literature support the conclusion that immunity against tetanus toxin can be overcome with a sufficiently high dose of toxin. This conclusion places the previous assumption, that widespread human vaccination against the toxin would make clinical use of tetanus toxin unfeasible, under serious challenge. Only further study will determine the potential clinical utility of tetanus toxin for a wide range of motor disorders.

Conflict of interest

None.

References


Newham DJ, Hsiao SF. Knee muscle isometric strength, voluntary activation and antagonist co-contraction in the first six months after stroke. Disabil Rehab 2001;23(9):379–86.


