Botulinum toxin for use in the treatment of acute injuries to skeletal muscles
Botulinum Toxin zur Behandlung von akuten Verletzungen der Skelettmuskeln
Toxine botulinique pour son utilisation dans le traitement de lésions aigues des muscles squelettiques

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muscle: intramuscular and intermuscular hematomas. Two different types of hematomas occur in the injured muscle by administration of a botulinum toxin to the injured muscle. [0002] Acute injuries to skeletal muscles include contusions (bruises), lacerations, ischemia, strains, and complete ruptures. These injuries may cause tremendous pain and can incapacitate the affected person, preventing them from being able to go to work or even to participate in normal daily activities. Of the acute injuries to skeletal muscles, strain (also known as stretch-induced injuries) is most common. For example, strains can account for up to 30% of all injuries treated by occupational or sports medicine professionals. Garret et al. Am J Sports Med, 24(6):S2-S8, 1996. [0003] A muscle strain injury is characterized by a disruption of a muscle-tendon unit. The disruption of the muscle-tendon unit may occur anywhere on the muscle. This type of injury most commonly occurs near the musculotendinous junction (MTJ) of the superficial muscles working across two joints, such as the rectus femoris, semitendinosus and gastrocnemius muscles. [0004] Muscle strain may result from an eccentric exercise, or uncommon use of the muscle. For example, eccentric contractions employ fewer active motor units to generate higher forces. In such case, the over-extended muscle units experience excessive tension during lengthening. The excessive tension may cause microscopic damages to the contractile element of the muscle, centering on what appears to be random disruptions of the Z-lines. When the muscle is damaged, the affected person may experience a delayed onset muscle soreness, characterized by pain, weakness and a limited range of motion. The pain is most intense for about 1 to 2 days after the muscle injury and the weakness and limited range of motion can last for a week or more. If a minor strain of the skeletal muscles is treated inappropriately, a more serious injury can occur. [0005] There are three classifications of muscle strains, based on the severity of the injury and the nature of the hematoma: (1) mild, (first degree) strain; a tear of a few muscle fibers; minor swelling and discomfort with no or only minimal loss of strength and restriction of movement; (2) moderate, (second degree) strain; a greater damage of muscle fibers with a clear loss of strength, and; (3) severe (third degree) strain; a tear extending across the whole muscle belly, resulting in a total loss of muscle function. [0006] Tearing of the intramuscular blood vessels during muscle strain can often result in a large hematoma. Two different types of hematomas occur in the injured muscle: intramuscular and intermuscular hematomas.

The first type, intramuscular hematomas, is limited in size by the intact muscle fascia. There, the extravasation of blood increases the intramuscular pressure, compressing and limiting the size of the hematoma. Such type of hematoma causes pain and loss of function of the muscle. The second type, intermuscular hematomas, develops when the muscle fascia is ruptured and extravasated blood spreads into the intermuscular spaces without significantly increasing the pressure within the muscle. This type of hematoma may not cause significant pain if the pressure within the muscle does not increase. [0007] For treatments of strain injuries, it is critical that the injured muscle be immobilized, especially during the first two to three days after the injury, since mobilization of the injured muscles immediately after the injury often causes re-rupturing at the original injury site. A re-rupturing may lead to more severe injuries, delayed healing and scarring of tissues. Jarvinen et al., Curr Opin Rheumatol, vol 12:155-161 (2000). [0008] Re-rupturing of the damaged site may be avoided by immobilizing the injured muscle, preferably immediately after the injury. Immobilization allows the newly formed granulation tissue to reach sufficient tensile strength to withstand the forces created by contracting muscle. [0009] A known method for immobilization of an injured/strained muscle requires use of a physical restraint or cast. For example, a cervical collar can be used to immobilize an injured cervical flexor or extensor. However, the use of a restraint is often cumbersome and uncomfortable. Moreover, for injuries of certain muscle groups, it is not practical or possible to use a physical restraint. For example, it is very difficult to immobilize a strained upper trapezius or gluteus maximus muscle with a restraint.

Botulinum toxin [0010] The anaerobic, gram positive bacterium Clostridium botulinum produces a potent polypeptide neurotoxin, botulinum toxin, which causes a neuropahtyic illness in humans and animals referred to as botulism. The spores of Clostridium botulinum are found in soil and can grow in improperly sterilized and sealed food containers of home based canneries, which are the cause of many of the cases of botulism. The effects of botulism typically appear 18 to 36 hours after eating the foodstuffs infected with a Clostridium botulinum culture or spores. The botulinum toxin can apparently pass unattenuated through the lining of the gut and attack peripheral motor neurons. Symptoms of botulinum toxin intoxication can progress from difficulty walking, swallowing, and speaking to paralysis of the respiratory muscles and death. [0011] Botulinum toxin type A ("BoNT/A") is the most lethal natural biological neurotoxin known to man. About 50 picograms of botulinum toxin (purified neurotoxin complex) serotype A is a LD$_{50}$ in mice. One unit (U) of
botulinum toxin is defined as the LD50 upon intraperitoneal injection into female Swiss Webster mice weighing 18-20 grams each. Seven immunologically distinct botulinum neurotoxins have been characterized, these being respectively botulinum neurotoxin serotypes A, B, C1, D, E, F and G each of which is distinguished by neutralization with serotype-specific antibodies. The different serotypes of botulinum toxin vary in the animal species that they affect and in the severity and duration of the paralysis they evoke. For example, it has been determined that BoNT/A is 500 times more potent, as measured by the rate of paralysis produced in the rat, than is botulinum toxin serotype B (BoNT/B). Additionally, BoNT/B has been determined to be non-toxic in primates at a dose of 480 U/kg - which is about 12 times the primate LD50 for BoNT/A. Botulinum toxin apparently binds with high affinity to cholinergic motor neurons, is translocated into the neuron and blocks the release of acetylcholine.

[0012] Botulinum toxins have been used in clinical settings for the treatment of neuromuscular disorders characterized by hyperactive skeletal muscles. BoNT/A has been approved by the U.S. Food and Drug Administration for the treatment of blepharospasm, strabismus and hemifacial spasm. Non-serotype A botulinum toxin serotypes apparently have a lower potency and/or a shorter duration of activity as compared to BoNT/A. Clinical effects of intramuscular injection of a botulinum toxin do, such as BoNT/A, can be noted in a matter of hours. Thus, it is important to note that most if not all of the botulinum toxins can, upon intramuscular injection, produce significant muscle paralysis within one day of the injection, as measured, for example, by the mouse Digit Abduction Score (DAS). Aoki K. R., Preclinical Update on BOTOX (Botulinum Toxin Type A)-Purified Neurotoxin Complex Relative to Other Botulinum Toxin Preparations, Eur J. Neu 1999, 6 (suppl 4):S3-S10. The typical duration of symptomatic relief from a single intramuscular injection of BoNT/A averages about three months. Botulinum toxins, including botulinum toxin type A, with reduced periods of in vivo biological activity are set forth in co-pending U.S. patent application serial number 09/620840, which application is incorporated herein by reference in its entirety.

[0013] Although all the botulinum toxins serotypes apparently inhibit release of the neurotransmitter acetylcholine at the neuromuscular junction, they do so by affecting different neurosecretory proteins and/or cleaving these proteins at different sites. For example, botulinum serotypes A and E both cleave the 25 kilo Dalton (kD) synapticosomal associated protein (SNAP-25), but they target different amino acid sequences within this protein. BoNT/B, D, F and G act on vesicle-associated protein (VAMP, also called synaptobrevin), with each serotype cleaving the protein at a different site. Finally, botulinum toxin serotype C1 (BoNT/C1) has been shown to cleave both syntaxin and SNAP-25. These differences in mechanism of action may affect the relative potency and/or duration of action of the various botulinum toxin serotypes.

[0014] Regardless of serotype, the molecular mechanism of toxin intoxication appears to be similar and to involve at least three steps or stages. In the first step of the process, the toxin binds with high affinity to the presynaptic membrane of the target neuron through a specific interaction between the H chain and a cell surface receptor; the receptor is thought to be different for each serotype of botulinum toxin and for tetanus toxin. The carboxy end segment of the H chain, H20 appears to be important for targeting of the toxin to the cell surface.

[0015] In the second step, the toxin crosses the plasma membrane of the poisoned cell. The toxin is first engulfed by the cell through receptor-mediated endocytosis, and an endosome containing the toxin is formed. The toxin then escapes the endosome into the cytoplasm of the cell. This last step is thought to be mediated by the amino end segment of the H chain, HN, which triggers a conformational change of the toxin in response to a pH of about 5.5 or lower. Endosomes are known to possess a proton pump which decreases intra endosomal pH. The conformational shift exposes hydrophobic residues in the toxin, which permits the toxin to embed itself in the endosomal membrane. The toxin then translocates through the endosomal membrane into the cytosol.

[0016] The last step of the mechanism of botulinum toxin activity appears to involve cleavage of the critical intracellular exocytosis proteins by the L chain. The entire toxic activity of botulinum and tetanus toxins is contained in the L chain of the holotoxin; the L chain is a zinc (Zn++) endopeptidase which selectively cleaves proteins essential for recognition and docking of neurotransmitter-containing vesicles with the cytoplasmic surface of the plasma membrane, and fusion of the vesicles with the plasma membrane. Tetanus neurotoxin, botulinum toxin/B/D/F, and/G cause degradation of synaptobrevin (also called vesicle-associated membrane protein (VAMP)), a synapticosomal membrane protein. Most of the VAMP present at the cytosolic surface of the synaptic vesicle is removed as a result of any one of these cleavage events. Each toxin specifically cleaves a different bond.

[0017] The molecular weight of the botulinum toxin protein molecule, for all seven of the known botulinum toxin serotypes, is about 150 kD. Interestingly, the botulinum toxins are released by Clostridial bacterium as complexes comprising the 150 kD botulinum toxin protein molecule along with associated non-toxin proteins. Thus, the BoNT/A complex can be produced by Clostridial bacterium as complexes comprising the 150 kD botulinum toxin protein molecule along with associated non-toxin proteins. Thus, the BoNT/A complex can be produced by Clostridial bacterium as complexes comprising the 150 kD botulinum toxin protein molecule along with associated non-toxin proteins. Thus, the BoNT/A complex can be produced by Clostridial bacterium as complexes comprising the 150 kD botulinum toxin protein molecule along with associated non-toxin proteins. Thus, the BoNT/A complex can be produced by Clostridial bacterium as complexes comprising the 150 kD botulinum toxin protein molecule along with associated non-toxin proteins. Thus, the BoNT/A complex can be produced by Clostridial bacterium as complexes comprising the 150 kD botulinum toxin protein molecule along with associated non-toxin proteins.
may act to provide stability against denaturation to the botulinum toxin molecule and protection against digestive acids when toxin is ingested. Additionally, it is possible that the larger (greater than about 150 kD molecular weight) botulinum toxin complexes may result in a slower rate of diffusion of the botulinum toxin away from a site of intramuscular injection of a botulinum toxin complex.

[0018] In vitro studies have indicated that botulinum toxin inhibits potassium cation release of both acetylcholine and norepinephrine from primary cell cultures of brainstem tissue. Additionally, it has been reported that botulinum toxin inhibits the evoked release of both acetylcholine and glutamate in primary cultures of spinal cord neurons and that in brain synaptosome preparations botulinum toxin inhibits the release of each of the neurotransmitters acetylcholine, dopamine, norepinephrine, CGRP and glutamate.

[0019] BoNT/A can be obtained by establishing and growing cultures of Clostridium botulinum in a fermenter and then harvesting and purifying the fermented mixture in accordance with known procedures. All the botulinum toxin serotypes are initially synthesized as inactive single chain proteins which must be cleaved or nicked by proteases to become neuroactive. The bacterial strains that make botulinum toxin serotypes A and G possess endogenous proteases and serotypes A and G can therefore be recovered from bacterial cultures in their active form. In contrast, botulinum toxin serotypes C1, D and E are synthesized by nonproteolytic strains and are therefore typically inactivated when recovered from culture. Serotypes B and F are produced by both proteolytic and nonproteolytic strains and therefore can be recovered in either the active or inactive form. However, even the proteolytic strains that produce, for example, the BoNT/B serotype only cleave a portion of the toxin produced. The exact proportion of nicked to unnicked molecules depends on the length of incubation and the temperature of the culture. Therefore, a certain percentage of any preparation of, for example, the BoNT/B toxin is likely to be inactive, possibly accounting for the known significantly lower potency of BoNT/B as compared to BoNT/A. The presence of inactive botulinum toxin molecules in a clinical preparation will contribute to the overall protein load of the preparation, which has been linked to increased antigenicity, without contributing to its clinical efficacy. Additionally, it is known that BoNT/B has, upon intramuscular injection, a shorter duration of activity and is also less potent than BoNT/A at the same dose level.

[0020] It has been reported that BoNT/A has been used in clinical settings as follows:

1. About 75-125 units of BOTOX® per intramuscular injection (multiple muscles) to treat cervical dystonia;
2. 5-10 units of BOTOX® per intramuscular injection to treat glabellar lines (brow furrows) (5 units injected intramuscularly into the procerus muscle and 10 units injected intramuscularly into each corrugator supercilii muscle);
3. About 30-80 units of BOTOX® to treat constipation by intrasphincter injection of the puborectalis muscle;
4. About 1-5 units per muscle of intramuscularly injected BOTOX® to treat blepharospasm by injecting the lateral pre-tarsal orbicularis oculi muscle of the upper lid and the lateral pre-tarsal orbicularis oculi of the lower lid.
5. To treat strabismus, extraocular muscles have been injected intramuscularly with between about 1-5 units of BOTOX®, the amount injected varying based upon both the size of the muscle to be injected and the antigenic potential. Botulinum toxin preparations were injected into mouse digit abduction scoring assay (DAS). ED50 values were calculated from dose response curves. Additional mice were given intramuscular injections to determine LD50 doses. The therapeutic index was calculated as LD50/ED50. Separate groups of mice received hind limb injections of BOTOX® (5.0 to 10.0 units/kg) or BoNT/B (50.0 to 400.0 units/kg), and were tested for muscle weakness and increased water consumption, the later being a putative model for dry mouth. Antigenic potential was assessed by monthly intramuscular injections in rabbits (2.0 or 8.7 Units/kg for Botox® or 3.0 Units/kg for Dysport®). Peak muscle weakness and duration were dose related for all serotypes. DAS ED50 values (units/kg) were as follows: BOTOX®: 6.7, Dysport®: 24.7, BoNT/B: 11.8 to 244.0, BoNT/F: 4.3. BOTOX® had a longer duration of action than BoNT/B or BoNT/F. Therapeutic index
Acetylcholine release from the axon of the affected neuron into the synapse, resulting in paralysis. The effect of intoxication on the affected neuron is long-lasting and until recently has been thought to be irreversible. The tetanus neurotoxin is known to exist in one immunologically distinct serotype.

**Acetylcholine**

Typically only a single type of small molecule neurotransmitter is released by each type of neuron in the mammalian nervous system. The neurotransmitter acetylcholine is secreted by neurons in many areas of the brain, specifically by the large pyramidal cells of the motor cortex, by several different neurons in the basal ganglia, by the motor neurons that innervate the skeletal muscles, by the preganglionic neurons of the autonomic nervous system (both sympathetic and parasympathetic), by the postganglionic neurons of the parasympathetic nervous system, and by some of the postganglionic neurons of the sympathetic nervous system. Essentially, only the postganglionic sympathetic nerve fibers to the sweat glands, the piloerector muscles and a few blood vessels are cholinergic and most of the postganglionic neurons of the sympathetic nervous system release the neurotransmitter norepinephrine. In most instances acetylcholine has an excitatory effect. However, acetylcholine is known to have inhibitory effects at some of the peripheral parasympathetic nerve endings, such as inhibition of the heart by the vagal nerve.
As discussed above, the present methods of treating injured muscles are still inadequate. There is a need to have improved methods of treating injured muscles.

### SUMMARY

In accordance with the present invention, a botulinum toxin for use as defined in claim 1 is provided. The treatment may include the step of in vivo, local administration of a therapeutically effective amount of a botulinum toxin into or to the vicinity of the injured muscle. The botulinum toxin functions to provide a temporary chemodenervation of the injured muscle and to reduce the muscle’s contractions. An objective of the present invention is therapy to facilitate healing and a speedy return to function of an injured muscle. The injured muscle may be, for example, a strained muscle. In one embodiment, the botulinum toxin is administered intramuscularly or subcutaneously. In another embodiment, the step of administering a botulinum toxin is preceded by and/or followed by physical therapy and/or surgery.

Further in accordance with the invention, the step of administering the botulinum toxin is immediately after the muscle is injured, or is as soon thereafter as is practical. In one embodiment, the botulinum toxin is effective to immobilize or to substantially immobilize the injured muscle during at least phase 1 and/or phase 2 of the repair process of the injured muscle.

In accordance with the invention, the botulinum toxin can include a targeting component, a therapeutic component and a translocation component. The targeting component can bind to a presynaptic motor neuron. In one embodiment, the targeting component can comprise a carboxyl end fragment of a heavy chain of a butyricum toxin, a tetani toxin, or of a botulinum toxin type A, B, C₁, D, E, F, G or a variant thereof. The therapeutic component can interfere with or modulate the release of a neurotransmitter from a neuron or its processes. In one embodiment, the therapeutic component comprises a light chain of a botulinum toxin type A, B, C₁, D, E, F, G or a variant thereof. The translocation component can facilitate the transfer of at least a part of the neurotoxin, for example the therapeutic component, into the cytoplasm of the target cell. In one embodiment, the translocation component can comprise an amino end fragment of a heavy chain of a botulinum toxin type A, B, C₁, D, E, F, G or variants thereof.

Still further in accordance with the invention, the botulinum toxin is a botulinum toxin type A, B, E and/or F. In a preferred embodiment, the botulinum toxin used to treat an injured muscle is botulinum toxin type A. In fact, the use of botulinum toxin type A is preferred because of its commercial availability, known clinical uses, and successful application to treat muscle injury according to the present invention, as disclosed herein. Use of from about 0.1 U/kg to about 30 U/kg of a botulinum toxin type A and from about 1 U/kg to about 150 U/kg of a botulinum toxin type B is within the scope of a method practiced according to the present disclosed invention. With regard to the other botulinum toxin serotypes (including toxin types E and F) the U/kg dosage to be used is within the range of about 0.1 U/kg to about 150 U/kg, as set forth herein.

Still further in accordance with the invention, the botulinum toxin can be recombinantly produced.

A detailed embodiment of the present invention is a method for treating (as by promoting the healing of) an injured muscle by in vivo, local administration of a therapeutically effective amount of a botulinum toxin to an injured muscle, thereby treating the injured muscle. The botulinum toxin can be botulinum toxin type A. Significantly, the present invention also can be used for treating pain associated with an injured muscle by in vivo, local administration of a therapeutically effective amount of a botulinum toxin to an injured muscle, thereby reducing the pain associated with an injured muscle.

Definitions

The following definitions are provided and apply herein.

"About" means approximately or nearly and in the context of a numerical value or range set forth herein means ± 10% of the numerical value or range recited or claimed.

"Heavy chain" means the heavy chain of a clostridial neurotoxin. It preferably has a molecular weight of about 100 kDa and may be referred to herein as H chain or as H.

"H₉" means a fragment (preferably having a molecular weight of about 50 kDa) derived from the H chain of a Clostridial neurotoxin which is approximately equivalent to the amino terminal segment of the H chain, or the portion corresponding to that fragment in the intact in the H chain. It is believed to contain the portion of the natural or wild type clostridial neurotoxin involved in the translocation of the L chain across an intracellular endosomal membrane.

"H₉⁻" means a fragment (about 50 kDa) derived from the H chain of a clostridial neurotoxin which is approximately equivalent to the carboxyl terminal segment of the H chain, or the portion corresponding to that fragment in the intact H chain. It is believed to be immunogenic and to contain the portion of the natural or wild type Clostridial neurotoxin involved in high affinity, presynaptic binding to motor neurons.

"Injured muscle" means an acutely injured skeletal muscle; this term includes a strained, torn or pulled muscle, as well as a muscle with a contusion (bruise), laceration, ischemia or rupture.

"Light chain" means the light chain of a clostridial neurotoxin. It preferably has a molecular weight of about 50 kDa, and can be referred to as L chain, L or as the proteolytic domain (amino acid sequence) of a clostridial neurotoxin. The light chain is believed to be...
effective as an inhibitor of neurotransmitter release when it is released into a cytoplasm of a target cell.

"Local administration" means direct administration of a pharmaceutical at or to the vicinity of a site on or within an animal body, at which site a biological effect of the pharmaceutical is desired. Local administration excludes systemic routes of administration, such as intravenous or oral administration.

"Neurotoxin" means a botulinum toxin.

"Variant" means a chemical entity which is slightly different from a parent chemical entity but which still has a biological effect. The biological effect of the variant may be substantially the same or better than that of the parent. For example, a variant light chain of a botulinum toxin having at least one amino acid replaced, modified, deleted or added, may have the same or better ability to prevent the release of neurotransmitter vesicles. Additionally, the biological effect of a variant may be decreased. For example, a variant light chain of a botulinum toxin type A having a leucine-cystine motif removed may have a shorter biological persistence than that of the parent (or native) botulinum toxin type A light chain.

An effective method for treating an injured muscle can include the step of locally administering a therapeutically effective amount of a neurotoxin into an injured muscle. Preferably, the injured muscle is a strained muscle.

A strain injury of the skeletal muscle may be classified as a shearing injury. In shearing injury, not only the myofibers but also the myosheath are torn. Almost immediately after the injury of the muscle, a repair process of muscle begins. The repair process of the shearing injury may be divided into three phases.

Phase 1 is the destruction phase, which is characterized by hematoma formation, myofiber necrosis, and inflammatory cell reaction. The site of rupture of an otherwise healthy muscle often occurs close to its distal myotendinous junction (MTJ) after a strain. The ruptured myofibers contract and a gap is formed between the stumps. Because skeletal muscle is richly vascularized, hemorrhage from the torn vessels is inescapable and the gap becomes filled with a hematoma, later replaced by scar tissue. In shearing injuries the mechanical force tears the entire myofiber, damaging the myofiber plasma membrane and leaving sarcoplasm open at the ends of the stumps. Because myofibers are very long, string-like cells, the necrosis initiated at this site extends all along the whole length of the ruptured myofiber. The blood vessels are also torn in shearing injuries; thus, blood-borne inflammatory cells gain immediate access to the injury site to induce an inflammation. Phase 1 persists for about 2 to 3 days following the injury.

Phase 2 is the repair phase, which consists of phagocytosis of the necrotized tissue, regeneration of the myofibers, production of connective tissue scar, and capillary ingrowth. The key step in the regeneration of injured muscle tissue is the vascularization of the injured area. The restoration of vascular supply is a necessary for the regeneration of an injured muscle. The new capillaries sprout from surviving trunks of blood vessels and pierce across the center of injured area. These new capillaries help provide adequate oxygen supply to the regenerating area.

Phase 3 is the remodeling phase, which consists of maturation of the regenerated myofibers, contraction and reorganization of the scar tissue, and restoration of the functional capacity of the repaired muscle. Phase 2 (repair) and 3 (remodeling) often occur simultaneously and persists for about 2 days to about six weeks following phase 1.

In one embodiment of the present invention, the neurotoxin is locally administered, intramuscularly, to immobilize the injured muscle to facilitate healing. Local administration of a neurotoxin according to the present disclosed invention can also reduce the pain experienced due to a muscle injury. Preferably, the administration of the neurotoxin is immediately at the time of injury or close thereafter. In one preferred embodiment, the neurotoxin is effective to immobilize the injured muscle during the destruction phase (phase 1) to prevent re-rupturing of the muscle.

Without wishing to limit the invention to any particular theory of mechanism of operation, it is believed that mobilization during the repair and/or remodeling phases is beneficial in that such mobilization induces more rapid and intensive capillary ingrowth to the injured area, as well as better muscle fiber regeneration and orientation. Therefore, in one embodiment, the immobilizing effect of the botulinum toxin is absent during the repair phase (phase 2) and/or remodeling phase (phase 3). In a more preferred embodiment, the botulinum toxin is administered and is effective to immobilize the injured muscle during phase 1, but not during phases 2 and 3 of the repair process. For example, if the botulinum toxin is injected, intramuscularly, immediately to the muscle following an injury, it is preferable that it immobilizes the injured muscle for about 3 days after the time of administration. Alternatively, the botulinum toxin can have its immobilization effect only up to the point where the patient experiences little or no pain in the use of the injured muscle in basic movements. When this critical point is reached, the patient should be encouraged to start active, progressive mobilization.

In another embodiment of the present invention, the botulinum toxin is effective to immobilize the injured muscle for all of the phase 1-3 periods and for a subsequent muscle injury recovery period thereafter.

Botulinum toxins, which can require from less than about one day to about seven days to exhibit significant clinical muscle paralysis effect and/or where the muscle paralysis effect is sustained post injection for a period of several months, are within the scope of the present invention, as they can be used to treat relatively serious or long lasting muscle injuries or where a long period of muscle immobilization is indicated for proper healing.
A botulinum toxin is used because of the known uses and clinical safety of a botulinum toxin, such as botulinum toxin type E to treat muscle disorders, such as muscle spasms. In a particularly preferred embodiment of the present invention, especially to severe, or third degree muscle injuries, the locally administered botulinum toxin is a botulinum toxin type E. Botulinum toxin type A can also be used in both these embodiments.

Although the botulinum toxins of the present invention treat injured muscles by immobilizing them, the botulinum toxin may reduce pain and/or spasm. The botulinum toxin is able to immobilize the injured muscle and to reduce pain associated with that injured muscle. In a preferred embodiment, a botulinum toxin, for example a botulinum toxin type E, or most preferably type A, is administered to a strained muscle to immobilize the muscle and optionally to reduce pain associated with that muscle.

Of course, an ordinarily skilled medical provider can determine the appropriate dose and frequency of administration(s) to achieve an optimum clinical result. That is, one of ordinary skill in medicine would be able to administer the appropriate amount of the neuromuscular blocking agent at the appropriate time(s) to effectively immobilize the injured muscle(s). The dose of the botulinum toxin to be administered depends upon a variety of factors, including the size of the muscle, the severity of the muscle injury. In a preferred embodiment, the dose of the botulinum toxin administered immobilizes the injured muscle(s) for no longer than the duration of phase 1 of the repair process. In the various methods of the present invention, from about 0.1 U/kg to about 15 U/kg, of botulinum toxin type A can be administered to the injured muscle. Preferably, about 1 U/kg to about 20 U/kg of botulinum toxin type A may be administered to the injured muscle. Use of from about 0.1 U/kg to about 30 U/kg of a botulinum toxin type A and from about 1 U/kg to about 150 U/kg of a botulinum toxin type B is within the scope of a method practiced according to the present disclosed invention. With regard to the other botulinum toxin serotypes (including toxin types E and F) the U/kg dosage to be used is within the range of about 0.1 U/kg to about 150 U/kg, as set forth herein.

Intramuscular injection is the route of administration.

In another broad embodiment, the method of treating injured muscle according to this invention further includes other steps described below. These other steps may be taken prior to, in conjunction with or following the step of administering a botulinum toxin, preferably to the injured muscle. For example, the present recommended treatment for strained muscle includes resting, icing, compression and elevating. These four steps (or procedures) have the same objective. They minimize bleeding from ruptured blood vessels to rupture site. This will prevent the formation of a large hematoma, which has a direct impact on the size of scar tissue at the end of the regeneration. A small hematoma and the limitation of interstitial edema accumulation on the rupture site also shorten the ischemic period in the granulation tissue, which in turn accelerates regeneration.

Other additional steps may be employed in the treatment of injured muscles. In one embodiment, the additional step include an administration of nonsteroidal anti-inflammatory drugs (NSAIDs), therapeutic ultrasound, hyperbaric oxygen, and in severe injuries, surgery may also be employed. NSAIDs should be a part of early treatment and should be started immediately after the injury. Short-term use of NSAIDs in the early phase of healing decreases the inflammatory cell reaction, and has no adverse effects on tensile or contractile properties of injured muscle.

In another embodiment, the additional step includes the use of therapeutic ultrasound. Therapeutic ultrasound is widely recommended and used in the treatment of muscle strains. It is thought that therapeutic ultrasound promotes the proliferation phase of myoregeneration.

In another embodiment, the additional step includes surgical intervention. Surgical treatment of muscle injuries should be reserved for the most serious injuries, because in most cases conservative treatment results in a good outcome. Surgical treatment is indicated only in cases of (1) large intramuscular hematomas, (2) third-degree strains or tears of muscles with few or no agonise muscles, and (3) second-degree strains, if more than half of the muscle belly is torn.

In another broad aspect of this invention, recombinant techniques are used to produce at least one of the components of the botulinum toxins. The technique includes steps of obtaining genetic materials from either DNA cloned from natural sources, or synthetic oligonucleotide sequences, which have codes for one of the components, for example the therapeutic, translocation and/or targeting component(s). The genetic constructs are incorporated into host cells for amplification by first fusing the genetic constructs with a cloning vectors, such as phages or plasmids. Then the cloning vectors are inserted into hosts, preferably E. coli’s. Following the expressions of the recombinant genes in host cells, the resultant proteins can be isolated using conventional techniques. The protein expressed may comprise all three components of the neurotoxin. For example, the protein expressed may include a light chain of botulinum toxin type E (the therapeutic component), a heavy chain, preferably the HN, of a botulinum toxin type B (the translocation component), and an H2 of botulinum toxin type A, which selectively binds to the motor neurons. In one embodiment, the protein expressed may include less than...
all three components of the neurotoxin. In such case, the components may be chemically joined using techniques known in the art.

There can be many advantages to producing these botulinum toxins recombinantly. For example, production of botulinum toxin from anaerobic Clostridium cultures is a cumbersome and time-consuming process including a multi-step purification protocol involving several protein precipitation steps and either prolonged and repeated crystallization of the toxin or several stages of column chromatography. Significantly, the high toxicity of the product dictates that the procedure must be performed under strict containment (BL-3). During the fermentation process, the folded single-chain neurotoxins are activated by endogenous Clostridial proteases through a process termed nicking. This involves the removal of approximately 10 amino acid residues from the single-chain to create the dichain form in which the two chains remain covalently linked through the intrachain disulfide bond.

The nicked botulinum toxin is much more active than the unnicked form. The amount and precise location of nicking varies with the serotypes of the bacteria producing the toxin. The differences in single-chain botulinum toxin activation and, hence, the yield of nicked toxin, are due to variations in the type and amounts of proteolytic activity produced by a given strain. For example, greater than 99% of Clostridial botulinum type A single-chain toxin is activated by the Hall A Clostridial botulinum strain, whereas type B and E strains produce toxins with lower amounts of activation (0 to 75% depending upon the fermentation time). Thus, the high toxicity of the mature toxin plays a major part in the commercial manufacture of toxins as therapeutic toxins.

The degree of activation of engineered Clostridial toxins is, therefore, an important consideration for manufacture of these materials. It would be a major advantage if botulinum toxin could be expressed, recombinantly, in high yield in rapidly-growing bacteria (such as heterologous E. coli cells) as relatively non-toxic single-chains (or single chains having reduced toxic activity) which are safe, easy to isolate and simple to convert to the fully-active form.

With safety being a prime concern, previous work has concentrated on the expression in E. coli and purification of individual H and L chains of tetanus and botulinum toxins; these isolated chains are, by themselves, non-toxic; see Li et al., Biochemistry 33: 7014-7020 (1994); Zhou et al., Biochemistry 34: 15175-15181 (1995), hereby incorporated by reference herein. Following the separate production of these peptide chains and under strictly controlled conditions the H and L subunits can be combined by oxidative disulphide linkage to form the neuroparalytic di-chains.

The following non-limiting examples provide preferred methods of treating injured muscles and producing recombinant botulinum toxins. The methods of producing recombinant botulinum toxins described in the below Examples 4-8 are drawn from and are similar to those described in Dolly et al. International Patent Application No. WO 95/32738.

Example 1

Treatment of a Ruptured Biceps Tendon

Ruptures of the biceps brachii commonly occur at the proximal end and involve the long head of the biceps. The muscle may rupture at the distal insertion onto the radius, but is rare. Most often, ruptures occur in adults older than age 40 years who have a long history of shoulder pain secondary to an impingement syndrome. Over time, the tendon becomes frayed and weak, and ultimately ruptures, partially or entirely. Regardless, the rupture is often caused by a trivial event. These ruptures are usually associated with a rotator cuff tear, especially among the elderly.

A 45 year old man presents with a bulge in the lower arm after lifting heavy boxes. He reports a history of sudden sharp pain in the upper arm, often accompanied by an audible snap. The man is diagnosed as having a ruptured biceps tendon and is at the beginning of phase 1 of the repair process. The rupture may be classified as a mild second degree strain.

The patient is treated by a bolus injection of between about 0.1 U/kg to about 25 U/kg of a neurotoxin intramuscularly to the biceps. Preferably the neurotoxin is botulinum toxin type E and/or F, more preferably type A. The particular dose and frequency of administrations depend upon a variety of factors, and are to be determined by the treating physician. The patient is further instructed to rest and apply ice and compression to the biceps. Within about three days after the administration of the neurotoxin, the patient is able to bend his arm. Also, after about three days, the patient experiences a reduction in inflammation, which is a sign that the patient is entering into phase 2 and 3 of the repair process. The patient also experiences a significant pain reduction. Local administration of from about 10 units to about 200 units of botulinum toxin type A can also be used for long term (2-4 months) muscle immobilization and pain reduction.

Example 2

Extensor Mechanism Rupture

Rupture of the extensor mechanism of the knee occurs in one of two ways: in younger patients as a result of a sudden or violent force (such as jumping, heavy lifting); and in older patients as a result of relatively trivial force. In either group, there may have been some prior arching. This condition affects older patients who have typically been somewhat sedentary and have suddenly increased their activity level, or patient who have had some preexisting or co-existing condition such as diabe-
tes mellitus, rheumatoid arthritis, and other systemic inflammatory disorders, or prior knee surgery.

A 22 year old female soccer player presents with an inability to extend her knee. The patient also is also unable to do straight leg raise, but is able to walk if she keeps a hand on her thigh and maintain her knee in extension. A plain radiograph shows that the patella is in a lower than usual location. The patient is diagnosed with a severe rupture of the quadriceps.

After determining the injury is severe (third degree), the patient agrees to undergo reparative surgery. Post-operationally, the patient is treated by a bolus injection of between about 0.1 U/kg to about 25 U/kg of a neurotoxin (such as about 10 units to about 400 units of botulinum toxin type A) intramuscularly to the quadriceps. Preferably the neurotoxin is botulinum toxin type A. The particular dose and frequency of administrations depend upon a variety of factors, and are to be determined by the treating physician. The patient is further instructed to rest and apply ice and compression to the quadriceps. Within about 15 days after the administration of the neurotoxin, gradual movement and activity of the injured muscle is possible. The patient is then encouraged to gently move the recovering muscle to strengthen it and the surrounding muscles. As the toxin effect wears off some more, the patient would then have the ability to rapidly participate in a physical therapy program or resume the general activity and/or sport. If this patient depended upon this sport for her livelihood, botulinum toxin therapy would facilitate her early return to this activity. Local administration of from about 10 units to about 200 units of botulinum toxin type A can be used for long term (2-4 months) muscle immobilization.

Example 3

Treatment of Shin Splints

Runners commonly experience shin splits in the lower limb which causes pain and restricts this activity. The lower leg pain resulting from shin splits is caused by very small tears in the leg muscles at their point of attachment to the shin. There are two types: 1. Anterior shin splints occur in the front portion of the shinbone (tibia). 2. Posterior shin splints occur on the inside (medial) part of the leg along the tibia.

Anterior shin splints are due to muscle imbalances, insufficient shock absorption or toe running. Excessive pronation contributes to both anterior and posterior shin splints.

In treating strained muscle, such as a shin splint, five steps are recommended: (1) Protect the injured muscle from further injury by using splints, pads and/or crutches; (2) Restrict activity, usually for 48 to 72 hours to allow the healing process to begin. The administration of a short acting botulinum toxin type E or F or a botulinum toxin type A modified so as to reduce the period of in vivo biological activity (i.e. a shorter period of flaccid muscle paralysis) of the type A toxin. Suitable botulinum toxins, including botulinum toxin type A, with reduced periods of in vivo biological activity suitable for use herein are set forth in co-pending U.S. patent application serial number 09/620840, which application is incorporated herein by reference in its entirety. In more severe strains restriction of activity can last for weeks to months. With a longer required restriction of activity, an administration of a longer acting botulinum toxin, for example (unmodified) botulinum toxin type B, or more preferably, type A toxin, can be appropriate. Without this treatment, patients could experience weeks of restricted activity. As the healing process begins, gentle motion and movement of the affected muscle is advised; (3) Ice should be applied for 15-20 minutes every hour; (4) Compression such as elastic bandage should be kept on between icing; and (5) Elevate the injured area to minimize swelling.
to achieve overlapping sequencing runs. The cloned sequence is found to be identical to the sequence disclosed by Binz, et al., in J. Biol. Chem. 265:9153 (1990), and Thompson et al., in Eur. J. Biochem. 189:73 (1990).

[0081] Site-directed mutants designed to compromise the enzymatic activity of the BoNT/A-L chain can also be created.

Example 5

Expression of the Botulinum Toxin Type A-L (BoNT/A-L) Chain Fusion Proteins

[0082] This Example describes the methods to verify expression of the wild-type L chains, which may serve as a therapeutic component, in bacteria harboring the pCA-L plasmids. Well isolated bacterial colonies harboring either pCAL are used to inoculate L-broth containing 100 µg/ml ampicillin and 2% (w/v) glucose, and grown overnight with shaking at 30°C. The overnight cultures are diluted 1:10 into fresh L-broth containing 100 µg/ml of ampicillin and incubated for 2 hours. Fusion protein expression is induced by addition of IPTG to a final concentration of 0.1 mM. After an additional 4 hour incubation at 30°C, bacteria are collected by centrifugation at 6,000 x g for 10 minutes.

[0083] A small-scale SDS-PAGE analysis confirmed the presence of a 90 kDa protein band in samples derived from IPTG-induced bacteria. This Mr is consistent with the predicted size of a fusion protein having MBP (~ 40 kDa) and BoNT/A-L chain (~ 50 kDa) components. Furthermore, when compared with samples isolated from control cultures, the IPTG-induced clones contained substantially larger amounts of the fusion protein.

[0084] The presence of the desired fusion proteins in IPTG-induced bacterial extracts is also confirmed by Western blotting using the polyclonal anti-L chain probe described by Cenci di Bello et al., in Eur. J. Biochem. 219:161 (1993). Reactive bands on PVDF membranes (Pharmacia; Milton Keynes, UK) are visualized using an anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Bio-Rad; Hemel Hempstead, UK) and the ECL detection system (Amersham, UK). Western blotting results confirmed the presence of the desired fusion protein together with several faint bands corresponding to proteins of lower Mr than the fully sized fusion protein. This observation suggested that limited degradation of the fusion protein occurred in the bacteria during or after the isolation procedure. Neither the use of 1 mM nor 10 mM benzamidine (Sigma; Poole, UK) during the isolation procedure eliminated this proteolytic breakdown.

[0085] The yield of intact fusion protein isolated by the above procedure remained fully adequate for all procedures described herein. Based on estimates from stained SDS-PAGE gels, the bacterial clones induced with IPTG yielded 5-10 mg of total MBP-wild-type or mutant L chain fusion protein per liter of culture. Thus, the method of producing BoNT/A-L chain fusion proteins disclosed herein is highly efficient, despite any limited proteolysis that did occur.

[0086] The MBP-L chain fusion proteins encoded by the pCAL and pCAL-TyrU7 expression plasmids are purified from bacteria by amylose affinity chromatography. Recombinant wild-type or mutant L chains are then separated from the sugar binding domains of the fusion proteins by site-specific cleavage with Factor X2. This cleavage procedure yielded free MBP, free L chains and a small amount of uncleaved fusion protein. While the resulting L chains present in such mixtures have been shown to possess the desired activities, we have also employed an additional purification step. Accordingly, the mixture of cleavage products is applied to a second amylose affinity column that bound both the MBP and uncleaved fusion protein. Free L chains are not retained on the affinity column, and are isolated for use in experiments described below.

Example 6

Purification of Fusion Proteins and Isolation of Recombinant BoNT/A-L Chains

[0087] This Example describes a method to produce and purify wild-type recombinant BoNT/A light chains from bacterial clones. Pellets from 1 liter cultures of bacteria expressing the wild-type BoNT/A-L chain proteins are resuspended in column buffer [10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EGTA and 1 mM DTT] containing 1 mM phenyl-methanesulfonyl fluoride (PMSF) and 10 mM benzamidine, and lysed by sonication. The lysates are cleared by centrifugation at 15,000 x g for 15 minutes at 4°C. Supernatants are applied to an amylose affinity column [2x10 cm, 30 ml resin] (New England Biolabs; Hitchin, UK). Unbound proteins are washed from the resin with column buffer until the eluate is free of protein as judged by a stable absorbance reading at 280 nm. The bound MBP-L chain fusion protein is subsequently eluted with column buffer containing 10 mM maltose. Fractions containing the fusion protein are pooled and dialyzed against 20 mM Tris-HCl (pH 8.0) supplemented with 150 mM NaCl, 2 mM CaCl2 and 1 mM DTT for 72 hours at 4°C.

[0088] Fusion proteins are cleaved with Factor X2 (Promega; Southampton, UK) at an enzyme:substrate ratio of 1:100 while dialyzing against a buffer of 20 mM Tris-HCl (pH 8.0) supplemented with 150 mM NaCl, 2 mM CaCl2 and 1 mM DTT. Dialysis is carried out for 24 hours at 4°C. The mixture of MBP and either wild-type or mutant L chain that resulted from the cleavage step is loaded onto a 10 ml amylose column equilibrated with column buffer. Aliquots of the flow through fractions are prepared for SDS-PAGE analysis to identify samples containing the L chains. Remaining portions of the flow through fractions are stored at -20°C. Total E. coli extract or the purified proteins are solubilized in SDS sample buffer and subjected to PAGE according to standard pro-
evaluation of the proteolytic activities of recombinant derivatives thereof. The foregoing results indicate that the approach to creating MBP-L chain fusion proteins described herein could be used to efficiently produce wild-type and mutant recombinant BoNT/A-L chains. Further, the results demonstrate that recombinant L chains could be separated from the maltose binding domains of the fusion proteins and purified thereafter.

A sensitive antibody-based assay is developed to compare the enzymatic activities of recombinant L chain products and their native counterparts. The assay employed an antibody having specificity for the intact C-terminal region of SNAP-25 that corresponded to the BoNT/A cleavage site. Western Blotting of the reaction products of BoNT/A cleavage of SNAP-25 indicated an inability of the antibody to bind SNAP-25 sub-fragments. Thus, the antibody reneurotoxin employed in the following Example detected only intact SNAP-25. The loss of antibody binding served as an indicator of SNAP-25 proteolysis mediated by added BoNT/A light chain or recombinant derivatives thereof.

**Example 7**

**Evaluation of the Proteolytic Activities of Recombinant L Chains Against a SNAP-25 Substrate**

This Example describes a method to demonstrate that both native and recombinant BoNT/A-L chains can proteolyze a SNAP-25 substrate. A quantitative assay is employed to compare the abilities of the wild-type and their recombinant analogs to cleave a SNAP-25 sub-fragment. The substrate utilized for this assay is obtained by preparing a glutathione-S-transferase (GST)-SNAP-25 fusion protein, containing a cleavage site for thrombin, expressed using the pGEX-2T vector and purified by affinity chromatography on glutathione agarose. The SNAP-25 is then cleaved from the fusion protein using thrombin in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 2.5 mM CaCl2 (Smith et al., Gene 67:31 (1988)) at an enzyme:substrate ratio of 1:100. Uncleaved fusion protein and the cleaved glutathione- binding domain bound to the gel. The recombinant SNAP-25 protein is eluted with the latter buffer and dialyzed against 100 mM HEPES (pH 7.5) for 24 hours at 4°C. The total protein concentration is determined by routine methods.

Rabbit polyclonal antibodies specific for the C-terminal region of SNAP-25 are raised against a synthetic peptide having the amino acid sequence, CAN-QRATKMLGSS (SEQ ID#3). This peptide corresponded to residues 195 to 206 of the synaptic plasma membrane protein and an N-terminal cysteine residue not found in native SNAP-25. The synthetic peptide is conjugated to bovine serum albumin (BSA) (Sigma; Poole, UK) using maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as a crosslinking neurotoxin (Sigma; Poole, UK) to prove antigenicity (Liu et al., Biochemistry 18:690 (1979) 1. Affinity purification of the anti-peptide antibodies is carried out using a column having the antigenic peptide conjugated via its N-terminal cysteine residue to an amioalkyl agarose resin (Bio-Rad; Hemel Hempstead, UK), activated with iodoacetic acid using the cross-linker ethyl 3-(3-dimethylpropyl) carbodiimide. After successive washes of the column with a buffer containing 25 mM Tris-HCl (pH 7.4) and 150 mM NaCl, the peptide-specific antibodies are eluted using a solution of 100 mM glycine (pH 2.5) and 200 mM NaCl, and collected in tubes containing 0.2 ml of 1 M Tris-HCl (pH 8.0) neutralizing buffer.

**Example 8**

Western blotting results indicate clear differences between the proteolytic activities of the purified mutant L chain and either native or recombinant wild-type BoNT/A-L chain. Specifically, recombinant wild-type L chain cleaves the SNAP-25 substrate, though somewhat less efficiently than the reduced BoNT/A native L chain that serves as the positive control in the procedure. Thus, an enzymatically active form of the BoNT/A-L chain is produced by recombinant means and subsequently isolated. Moreover, substitution of a single amino acid in the L chain protein abrogated the ability of the recombinant protein to degrade the synaptic terminal protein.

As a preliminary test of the biological activity of the wild-type recombinant BoNT/A-L chain, the ability of the MBP-L chain fusion protein to diminish Ca2+-evoked catecholamine release from digitonin-permeabilized bovine adrenochromaffin cells is examined. Consistently, wild-type recombinant L chain fusion protein, either intact or cleaved with Factor X2 to produce a mixture containing free MBP and recombinant L chain, induced a dose-dependent inhibition of Ca2+-stimulated release equivalent to the inhibition caused by native BoNT/A.
Reconstitution of Native L Chain, Recombinant Wild-Type L Chain with Purified H Chain

[0097] Native H and L chains are dissociated from BoNT/A (List Biologicals Inc.; Campbell, USA) with 2 M urea, reduced with 100 mM DTT and then purified according to established chromatographic procedures (Kozaki et al., Japan J. Med. Sci. Biol. 34:61 (1981); Maisey et al., Eur. J. Biochem. 177:683 (1988)). Purified H chain is combined with an equimolar amount of either native L chain or recombinant wild-type L chain. Reconstitution is carried out by dialyzing the samples against a buffer consisting of 25 mM Tris (pH 8.0), 50 μM zinc acetate and 150 mM NaCl over 4 days at 4°C. Following dialysis, the association of the recombinant L chain and native H chain to form disulfide-linked 150 kDa dichains is monitored by SDS-PAGE and quantified by densitometric scanning. The proportion of dichain molecules formed with the recombinant L chains is lower than that obtained when native L chain is employed. Indeed, only about 30% of the recombinant wild-type or mutant L chain is reconstituted while >90% of the native L chain reassociated with the H chain. In spite of this lower efficiency of reconstitution, sufficient material incorporating the recombinant L chains is easily produced for use in subsequent functional studies.

[0098] Other embodiments, versions, and modifications within the scope of the present invention are possible. For example, from about 500 units to about 4,000 units of a botulinum toxin type B can be used to treat an injured muscle according to the present disclosed invention.

Claims

1. A botulinum toxin for use in the treatment of acute injuries to skeletal muscles by local administration, by intramuscular injection, which substantially immobilizes the injured muscle.

2. A botulinum toxin for use according to claim 1, which is effective to immobilize the injured muscle during phase 1 and phase 2 of a repair process of the injured muscle.

3. A botulinum toxin for use according to any one of the preceding claims, which is effective to immobilize the injured muscle during phase 1 of a repair process of the injured muscle.

4. A botulinum toxin for use according to any one of the preceding claims, which is a botulinum toxin type A, B, C₁, D, E, F or G.

5. A botulinum toxin for use according to any one of claims 1 to 4, which is recombinantly made.
Revendications

1. Toxine botulinique à utiliser dans le traitement de lésions aiguës des muscles squelettiques par administration locale, par injection intramusculaire, qui immobilise sensiblement le muscle lésé.

2. Toxine botulinique à utiliser selon la revendication 1, qui est efficace pour immobiliser le muscle lésé pendant la phase 1 et la phase 2 d’un processus de réparation du muscle lésé.

3. Toxine botulinique à utiliser selon l’une quelconque des revendications précédentes, qui est efficace pour immobiliser le muscle lésé pendant la phase 1 d’un processus de réparation du muscle lésé.

4. Toxine botulinique à utiliser selon l’une quelconque des revendications précédentes, qui est une toxine botulinique de type A, B, C1, D, E, F ou G.

5. Toxine botulinique à utiliser selon l’une quelconque des revendications 1 à 4, qui est fabriquée par recombinaison.

6. Toxine botulinique à utiliser selon l’une quelconque des revendications précédentes, qui est destinée à une administration en plus d’une thérapie physique et/ou d’une opération chirurgicale.

7. Toxine botulinique à utiliser selon l’une quelconque des revendications précédentes, qui est une toxine botulinique de type A.

8. Toxine botulinique à utiliser selon la revendication 7, qui est administrée de façon à favoriser la guérison du muscle lésé.

9. Toxine botulinique à utiliser selon l’une quelconque des revendications précédentes, qui atténue également la douleur associée au muscle lésé.
REFERENCES CITED IN THE DESCRIPTION

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