Acute Effects of the Skeletal Muscle-Specific Immunotoxin Ricin-mAb 35 on Extraocular Muscles of Rabbits

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PURPOSE. To determine the acute histologic and ultrastructural effects of a recently developed muscle-specific immunotoxin, ricin-mAb 35.

METHODS. Graduated doses of ricin-mAb 35, composed of ricin conjugated to a monoclonal antibody against the nicotinic acetylcholine receptor of skeletal muscle, were injected into one superior rectus muscle in rabbits. After 3, 7, and 14 days, both superior rectus muscles were removed and prepared for electron microscopy and histologic examination, by using a number of immunohistochemical markers to identify inflammatory cell infiltration, muscle fiber loss, and muscle regeneration.

RESULTS. Myotoxicity of the ricin-mAb 35 was focal and dose related. At the highest dose tested, there was substantial inflammatory cell infiltrate by 3 days, which largely disappeared by 7 days. Significant muscle loss was apparent by 7 days after ricin-mAb 35 treatment. Both the inflammatory reaction and muscle fiber loss were confined to the immediate injection site. Surrounding muscle appeared to be normal. At 14 days after treatment, early signs of muscle regeneration were evident within the tissue sections. No evidence of orbital or systemic toxicity was seen in any animal.

CONCLUSIONS. Direct injection of ricin-mAb 35 into the extraocular muscles of rabbits results in a dose-related focal injury to the muscles, with a self-limited inflammatory component and significant muscle fiber loss. This novel immunotoxin may be useful in the treatment of strabismus if chronic studies show a sustained histologic and electrophysiologic effect. (Invest Ophthalmol Vis Sci. 2000; 41:3402–3409)

The goal of surgery for nonrestrictive forms of strabismus is to adjust extraocular muscle force generation so that, in the presence of abnormal efferent motor signals, normal binocular alignment can be achieved. Typically, one or more of the extraocular muscles are weakened or strengthened by altering the length of the muscle (e.g., resection) or the position of the muscle on the globe (e.g., recession). Although effective at changing the rotational position of the globe, incisional surgery compromises normal muscle dynamics. The arc of contact of the muscle with the globe, the intrinsic elasticity of the muscles involved in the surgery, the resting tension on the agonist–antagonist pair,1 and generated twitch tension2 all change after surgery. In addition, surgery unavoidably induces scarring that can alter extraocular muscle function further. Although the effects of these changes are usually subclinical, large recessions and resections may result in incomitant gaze and, occasionally, in secondary forms of strabismus.

The use of botulinum toxin addresses some of these concerns by permitting a titratable means of weakening extraocular muscle without altering the insertion of the muscle and without inducing the degree of scarring that would be expected with an incisional procedure. However, botulinum toxin has a number of characteristics that limit its effectiveness in the treatment of strabismus. These include a relatively short duration of action, orbital leakage with collateral effects on other extraocular muscles,3 systemic absorption with measurable effects on distant skeletal muscle and the central nervous system,4–6 and resistance that develops after repeated injections.7 Despite these limitations, the concept of a pharmacologic treatment for strabismus is interesting and may be feasible if new agents can be developed that permit precise targeting and that have a longer duration of action than botulinum toxin.

A new muscle-specific immunotoxin, ricin-mAb 35, has been developed that may be a candidate for the treatment of strabismus. It appears to cause long-lasting weakening of skeletal muscle by direct myopathic effects.8 This immunotoxin consists of ricin linked to a monoclonal antibody (mAb), mAb 35, that binds to the α-subunit of the nicotinic acetylcholine receptor in skeletal muscle. Ricin enters the myofiber and inactivates protein synthesis, destroying the fiber. Preliminary evidence in limb muscle suggests that this immunotoxin may be more effective and have a longer duration of action than botulinum toxin.8 If this can be confirmed in extraocular mus-
cle, ricin-mAb 35 may allow long-term adjustments in the force generation of specific muscles and, as a consequence, create long-term changes in eye alignment.

The purpose of this study was to investigate the short-term effects of ricin-mAb 35 on extraocular muscle to establish minimum effective doses that would result in a histologic effect.

MATERIALS AND METHODS

New Zealand White rabbits were obtained from Birchwood Farms (Red Wing, MN) and housed with Research Animal Resources at the University of Minnesota. All experimental procedures conformed to the National Institutes of Health guidelines for use of animals in research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation and purification of ricin-mAb 35 has been described previously by Hott et al.8 The maximum tolerated dose (the highest dose at which all treated animals survive) of ricin-mAb 35 is 2 μg/kg for mice. Thus, for a 1.6-kg rabbit, 1/100 and 1/300 of the maximum tolerated dose (rMTD) was estimated to be 31 ng and 10.7 ng, respectively.

Rabbits were anesthetized with an intramuscular injection of ketamine and xylazine, 10 mg/kg and 2 mg/kg, respectively. Proparacaine drops were placed in the conjunctival cul-de-sac to reduce the blink reflex before injection into the extraocular muscles. The superior rectus muscles of each rabbit were randomly assigned to treatment and control groups. These muscles were chosen because of their proximity to the limbus and ease of surgical exposure. Using an aseptic technique, a superior conjunctival peritomy was performed, and the conjunctiva was retracted, exposing the distal superior rectus muscle. Under direct observation, one superior rectus muscle of each rabbit was injected with ricin-mAb 35, diluted with sterile isotonic saline to 1:1000, 1:300, 1:100, 1:50, or 1:10 rMTD in a volume of 100 μL. Injections were made slowly through a 30-gauge needle that was left in place for an additional 30 seconds after completion of injection to reduce leakage into the orbit. The control (contralateral) superior rectus muscle was injected with 100 μL of sterile isotonic saline alone.

Initially, one set of muscles was injected at each of the doses, and the muscles were examined 7 days after treatment. No histologic changes were seen in treated muscles at doses lower than 1:100 rMTD. A myopathic effect was seen at both 1:100 rMTD and 1:50 rMTD, and two additional superior rectus muscles were treated at each dose. Because the greatest myotoxic effect was observed after the 1:10-rMTD treatments, all subsequent injections were made at this dose.

The animals that received ricin-mAb 35 injections of 1:10 rMTD were killed with an overdose of barbiturate anesthesia 3, 7, or 14 days after injection. The superior rectus muscle from each orbit was removed from origin to insertion. The extirpated muscle was stretched to its tether length, embedded in either optimal temperature cutting compound (OCT; Miles, Elkhart, IN) or tragacanth gum, quick frozen in 2-methylbutane chilled to a slurry on liquid nitrogen, and serially sectioned at 12 μm on a cryostat. One set of sections was stained with hematoxylin and eosin. A second set of muscle sections was stained immunohistochemically with an antibody against CD11b, an antibody that allows visualization of neutrophils, monocytes, and macrophages. The tissue sections were fixed for 10 minutes in 10% formalin and quenched in hydrogen peroxide to remove endogenous peroxidase. After a phosphate-buffered saline (PBS) rinse, the sections were blocked with normal horse serum and incubated with an antibody to CD11b at a dilution of 1:40 (Harlan Sprague-Dawley, Indianapolis, IN). The tissue was incubated using a peroxidase ABC kit (Vectastain Elite; Vector, Burlingame, CA) which was visualized by incubation with 3,3′-diaminobenzidine (DAB) and heavy metals. Additional muscle cross sections were stained immunohistochemically with antibodies to fast, slow, developmental, and neonatal myosin heavy chain (MHC) isoforms (NovoCastra; Vector). Immunohistochemistry was performed without fixation or quenching on frozen sections, which were processed simultaneously as the first two sets. Antibodies were diluted at 1:40 for fast and slow MHC isoforms and 1:20 for neonatal and developmental MHC antibodies. Sections from each muscle were examined by light microscopy.

Two additional superior rectus muscles, injected with ricin-mAb 35 at 1:10 rMTD, and two additional control muscles, were prepared for electron microscopic examination of the acute effects of the toxin on individual myofibers. Three and 14 days after injection, a rabbit was killed with an overdose of barbiturate and perfused through the heart with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The rectus muscles were dissected from the orbits, trimmed, and postfixed in 1% osmium tetroxide in phosphate buffer. Before dehydration, the specimens were stained with freshly prepared 1% β-phenylenediamine in 70% alcohol for 1.5 hours. The superior rectus muscles were dehydrated in a graded series of alcohols, embedded in Epon, and sectioned at both 1 μm and ultrathin for examination using a transmission electron microscope (model 100CX; JEOL, Peabody, MA).

RESULTS

The rabbits were monitored daily for changes within the orbit. All the rabbits appeared to be healthy and ate normally. Mild conjunctival redness developed at the surgical site in some of the eyes. However, no significant ocular, periocular, or orbital changes were noted in the treated eyes, and no gross changes were visible in the treated muscles at necropsy. There was no evidence of systemic toxicity seen in any animal.

Three Days after Injection

Cross sections stained with hematoxylin and eosin and for CD11b were examined to assess the acute histologic effects of ricin-mAb 35. No effect was seen at any dose lower than 1:100 rMTD. At lower doses of 1:100 and 1:50 rMTD, a very diffuse CD11b-positive cellular infiltrate surrounded the injection site 3 days after intramuscular injection (Fig. 1).

In contrast, at the center of the injection site at the highest dose of 1:10 rMTD, inflammatory cell infiltration was intense and involved the entire muscle cross section (Fig. 2). The cellular infiltrate surrounded the myofibers and was localized deep to the epimysium. The extent of the inflammatory infiltrate was dose related. At doses of 1:100, 1:50, and 1:10 rMTD, the mean diameter of the zone of inflammatory cells was 1.0, 3.6, and 7.0 mm, respectively. The severity of inflammation diminished with increasing distance from the center of the
injection (Figs. 2, 3). Areas of the muscle distant from the injection site were devoid of inflammatory cells. Even in areas of dense inflammatory cell infiltrate, surviving muscle fibers were still apparent at the 3-day interval. However, most fibers were irregular in contour and in various stages of degeneration (Fig. 4).

Seven Days after Injection
At doses of 1:100 and 1:50 rMTD, no inflammatory cell infiltration was seen in any of the muscle cross sections examined at 7 days after intramuscular injection. At 1:10 rMTD, the CD11b-positive cellular infiltrate was markedly reduced compared with that seen at 3 days, and tissue inflammation often appeared as an isolated focus of inflammatory cells (Fig. 5). The region of the muscle that retained inflammatory cell infiltrate was largely devoid of myofibers (Fig. 6). However, cross-sectional architecture normalized in sections distant from the injection site when compared with saline-injected control superior rectus muscles.

The MHC isoform expression patterns for fast and slow MHC isoforms were not strikingly different in the 7-day ricin-mAb 35-treated muscles compared with normal, but there was a clear increase in the numbers of neonatal and developmental MHC-positive myofibers (Fig. 7) compared with the number in control superior rectus muscles.

Fourteen Days after Injection
Fourteen days after ricin-mAb 35 injection at the 1:10-rMTD dose, CD11b-positive inflammatory cells were no longer present in the treated muscle cross sections. Regenerating muscle fibers were abundant at the site of injection. The increase in proportion of myofibers staining positive for developmental and neonatal MHC isoforms, noted at 7 days, persisted at the 14-day interval (not shown). Both hematoxylin and eosin (Fig. 8C) and transmission electron micrographic examination of the ricin-mAb 35-treated muscle cross sections demonstrated the presence of myoblasts and myotubes with central
nuclei (Fig. 8a). Peripheral nerve (Fig. 8b) and vasculature was normal in appearance after ricin-mAb 35 treatment of these muscles.

**DISCUSSION**

Ricin, one of the most potent toxins known, is derived from the seeds of the castor bean plant. It is a cytotoxic protein composed of a ribosome-inactivating enzyme, the A chain, linked by a disulfide bond to a galactose-\(N\)-acetylgalactosamine-binding lectin, the B chain. Ricin, by irreversibly inactivating ribosomes, inhibits protein synthesis and kills affected cells. Chemically linked to an mAb against nicotinic acetylcholine receptors, ricin becomes a skeletal muscle-specific cytotoxic agent that may have utility in the treatment of focal muscle dystonias. If the myotoxic effects of ricin-mAb 35 are discrete, titratable, sustained, and well tolerated, it may also be useful in the treatment of strabismus.

In this study, we have shown that injection of ricin-mAb 35 into the superior rectus muscles of rabbits results in an acute, dose-related, focal inflammatory reaction within the muscle that begins within 3 days of exposure and is accompanied by muscle fiber destruction. Areas of the muscle distant from the injection site appeared to be unaffected with little inflammatory response and normal myofiber size and MHC isoform composition. By 7 days, muscle fiber loss was readily apparent at the injection site, but the inflammatory cell infiltrate was greatly reduced. Fourteen days after injection, there was evidence for muscle regeneration within the treated muscles. Thus, ricin-mAb 35 was myotoxic in extraocular muscles at doses between 1:100 and 1:10 rMTD, with the most muscle destruction occurring at the highest dose.

The toxin appeared to stay localized within the muscle epimysium after a direct muscle injection. In contrast, botulinum toxin is known to spread easily within injected tissues and can cause unwanted paralysis or paresis of nearby muscles as a side effect of treatment.\(^5,6\) Presumably due to orbital diffusion of the injected drug.\(^10\) The apparent localization of ricin-mAb 35 within the injected muscle may decrease the likelihood of...
unwanted myotoxic effects on nearby extraocular muscles. The muscle-specific binding property of the ricin-mAb 35 molecule presumably plays an important role in the containment of the toxin after a direct muscle injection.8

Tissue destruction, in general, caused inflammatory cell infiltration. The time course of muscle injury and subsequent muscle loss occurred within 7 days of the ricin-mAb 35 injections. The inflammatory cell infiltration very closely mirrored the location of muscle injury in the tissue sections. The ricin-mAb 35 treatment did not cause a generalized inflammatory reaction in the nearby orbital tissues. This is in direct contrast to other types of muscle injury, such as crush or freezing injuries, which result in generalized tissue inflammation, including edema, hyperalgesia, and increased vascular permeability.11 Although widespread inflammation within the orbit would have been a negative sequela to ricin-mAb 35 injections had it occurred, the inflammatory cells that invade the injured muscle are thought to play an important role in muscle fiber regeneration.12

Muscle fiber loss in the ricin-mAb 35-treated muscles occurred relatively slowly. Although myofiber degeneration was seen as early as 3 days after injection, it took up to 7 days for maximum fiber loss to occur. In contrast, other myotoxic agents such as doxorubicin,13 bupivacaine,14,15 and anticholinesterase agents16 cause rapid necrotic muscle loss. After injection of doxorubicin into facial muscles, for example, muscle loss is relatively complete by 24 hours.13

In contrast to the permanent muscle loss caused by direct injection of doxorubicin into skeletal muscles,17 signs of regeneration in ricin-mAb 35-treated muscles were evident as early as 14 days after injection. Despite this, preliminary evidence suggests that even 105 days after ricin-mAb 35 treatment of the superior rectus, there were decreased numbers of myofibers, decreased total muscle cross-sectional areas, and an increase in the proportion of myofibers expressing immature myosin heavy chain isoforms compared with normal extraocular muscle.18 Thus, myopathic effects may persist after ricin-mAb 35 injection, despite early regeneration, even at relatively long posttreatment intervals.

Studies in which botulinum toxin injection was used for treatment of focal dystonias indicate that it takes an average of 3 days for onset of the relief of motor symptoms, an average of 7 days for maximum effect, and that sufficient relief of symptoms continues for approximately 7 to 12 weeks.19 One long-term effect of botulinum toxin has been described that results in long-term atrophy of the orbital, singly innervated myofibers, and a decrease in mean vascular density.20 These changes have been postulated to explain single Botox injection reversal of congenital esotropia. Botulinum toxin acts at the neuromuscular junction, and recovery occurs as a result of axonal sprouting and formation of new neuromuscular junctions.21 Tissue

**Figure 5.** Montage of a cross section through the distal end of a superior rectus muscle 7 days after an injection of ricin-mAb 35 at 1:10 rMTD, with immunostaining for the presence of CD11b-positive inflammatory cell infiltrate. There was a great reduction in inflammation, and only a small locus of inflammatory cell infiltration was apparent. Bar, 100 μm.

**Figure 6.** Cross section through distal superior rectus muscle injected with ricin-mAb 35 (1:10 rMTD) at 7 days. A large region of the muscle cross section was almost completely devoid of myofibers, containing only connective tissue and inflammatory cells. (a) Section immunostained for CD11b-positive inflammatory cells. (b) Section immunostained for neonatal MHC isoform. Bar, 100 μm.
Culture studies show that this process begins as early as 4 days after botulinum toxin exposure. We have shown that injection of ricin-mAb results in myofiber loss, and that the treated muscles slowly recover by myofiber regeneration. In general, muscle regeneration is a slow process. Other studies have demonstrated that despite an apparent normal morphologic appearance 90 days after a muscle injury, the regenerated muscles still produce tetanic tensions that are below normal. In the ricin-mAb treated gastrocnemius, strength testing demonstrated that these muscles were weaker and that the myotoxic effect was of longer duration than equivalent botulinum toxin injections. Thus, with the exception of the changes in singly innervated orbital myofibers, the effect of ricin-mAb 35 on the extraocular muscle regeneration.
cles is predicted generally to be more inclusive of all fibers types and more long-lived than that seen after botulinum toxin treatment.

Ricin-mAb 35 appeared to be selectively myotoxic. Both light and electron microscopic examination demonstrated that the nerves and capillaries within the ricin-mAb 35-treated tissue were normal. This suggests that neurotoxicity does not play a significant role in the decline in muscle strength seen in limb muscle after the injection of immunotoxin. It also suggests that if leakage occurs after injection, other nonmuscle tissues in the orbit are at minimal risk for unwanted cytotoxicity.

If pharmacologic treatment of strabismus is to become a widely acceptable option, agents with longer duration of action than botulinum toxin will be needed. Our results show that dose-related myotoxicity can be achieved with the novel immunotoxin, ricin-mAb 35. If future studies demonstrate long-term fiber loss with concomitant decreases in extraocular mus-

**Figure 8.** Electron microscopic examination 14 days after injection of ricin-mAb 35, 1:10 rMTD. (a) Regenerating muscle cells were present with multiple centrally located nuclei, indicating regeneration. (b) Peripher al nerve in the treated muscles had a normal appearance, indicating that the effects of ricin-mAb 35 were muscle specific. (c) Fourteen days after injection of ricin-mAb 35, many myofiber profiles in cross-section had centrally located nuclei (arrows) indicating ongoing regeneration. Muscle section was stained with hematoxylin and eosin. Bar, 100 μm.
cle contractility, ricin-mAb 35 may be an effective alternative to muscle recession in the treatment of certain types of strabismus.

References