

# Botulinum toxin pretreatment augments the weakening effect of injection with ricin-mAb35 in rabbit extraocular muscle

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<b>PURPOSE</b>	To examine force generation and duration of effect of botulinum toxin pretreatment, followed by injection of ricin-mAb35 in extraocular muscle.
<b>METHODS</b>	In normal adult rabbits, one superior rectus muscle was injected with either 5 units botulinum toxin or 1/50 maximally tolerated dose for rats (rMTD) of ricin-mAb35. Additional rabbits were first injected with 5 units botulinum toxin, and after 1, 2, or 4 weeks the same muscle was injected with either 1/10 or 1/50 rMTD ricin-mAb35. In each treatment group, the contralateral muscle was injected with an equal volume of saline. After 12 weeks (1/50 rMTD) or 6 months (1/10 rMTD), the rabbits were euthanized. Both SR muscles were removed and assayed physiologically, using an in vitro apparatus.
<b>RESULTS</b>	Twelve weeks after treatment with either botulinum toxin or immunotoxin alone, only ricin-mAb35-treated muscles were weaker than control muscles at tetanic stimulation frequencies. Pretreatment with botulinum toxin prior to injection with immunotoxin, especially at shorter intervals between injections, resulted in significant decreases in twitch and tetanic force generation compared with controls and muscles treated with ricin-mAb35 only or botulinum toxin only. At 6 months, force generation was decreased from control only in muscles treated with the higher dose of ricin-mAb35. Botulinum toxin pretreatment did not augment this effect at 6 months.
<b>CONCLUSIONS</b>	Upregulation of postsynaptic nicotinic acetylcholine receptors caused by botulinum toxin pretreatment amplifies the reduction of force generation in extraocular muscle following secondary injection of the immunotoxin ricin-mAb35 within 3 months of treatment. (J AAPOS 2007;xx:xxx)

Strabismus is associated with abnormal binocular function, and its etiology is poorly understood. Currently available treatments include patching, injection of botulinum toxin, or surgically adjusting the extraocular muscles to improve the alignment of the eyes. Recession and resection surgery can be used to alter muscle force by moving the insertion of the extraocular muscle on the globe or by shortening the muscle. However, surgery disrupts normal muscle and orbital biomechanics, particularly when large-angle corrections are needed, and

has a failure rate of 25% to 69%, depending on the study.<sup>1,2</sup>

Botulinum toxin was developed in the 1970s and was the first pharmacologic approach for the treatment of strabismus.<sup>3</sup> It has the theoretical advantage of maintaining normal muscle-globe relationships and successfully treats some forms of adult and childhood strabismus. However, its long-term outcome can be limited by the duration of its muscle-weakening effects.<sup>4</sup> In young children, the duration of treatment effect is particularly important because of the presence of a critical period for binocular sensory development in the visual cortex<sup>5</sup>; a period when cortical changes are extremely rapid.<sup>6</sup> In infantile forms of strabismus, misalignment of the eyes causes anomalous binocular interaction and irreversible loss of cortical binocular function.<sup>7</sup> The duration of this period of abnormal binocular interaction is critical in that the longer the period of misalignment, the poorer the long-term stereoacuity.<sup>8-10</sup> Therefore, whatever treatment we use must be rapid enough to allow reestablishment of binocularity and of sufficient duration that this binocularity can be maintained until long-term adaptations in the central nervous system and ocular motor plant take effect.

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Ricin-mAb35 is an immunotoxin composed of ricin, a toxin that inhibits protein synthesis, which is conjugated to a monoclonal antibody to the nicotinic acetylcholine receptor.<sup>11-14</sup> Thus, the immunotoxin is targeted to only those cells with nicotinic acetylcholine receptors. In the extraocular muscles these receptors are found only on mature myofibers.<sup>11</sup> Previous studies showed that the immunotoxin, ricin-mAb35, weakens muscle and reduces muscle cross-sectional area for an extended period of time but has a narrow therapeutic window.<sup>12,13</sup>

Botulinum toxin results in paralysis at the neuromuscular junction, followed by upregulation in the number of acetylcholine receptors in treated limb skeletal muscle,<sup>15,16</sup> and craniofacial muscles, including the extraocular muscles.<sup>17,18</sup> In fact, botulinum toxin injection into rabbit extraocular muscles results in a twofold increase in the density of neuromuscular junctions in the treated muscles.<sup>18</sup> In an effort to improve the therapeutic profile of ricin-mAb35, it seemed feasible that botulinum toxin could be injected prior to the immunotoxin to increase the number of neuromuscular junctions on the muscle surface. This would increase the number of possible binding sites for the immunotoxin, thereby increasing its myotoxic effect and potentially increasing its duration. Three separate injection protocols were compared: botulinum toxin only versus ricin-mAb35 only; botulinum toxin injection followed by injection of ricin-mAb35 at differing intervals and then examined 12 weeks after the final injection; and botulinum toxin injection followed by injection of higher dose ricin-mAb35 at differing intervals and assessed at 6 months after the final injection for alteration from controls in force generation and time to fatigue. Physiological force measurements give a good estimate of whether the muscles are significantly weakened compared with normal control values, essential if correction of eye position is to be maintained. The ultimate goal is to maximize the effectiveness of both botulinum toxin and ricin-mAb35 by using coinjection strategies.

## Methods

Adult New Zealand white rabbits were obtained from Bakkon Farms (Red Wing, MN) and housed with Research Animal Resources. All studies were approved by the University of Minnesota Institutional Animal Care and Use Committee and were in compliance with National Institutes of Health guidelines for the use of animals in research.

Rabbits were anesthetized by an intramuscular injection of ketamine, (Phoenix, St. Joseph, MO) 50 mg/kg, and xylazine (Phoenix, St. Joseph, MO) 2 mg/kg. All muscle injections were performed with direct visualization of the superior rectus muscles. Under sterile conditions, the conjunctiva was opened, and the muscle was exposed and hooked with a muscle hook. Tenon's capsule was bluntly dissected from the muscle surface, and a 30-gauge needle was inserted into the posterior belly of the muscle. The volume was slowly injected into the muscle, while pulling the needle anteriorly. Injections were confirmed to be within the

muscle when the injection volume stayed within the muscle epimysium without leakage. Previous studies demonstrated that the ricin-mAb35 did not spread outside the injected muscle.<sup>11,12</sup> One randomly selected superior rectus muscle was injected with 5 units of botulinum toxin (Botox, Allergan, Irvine, CA) in 100  $\mu$ L of sterile isotonic saline in six rabbits. This dose represents the upper end of the doses used in human strabismic patients to ensure a maximal effect from the treatment. The rabbits were allowed to survive 12 weeks. Another six rabbits were injected with 1/50 the maximally tolerated dose for rats (rMTD) of ricin-mAb35 in one superior rectus muscle and were allowed to survive 12 weeks. In another 18 rabbits, 5 units of Botox was injected into one superior rectus muscle. After 1 week, 2 weeks, or 4 weeks, the same muscle was injected with 1/50 rMTD ricin-mAb35 and allowed to survive 12 weeks after the second injection in the series. When these results were analyzed, an additional set of 18 rabbits was similarly treated with Botox followed 2 weeks later by immunotoxin, using the same parameters as above except that the dose of ricin-mAb35 was increased to 1/10 rMTD. This second set of 18 rabbits was allowed to survive 6 months after the last injection. In all animals, the contralateral superior rectus muscle received an injection of 0.1 mL sterile saline to serve as a control.

An *in vitro* system of measuring muscle force was used.<sup>13</sup> To verify the physiological health of the EOMs during testing, we compared twitch tension generation at the beginning, midway, and again after the final measurement was taken. We previously determined that there was less than a 5% variability in forces measured from right and left orbits in the same untreated animal.<sup>13</sup> At the appropriate postinjection survival interval, the rabbits were deeply anesthetized with ketamine and xylazine as indicated above. The chest was surgically opened, and a bilateral thoracotomy followed by exsanguination was performed.<sup>13</sup> Both superior rectus muscles were removed in their entirety from the orbits and placed in warm, oxygenated Ringer's solution. A suture loop was placed on the proximal and distal ends of the muscles to allow them to be mounted in *in vitro* incubation chambers, with the upper loop attached to a force transducer on a lever arm. For the entire experiment, the muscles were continually bathed in oxygenated Ringer's at 30°C. Generated force in grams was recorded using the 1205 Intact Muscle Test System and Dynamic Muscle Control software (Aurora Scientific, Aurora, Ontario, Canada). The muscle mass and length were determined. These measurements were used to determine force per cross-sectional area. This is obtained by dividing muscle mass (g) by the product of muscle length (cm) times a muscle density of 1.056 g/cm<sup>3</sup>. This yields force in g/cm<sup>2</sup>, which is converted to mN/cm<sup>2</sup>.

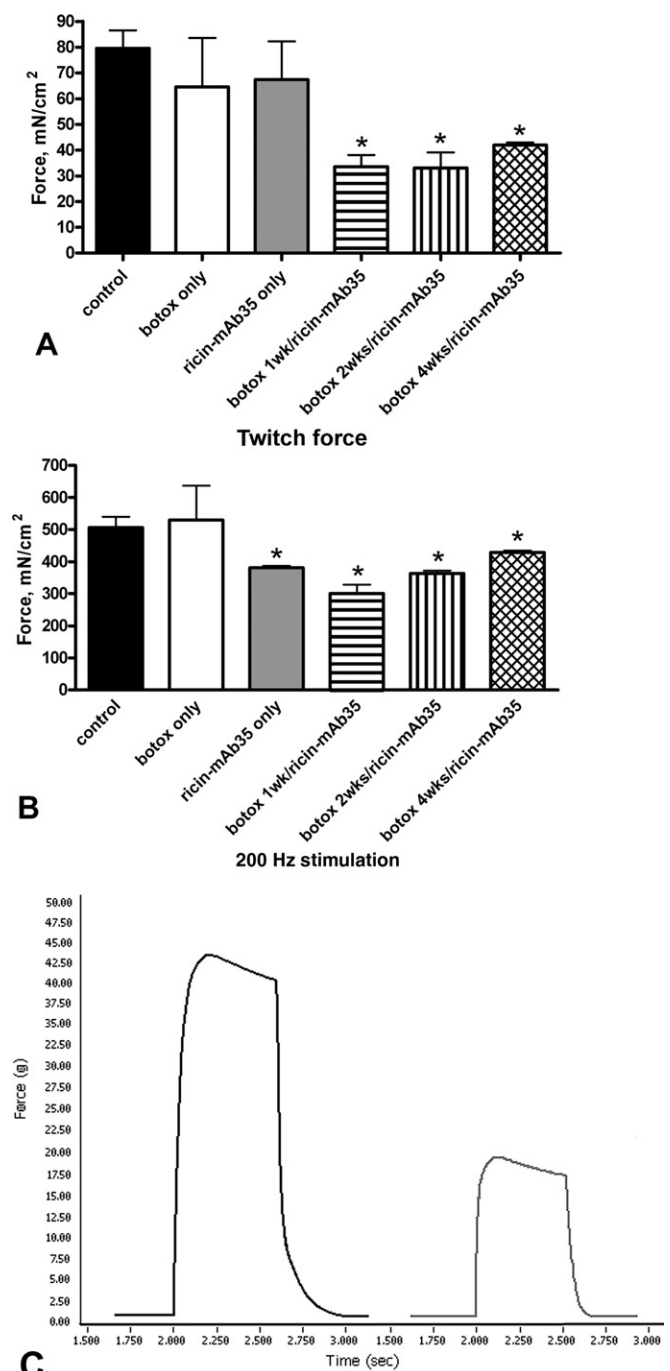
Both control and treated superior rectus muscles were tested simultaneously using our standard physiological protocol. Briefly, supramaximal stimulation intensity was determined by increasing voltage until maximal contraction was achieved using square-wave pulses of 0.5 ms duration (Aurora Scientific 701B bi-phase current stimulator) and delivered to the muscles via flanking platinum electrodes. Isometric length-tension curves were determined by stimulating each muscle at supramaximal intensity (500 mA, 0.5 ms) while varying the preload (resting

length) over a range of 0.5 g to 10.0 g. The optimal preload was determined by incrementally increasing the resting muscle length to achieve maximum isometric twitch force, allowing 60 seconds of rest between stimuli. All further tests were performed with supramaximal stimulus intensities at optimal preload. After two stabilizing near-tetanic stimulations (150 Hz, 500 mA, 0.5 ms) with 2 minutes rest between stimuli and 5 minutes rest after two consecutive stimulations, force development was determined for single, double, and triple pulses (0.5 ms pulse duration) with 2 minutes of rest between stimuli. Muscles were then stimulated at frequencies of 10, 20, 40, 100, 150, and 200 Hz at a train duration of 500 ms with a 2 minute rest between each stimulation. After 2 minutes rest, the muscles were then subjected to a fatigue protocol where a tetanic stimulus, consisting of a 1 second train at 150 Hz, was delivered every 2 seconds. The muscles were stimulated for 600 seconds, and the time to 50% reduction in generated muscle force was determined. Data from treated and control muscles were pooled at each postinjection interval and compared with the paired *t*-test. A *p*-value  $\leq 0.05$  was considered statistically significant.

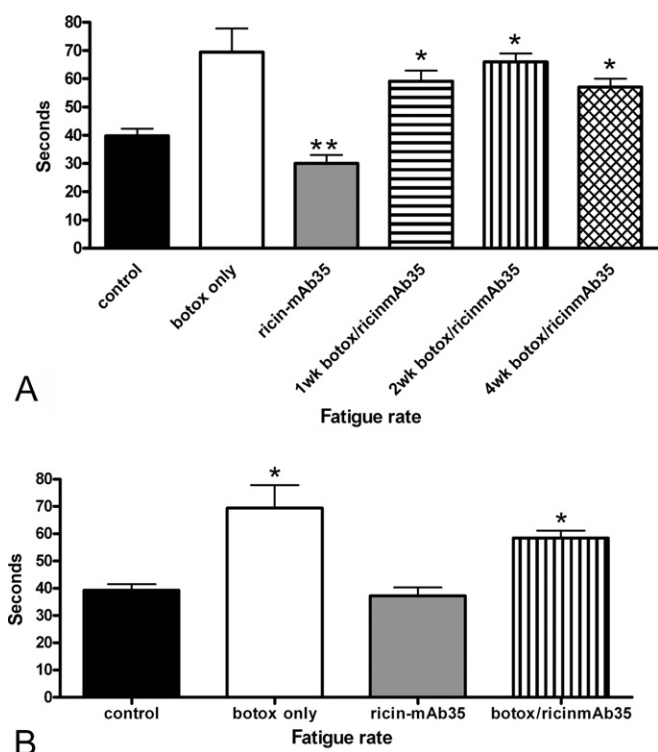
## Results

After 12 weeks, there was no statistical difference between the twitch force generated by the superior rectus muscles treated with either Botox alone or 1/50 rMTD ricin-mAb35 alone and the contralateral control muscles injected with saline (Figure 1A). However, in the superior rectus muscles pretreated with botulinum toxin 1 week, 2 weeks, or 4 weeks prior to injection with 1/50 rMTD ricin-mAb35, all the treated muscles showed significantly reduced twitch force generation (Figure 1A). At 200 Hz, tetanic force generation for the superior rectus muscles treated with Botox alone was not different from control, whereas ricin-mAb35-treated muscles generated less tetanic force (Figure 1B). Pretreatment with botulinum toxin augmented this effect, particularly when the Botox and ricin-mAb35 injections were separated by only 1 week (Figure 1B and C). When the interval between Botox and ricin-mAb35 injections was increased to 2 or 4 weeks, the augmentation effect lessened. Botulinum toxin significantly decreased the rate of fatigue, defined as time to 50% of the maximum tetanic force, either when given alone or when used for pretreatment prior to ricin-mAb35 injection when compared with normal control extraocular muscles (Figure 2A). This effect persisted at 6 months (Figure 2B). Ricin-mAb35, by contrast, when used alone, increased time to fatigue at 12 weeks but not at 6 months.

In the next set of experiments, a higher dose of ricin-mAb35 was administered. Previous studies<sup>12</sup> demonstrated greater force reduction with the higher dose of ricin-mAb35 at 12 weeks than seen in Figure 1. Thus, for the 6-month studies, we used the higher dose of 1/10 rMTD of immunotoxin. When single twitch force was examined 6 months after treatment, both the immunotoxin only and the botulinum toxin/ricin mAb35 injected animals showed reduced twitch force generation (Figure 3A). Twitch force



**FIG 1.** Twitch and tetanic force per cross-sectional area 12 weeks after superior rectus injection. (A) Force in mN/cm<sup>2</sup> after stimulation at twitch frequency, 12 weeks after single injections of botulinum toxin only, 1/50 rMTD ricin-mAb35 only, or botulinum toxin followed 1, 2, or 4 weeks later by injection of 1/50 rMTD ricin-mAb35 was assessed and compared with contralateral saline-injected controls. (B) Force in mN/cm<sup>2</sup> after stimulation at 200 Hz. Asterisks indicate significant difference from control muscles. (C) Representative force response, in grams, to a single stimulation at 200 Hz in control (black) and Botox-ricin-mAb35 (gray)-treated superior rectus muscles demonstrating muscle fusion at this stimulation frequency. Asterisks indicate a significant difference from control muscles.

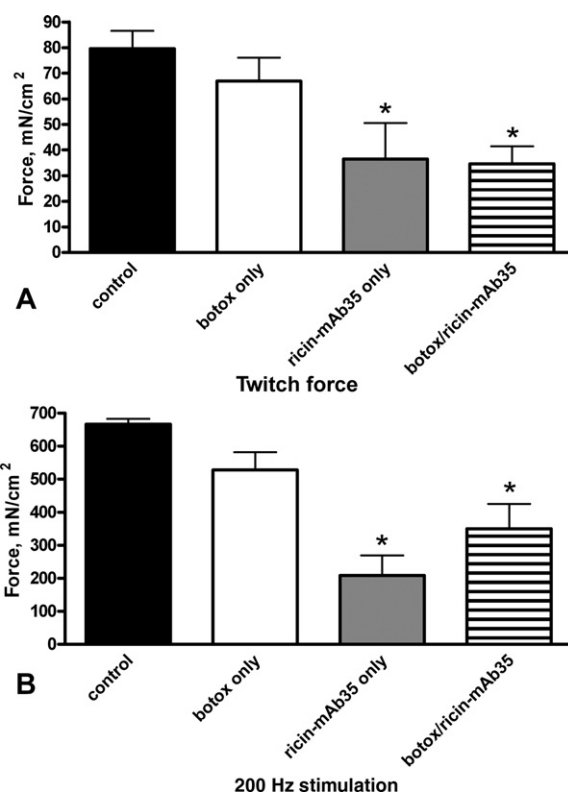


**FIG 2.** Time to 50% of maximum force at 12 weeks and 6 months. Time to fatigue was determined as the time at which there was a 50% reduction in generated muscle force and calculated for: (A) control and superior rectus muscles after single injections of botulinum toxin, 1/50 rMTD ricin-mAb35, or botulinum toxin followed 1, 2, or 4 weeks later by injection of 1/50 rMTD ricin-mAb35 and (B) control and superior rectus muscles after single injections of botulinum toxin, 1/10 rMTD ricin-mAb35, or botulinum toxin followed 2 weeks later by injection of 1/10 rMTD ricin-mAb35. \* Significant increase from control muscles. \*\* Significant decrease from control muscles.

generation following only botulinum toxin injection was also reduced in these long-term animals compared with the contralateral control, although it did not reach statistical significance. This effect persisted at 6 months after treatment. When tetanic force was examined at a stimulation frequency of 200 Hz, both the ricin-mAb35-treated muscles and the botulinum toxin/ricin-mAb35-cotreated muscles 6 months after treatment were significantly weaker than the control muscles (Figure 3B), although there was no augmentation effect from the botulinum toxin pretreatment. Despite the trend toward decreased force, botulinum toxin only were not significantly different from control.

## Discussion

When ricin-mAb35 is administered at a relatively low dose of 1/50 rMTD, decreased force generation is seen in the treated muscles only at high stimulation frequencies. However, in the muscles that were pretreated with botulinum toxin 1, 2, or 4 weeks before low-dose ricin-mAb35 injection, both twitch and tetanic force generation were significantly reduced compared with control at 12 weeks



**FIG 3.** Twitch and tetanic force per cross-sectional area 6 months after superior rectus injection. (A) Force in mN/cm<sup>2</sup> after stimulation at twitch frequency 6 months after single injections of botulinum toxin only, 1/10 rMTD ricin-mAb35 only, or botulinum toxin followed 1, 2, or 4 weeks later by injection of 1/10 rMTD ricin-mAb35 force was assessed and compared with contralateral saline-injected controls. (B) Force in mN/cm<sup>2</sup> after stimulation at 200 Hz. Asterisks indicate significant difference from control muscles.

postinjection. Thus, if a lower dose of ricin-mAb35 is required for patient safety, its effectiveness can be increased by preinjection of the extraocular muscle (EOM) with botulinum toxin. When a higher dose of ricin-mAb35 is administered, preinjection of botulinum toxin does not either increase or decrease its effect in the long term.

One concern with the use of the ricin-mAb35 was the relatively high dosage needed for long-term maintenance of muscle weakness. Botulinum toxin treatment results in increased neuromuscular junction density in treated extraocular muscles 1, 2, and 4 weeks later.<sup>18</sup> The ricin-mAb35 immunotoxin acts by specific binding of the antibody to the nicotinic acetylcholine receptor.<sup>14</sup> Thus, it was hypothesized that increasing the number of neuromuscular junctions could result in increased myotoxicity of the ricin-mAb35. Previous studies showed that ricin-mAb35 injected 3 days after a botulinum toxin injection did not result in an amplification of effect (SP Christiansen, B Anderson, LK McLoon, Combining ricin-mAb35 and botulinum toxin to weaken extraocular muscle [Invest Ophthalmol Vis Sci 2006;47:E-Abstract 5395]). As the lower dose of the immunotoxin, at the longer post-Botox treatment intervals of 1-4 weeks, the weakening effect was

amplified. This suggests that the effectiveness of lower doses of ricin-mAb35 may be enhanced using this approach. At the higher dose of ricin-mAb35 (1/10 rMTD) used in our previous studies,<sup>11-13</sup> pretreatment with botulinum toxin prior to immunotoxin administration does not amplify its toxic effects. Presumably, this is due to fact that the increased immunotoxin potency results in sufficiently more muscle damage such that more neuromuscular junction binding sites on remaining muscle fibers do not increase its effect.

The variable amount of force recovery between individual patients and between individual animals after botulinum toxin treatment has been described in the literature. Differences in effectiveness in patient and animal models have been ascribed to spread of the toxin within the injected muscles.<sup>19</sup> One of the more surprising results from our study was the effect botulinum toxin had on fatigue rate; botulinum toxin treated muscles fatigued more slowly than control or ricin-mAb35-treated muscles. In short-term experiments on the effect of botulinum toxin on tibialis anterior muscles in rats, time to fatigue was seen to increase in the treated animals.<sup>19,20</sup> It should be pointed out that in the short time periods of these studies, return of muscle function is solely due to nerve sprouts and formation of new neuromuscular junctions.<sup>16</sup> However, in the long term, recovery is due to return of synaptic activity at the original nerve terminals, and with that recovery the new nerve sprouts and newly formed neuromuscular junctions disappear.<sup>21</sup> Thus, there may be long-term changes in the original neuromuscular junctions of botulinum toxin treated muscles that have not been characterized previously. There are very few long-term changes known to occur in extraocular muscle as a result of botulinum toxin injection. Certainly in the short term, myofiber hypertrophy occurs.<sup>22,23</sup> Long-term analysis of myosin heavy chain isoform (MyHC) composition after botulinum toxin treatment shows a long-lasting decrease in myofibers positive for MyHC2b and a complete disappearance of the EOM-specific MyHC 8 months after a botulinum toxin injection, fibers that are responsible for the fastest contractile properties in the EOM.<sup>23</sup> Long-term changes caused by botulinum toxin mimic those seen with long-term nerve denervation, and concomitant to those MyHC isoform changes in denervated muscle models are significant physiological changes, including slower contraction rates and decreased contraction velocities.<sup>24,25</sup> Further study is needed to determine how botulinum toxin alters the original neuromuscular junctions to result in a longer time to fatigue.

To use botulinum toxin to increase the duration of effect of lower doses of ricin-mAb35, some method for accurate intramuscular injection will be needed. It is well known that botulinum toxin results in rapid muscle paralysis, evident within 18 hours of injection.<sup>26</sup> While some physicians do not use electromyographic guidance for extraocular muscle injections, for those that do, electromyographic-guided injection of the EOM would be more dif-

ficult after Botox injection, because the electrical signal would likely be either markedly diminished or absent. The present study suggests that sequential slow release or sequential delivery systems with the ricin mAb-35 in a slow release mode might be an effective strategy for increasing the duration of its toxicity when a lower dose is used. Sustained release of IGF-1 has been used effectively in animal models to increase muscle force for 3 months and is a viable approach for the development of long-term treatments.<sup>27</sup> This strategy would allow a lower dose of the immunotoxin to be administered. It should be pointed out, however, that the highest dose of ricin-mAb35 tested thus far results in long-term decreases in muscle force generation, as evidenced by our 6 month time point. Further studies in the nonhuman primate are needed to determine local and systemic safety profiles of 1/10 rMTD ricin-mAb35, and minimum effective doses in higher animals remain to be determined. If lower doses are required, sequential treatment with botulinum toxin and immunotoxin may be an effective adjunct to current treatment strategies.

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