Long-term Effects of Ricin-mAb 35 on Extraocular Muscles of Rabbits: Potential Treatment for Strabismus

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PURPOSE. The immunotoxin, ricin-mAb 35, composed of ricin conjugated to a monoclonal antibody against the nicotinic acetylcholine receptor of skeletal muscle, has been proposed as a potential new agent for treatment of focal muscle dystonias. It has been demonstrated that direct injection of ricin-mAb 35 into rabbit extraocular muscle (EOM) results in significant muscle loss within 1 week. In this study, the long-term myopathic effects of ricin-mAb 35 on extraocular muscle were investigated.

METHODS. Rabbit superior rectus muscles were injected with ricin-mAb 35 at a dose of 0.2 μg/kg, with the contralateral superior rectus muscle serving as the control. After 56 days, 105 days, and 1 year, the superior rectus muscles were removed and prepared for light or electron microscopy. Postinjection changes in muscle fiber morphometry and ultrastructure were examined. Immunohistochemical markers were used to identify inflammatory cellular infiltrate and myosin heavy chain (MHC) isoform expression.

RESULTS. Despite evidence of ongoing regeneration, treated muscles continued to show a decrease in both myofiber number and in total cross-sectional area 56 and 105 days after injection. Individual myofiber cross-sectional areas were markedly heterogeneous at 56 days. Myofiber number and muscle cross-sectional area returned to normal 1 year after injection, but pronounced heterogeneity of myofiber size remained. The most significant changes in myosin heavy chain (MHC) isoform expression occurred in the orbital layer, where, at 56 and 105 days, there were increased numbers of fast and neonatal myofibers and decreased numbers of slow myofibers. In the global layer, after both 105 days and 1 year, there was a decrease in myofibers that were positive for slow, neonatal, and development MHC expression.

CONCLUSIONS. EOM injection with ricin-mAb 35 results in a sustained decrease in muscle mass at 105 days after injection, with subtler morphometric changes persisting even to 1 year. Changes in muscle force development as a result of ricin-mAb 35 injection are currently under investigation. This novel immunotoxin may be useful in the treatment of strabismus if these studies show sustained weakness in treated muscles.

Ricin is a cytotoxic protein composed of a ribosome-inactivating enzyme (A chain), linked by a disulfide bond to a galactose-Vacuetylgalactosamine-binding lectin (B chain). Ricin, by irreversibly inactivating ribosomes, inhibits protein synthesis and kills affected cells. A newly developed immunotoxin, ricin-mAb 35, consists of ricin chemically linked to a monoclonal antibody against nicotinic acetylcholine receptors. This skeletal muscle-specific immunotoxin appears to cause long-lasting weakness in limb muscle as a consequence of local direct myotoxicity and has been suggested to be a potential treatment for focal muscle dystonias. The antibody to the nicotinic acetylcholine receptor specifically targets the ricin to mature myofibers that express that receptor and appears to spare other cells, including myoblasts. This permits the muscle to regenerate after the injury induced by ricin-mAb 35 injection.

If the effects of ricin-mAb 35 are similar in extraocular muscle, it may also be useful in the treatment of strabismus or nystagmus, by permitting long-term, dose-related adjustments in the force generation of specific extraocular muscles that could, in turn, result in changes in eye alignment. It may also have some advantages over currently available modalities. Incisional surgery, for example, compromises normal muscle dynamics by altering the arc of contact of the muscle with the globe, the intrinsic elasticity of the surgically altered muscles, the resting tension on the agonist-antagonist pair and generated twitch tension. In addition, surgery unavoidably induces scarring and often disrupts muscle relationships with soft-tissue pulleys that could further alter extraocular muscle function.

Botulinum toxin, approved for clinical use more than a decade ago, has been used effectively in both childhood and adult strabismus. However, the treatment of congenital strabismus with botulinum toxin often yields inconsistent results, particularly when the initial deviation is large. Reinjections are commonly necessary to achieve a stable result. The principle limitation of botulinum toxin injection has been its relatively short duration of action. The possibility that ricin-mAb 35, a targeted myotoxic agent, may have long-term effects on muscle strength prompted this investigation.

Initial studies of the acute response of rabbit extraocular muscles to direct injection with ricin-mAb 35 demonstrated that there was a dose-related focal injury to the muscles, with a self-limited inflammatory component and significant muscle fiber loss. In this study, we examined the long-term effects of ricin-mAb 35 on extraocular muscle morphometry, ultrastructure, and immunohistochemistry.

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 NIH guidelines for use of animals in research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ricin-mAb 35 was prepared and purified as previously described. It is free of unbound ricin as indicated by HPLC data (Fig. 1A). The HPLC procedure separates ricin-mAb 35 from 99.99% of free ricin and 80% of the excess antibody. The ricin-mAb 35 fractions from the HPLC procedure were characterized by gel filtration chromatography, SDS-PAGE, and Western blotting.
column were examined by Western blot analysis. The construct band is indicated by the arrow (Fig. 1B). Further purification was achieved with an affinity column that removes essentially all the remaining free antibody, as demonstrated by the Coomassie-stained gel (Fig. 1C; arrow).

Toxicity testing by Hott et al. showed that the maximum tolerated dose (MTD) of ricin-mAb 35, the highest dose at which all treated animals survived, is 2 \( \mu \)g/kg for mice. Unbound ricin, however, is far more toxic. In rabbits, the MTD for free ricin is 0.22 \( \mu \)g/kg. The minimum lethal dose of free ricin in rabbits is 0.44 \( \mu \)g/kg, which results in a precipitous decline in both systolic and diastolic blood pressure. Our rabbits remained healthy and active after the ricin-mAb 35 injections, again suggesting minimal exposure to free ricin. In previously published work, we found that to achieve a consistent histologic effect, an injected dose of 1/10 of the mouse MTD was required in rabbit extraocular muscle. All animals in the present study received an injection of ricin-mAb 35 at a dose of 1/10 MTD, or 0.2 \( \mu \)g/kg, in one superior rectus muscle.

Each rabbit was anesthetized with an intramuscular injection of ketamine (10 mg/kg) and xylazine (2 mg/kg). Proparacaine solution was placed in the conjunctival cul-de-sac of both eyes to reduce the blink reflex. Treatment and control eyes were randomized before surgery. The superior rectus muscles were exposed through a conjunctival peritomy, using aseptic technique. These muscles were chosen because of their proximity to the limbus and ease of surgical exposure. Under direct observation, one superior rectus muscle of
each rabbit was injected with ricin-mAb 35, diluted with sterile isotonic saline to a dose of 1/10-MTD in a volume of 100 μL. Injections were made slowly through a 30-gauge needle that was directed posteriorly into the muscle belly. The needle was left in place for 30 seconds after completion of injection to reduce leakage into the orbit. The control (contralateral) superior rectus muscle was injected with 100 μL sterile isotonic saline alone. As in both previous studies involving the ricin-mAb 35 construct, no signs of systemic toxicity were seen. No animals ever appeared to be sick or lethargic. All appeared to be completely healthy. No animals died as a result of the ricin-mAb 35 injections.

The animals were killed with an overdose of barbiturate anesthesia 56 days (n = 2), 105 days (n = 3), or 1 year (n = 2) after the ricin-mAb 35 injections. The injected superior rectus muscles were excised in their entirety. Each muscle was embedded in either optimal cutting temperature compound (OCT) or tragacanth gum, frozen on 2-methylbutane chilled to a slurry in liquid nitrogen, and sectioned at 12 μm on a cryostat. The muscle sections were stained immunohistochemically with antibodies to fast, slow, developmental, and neonatal myosin heavy chain (MHC) isoforms (NovoCastra-Vector Laboratories, Burlingame, CA). Immunohistochemistry was performed without fixation or quenching on frozen sections. Sections were rinsed in phosphate-buffered saline (PBS; pH 7.4), incubated in normal horse serum for 15 minutes, and then incubated in the appropriate primary antibody for 1 hour at room temperature. Antibodies were diluted 1:40 for fast and

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\text{FIGURE 2. (A)} \hspace{1cm} \text{FIGURE 3. (A)} \hspace{1cm} \text{FIGURE 3. (B)} \hspace{1cm} \text{FIGURE 3. (C)}
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*Statistically significant.
slow MHC isoforms and 1:20 for developmental and neonatal MHC antibodies. The sections were rinsed in PBS and incubated using an avidin-biotin peroxidase kit (Vectastain ABC; Vector Laboratories). The peroxidase was visualized by incubation with diaminobenzidine and heavy metals. Sections were immunostained for the presence of cd11b-positive neutrophils, lymphocytes, and macrophages by methods described previously.10

The long-term effects on MHC isoform expression were examined by counting contiguous fibers in three to four random fields in each of the three layers of the superior rectus cross sections. The number of myofibers that were positive or negative for the expression of the four MHC isoforms was determined, and calculations were performed to determine the percentage of fibers positive for each of the isoforms. All muscles were analyzed using an image analysis system (Bioquant; R and M Biometrics, Nashville, TN). Muscle loss after ricin-mAb 35 treatment was determined by comparing means for total muscle cross-sectional area in square millimeters and total myofiber number measured in three cross-sections from the midbelly of treated and control muscles at each postinjection interval. Individual myofiber cross-sectional areas were determined under bright-field microscopy by manual tracing of three to four representative fields in each of the three layers of the muscles (outer orbital, inner orbital, and global) of all muscles. Histograms were prepared of individual control- and ricin-mAb 35–treated muscles at each of the three postinjection intervals. The cross-sectional areas were divided into bins at 200-μm increments, and the histograms were constructed using biostatistics software (Prism and Statmate; Graphpad, San Diego, CA). Although only single muscles of each age are shown, the results for identically treated muscles were similar. Statistical significance is defined as P < 0.05. An F test was used to indicate that the variances of the control and injected groups were not statistically different.

One pair of ricin-mAb 35–injected superior rectus muscles treated with a dose of 1/10 MTD was prepared for electron microscopic examination of the long-term effects of ricin-mAb 35 on the treated muscle. After a postinjection interval of 105 days, the rabbit was killed with an overdose of barbiturate and perfused through the heart with 1% paraformaldehyde-1.25% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). The superior rectus muscles were dissected from the orbits, trimmed, and postfixed in 1% osmium tetroxide in phosphate buffer. The muscles were dehydrated in a graded series of alcohols, embedded in Epon, and sectioned in both 1-μm and ultrathin sections for examination under the electron microscope.

RESULTS

Before necropsy, the periorbita and conjunctiva appeared quiet in each animal. No significant scarring was encountered during removal of the superior rectus muscles, each of which appeared grossly normal. All sections were almost completely devoid of inflammatory cells in sections immunostained for cd11b (not shown).

Muscle Loss

There was a significant decrease in total muscle cross-sectional area both 56 and 105 days after ricin-mAb 35 injection (Fig. 2A). Because connective tissue replacement as a result of myofiber loss could give an inaccurate picture of muscle loss.
when measured as a single total area measurement, total myo-
fiber counts were also made in representative sections in the
midbelly region of the control and ricin-mAb 35–
injected mus-
cles. After both 56 and 105 days, there was a signi-
ficant loss in
total myo-
fiber number as a result of ricin-mAb 35 exposure
(Fig. 2B). Muscle
fiber number returned to normal by 1 year
after toxin treatment.

MHC Expression Patterns
There was an alteration in the patterns of MHC isoform myo-
fiber expression in the superior rectus muscles that was most
pronounced at the 56- and 105-day postinjection intervals (Figs. 3,
4). After both 56 and 105 days, in the outer orbital layer, there was
an increase in the number of myofibers positive for the fast MHC
isoform and a decrease in the number of myofibers positive for
the slow MHC isoform (Figs. 4A, 4B). An interesting observation
was that, at the longer survival times, there was a decrease in the
number of myofibers positive for the neonatal MHC isoform
compared with normal in the inner orbital and global layers and
in the outer orbital layer by 1 year (Fig. 4D). The most striking
differences seen 1 year after the initial injury with ricin-mAb 35
were large decreases in neonatal MHC expression in all layers and
a decrease in slow and developmental MHC isoform expression in
the global layers (Fig. 4).

Myofiber Heterogeneity
The most striking change noted in the muscles treated with
ricin-mAb 35 was in single-fiber cross-sectional area as the
muscle recovered from injury and muscle fibers regenerated.
For ease of presentation, only a single control and a single
ricin-mAb 35–treated muscle are shown in each of the histograms, although other muscles treated similarly yielded similar
histograms.

Electron Microscopic Examination
Transmission electron micrographic examination of the ricin-
mAb 35–treated muscle cross-sections 105 days after treatment
showed that the muscle fibers were largely normal in appear-
ance. However, myofibers could be found that showed disruption
of their myofibrillar arrays and dilatations of the sarcoplasmic
reticulum (Fig. 7A). Myofibers with centrally placed nuclei
were also present (Fig. 7B). The nerves and capillaries within
the ricin-mAb 35–treated muscles were normal in appearance
(Fig. 7C).
DISCUSSION

Injection of ricin-mAb 35 appears to have long-lasting, discrete, sustained, and well-tolerated myotoxic effects on extraocular muscles of rabbits. The toxic effects of the ricin-mAb 35 appeared to be directed at muscle only. It did not appear to spread to neighboring structures within the orbit. This decreases the likelihood of unwanted myotoxic effects on nearby extraocular muscles or of nonspecific toxicity to other orbital tissues. Ricin-mAb 35 did not appear to be acutely toxic to peripheral nerves or capillaries within the treated muscles nor at the long postinjection intervals in the present study (Fig. 5C). The muscle-specific binding property of the ricin-mAb 35 molecules presumably plays an important role in the containment of the toxin after a direct muscle injection.

The myotoxic effects of ricin-mAb 35 injection are not likely to be caused by contamination with free ricin for two reasons. First, the purification method used in the production of ricin-mAb 35 ensures that the injected material is essentially devoid of free ricin. Second, free ricin exerts its toxic effects systemically. Direct injection of free ricin into leg muscles, even at lethal doses, does not result in muscle injury (Youle, unpublished data, 1999). An important benefit of the ricin-mAb 35 conjugate appears to be the specific targeting of the toxin to myofibers, which permits myotoxicity without appreciable systemic toxicity.

FIGURE 6. Increased heterogeneity of myofiber cross-sectional areas present in the ricin-mAb 35–treated superior rectus muscle compared with normal controls in the (top) outer orbital and (bottom) global layers 1 year after injection of ricin-mAb 35 into the superior rectus. For ease of presentation, only a single control and a single ricin-mAb 35–treated muscle are shown in each of the histograms, although other muscles treated similarly yielded similar histograms.

FIGURE 7. A ricin-mAb 35–treated superior rectus muscle was examined 105 days after injection, by transmission electron microscopy. (A) The myofibers were generally normal in appearance, except many fibers contained regions with dilated endoplasmic reticulum. (B) Myofibers were also found with centrally placed myonuclei, a finding typical of muscle that has undergone regeneration. (C) The peripheral nerves and capillaries appeared normal within these muscle cross sections.
Several studies have shown that ricin conjugated to antibody molecules, such as CD22 or CD19, targets the toxicity of ricin to specific cancer or immune system cells, reducing the risk to patients of systemic toxicity. Several clinical trials using immunotoxin therapy in patients with brain cancer are in progress, testing either ricin A or a genetic mutant of diphtheria toxin conjugated to transferrin. These clinical trials have been very successful, and the patients have shown no evidence of systemic toxicity with these targeted immunotoxins. Thus, the strategy of linking a potent toxin to a targeting antibody or molecule is a successful approach for the direct, pharmacologic treatment of a number of diseases.

In contrast to botulinum toxin, with which functional recovery occurs by means of axonal sprouting and formation of new neuromuscular junctions, muscle recovery after injection of ricin-mAb 35 occurred slowly, by regeneration of the muscle. The initial work by Hott et al. showed that ricin-mAb 35 also spares the satellite cells within treated extraocular muscles. At 105 days after toxin treatment, myofiber number and overall muscle cross-sectional areas were still reduced, compared with control muscles. In addition, ultrastructural abnormalities in treated muscle persisted at 105 days including myofibers with central nuclei that are characteristic of regenerating muscle. There was a slow return, over the course of 1 year, to the normal number of myofibers in the treated muscles. Even at 1 year, however, there was a more heterogeneous range of myofiber cross-sectional areas than that seen in control muscles.

Muscle regeneration is normally a slow process. Previous studies have shown that even in muscles with an apparently normal morphologic appearance 90 days after a muscle injury, the regenerated muscles still produce tetanic tensions that are below normal. This suggests that the long-lasting morphologic alterations in extraocular muscles injected with ricin-mAb 35 could result in long-term physiological changes in the treated EOM as well. Future work will test this hypothesis.

Although the clinical use of botulinum toxin is now well established, the development of new pharmacologic agents for the treatment of strabismus has not been pursued, despite the potential advantages of ease of administration, limitation of postoperative scarring, and preservation of normal extraocular muscle–globe mechanical dynamics. There are other myotoxins that produce localized muscle weakness that have been investigated as potential treatments for focal muscle dystonias and contracture. None seems satisfactory for treatment of strabismus, however. For example, in animals with passive immunity to tetanus toxin, injections of tetanus toxin into orbicularis oculi resulted in weakness in the treated muscles. However, local spread of this toxin resulted in unwanted muscle weakness in neighboring muscles. Dooxorubicin, another potent myotoxin, has permanent effects on muscle, and muscle loss is profound. Ideally, injected agents should allow titratable adjustment of extraocular muscle force generation so that, in the presence of abnormal efferent motor signals, binocular alignment can be achieved. These effects must last sufficiently long that sensory and motor adaptation can occur to create a permanent change in the rotational position of the globe. The treatment must not create significant inflammation or cause unwanted collateral damage to adjacent extraocular muscles or other orbital structures.

Our studies suggest that ricin-mAb 35 may have some advantages over other pharmacologic approaches currently in clinical use or under investigation in laboratory studies. It appears to have a long-lasting and muscle-specific effect, presumably because it is specifically targeted to mature myofibers.

References

17. Louboutin JP, Fichter-Gegnepain V, Noireaud J. External calcium distribution of the targeted toxin TF-CRM107 in patients with cancer or immune system cells, reducing the inflammatory response, suggests that ricin-mAb 35 may be a potent and well-tolerated new treatment for strabismus.

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It is important to note that treatment with ricin-mAb 35 does not result in permanent muscle loss. This, combined with the self-limited and short-lived inflammatory response, suggests that ricin-mAb 35 may be a potent and well-tolerated new treatment for strabismus.