Morphopathological changes induced in the extraocular muscles by the local anesthetic agent bupivacaine hydrochloride were studied in the monkey using light and electron microscopy. Retrobulbar anesthetic blocks, using 0.75% bupivacaine hydrochloride, were performed in five adult cynomolgus monkeys. Morphological alterations in extraocular muscle fiber types were examined following survival periods of 3–27 days. Bupivacaine injections produced a mild and very limited myopathic response, with changes largely restricted to the global layer singly-innervated muscle fiber type which is characterized by low mitochondrial content. For survival times beyond 3 days, this fiber type exhibited peripheral migration and swelling of mitochondria and an outside-in pattern of myofibril dissolution. Some affected fibers also exhibited the Ringbinden or ring fiber pathology. Maximal myotoxic response was observed at 14 days after injections, and pathological changes were largely resolved by 27 days. A more limited analysis of the effects of retrobulbar injection of lidocaine revealed similar morphopathological responses, thereby suggesting that these effects are a property common to the entire class of aminoacyl anesthetics. In contrast to previous observations in other skeletal muscle, the extraocular muscles proved to be unexpectedly resistant to local anesthetic treatment as only limited alterations were observed. The observed muscle fiber type specificity was interpreted to result from differences in the relative ability of muscle fiber types to adapt to anesthetic-induced elevations of intracellular free calcium levels. Invest Ophthalmol Vis Sci 29:163–174, 1988

Bupivacaine hydrochloride (Marcaine®, Winthrop-Breon Laboratories, New York, NY) is an aminoacyl type local anesthetic commonly used in ophthalmic procedures due to its prolonged anesthetic and akinetic properties. It is used either alone, or in conjunction with more rapidly acting agents of the same class (eg, lidocaine). While local anesthetics serve to block generation and conduction of action potentials by decreasing the transient voltage-dependent opening of sodium channels, these agents also competitively displace calcium from intracellular binding sites. An undesired side effect of many local anesthetics has been the induction of skeletal muscle damage in the vicinity of the application site. A bupivacaine-induced elevation of muscle fiber free calcium concentration is most likely responsible for the agent's myotoxic activity. Elevated intracellular free calcium levels trigger calcium-activated neutral proteases (calpain, EC 3.4.22.17) and, to a lesser extent, lysosomal enzymes, thereby leading to myofibrillar degradation. Bupivacaine myotoxicity, in many respects, mimics those myopathic changes which occur in Duchenne muscular dystrophy.

Recent reports have suggested that the myotoxic properties of retrobulbar anesthetics may be responsible for occasional postoperative diplopia and ptosis. Presumably, patients experience transient motor deficits until the anesthetic-induced extraocular muscle damage is repaired. The present study was designed to examine this hypothesis in the cynomolgus monkey, a species in which extraocular muscle organization and fiber types are virtually identical to that of man. Specifically, these studies examined light and electron microscopic alterations induced in primate extraocular muscle fiber types by single retrobulbar injections of bupivacaine hydro-
chloride or lidocaine hydrochloride. These local anesthetics produced an unexpectedly mild myotoxic response in the extraocular muscles, consisting of the breakdown of myofilaments in a particular muscle fiber type. Furthermore, morphopathological changes were correlated with muscle fiber mitochondrial and internal membrane system content. Fiber types having high mitochondrial content were resistant to anesthetic-induced damage, while those fiber types with low mitochondrial and high sarcoplasmic reticulum were subject to disruption by aminoacyl anesthetics. Myotoxicity also correlated with the innervation pattern of extraocular muscle fiber types, in that only singly-innervated fibers were affected.

Materials and Methods

Retrobulbar anesthetic blocks were performed in five adult cynomolgus monkeys (Macaca fascicularis). Monkeys were immobilized with ketamine hydrochloride, and 0.75% bupivacaine hydrochloride (Marcaine®, Winthrop-Breon Laboratories) containing 75 units/ml hyaluronidase (Wydase®, Wyeth Laboratories, Philadelphia, PA) was slowly infused behind the left eye until complete anesthesia of the orbit was obtained. Typically, 1–2 ml of bupivacaine hydrochloride was required to produce pupillary dilatation and loss of the corneal reflex. Anesthetic dosage chosen was consistent with that in clinical use at this and other institutions, although a cocktail containing two aminoacyl anesthetics (eg, one part 0.75% bupivacaine to one part 2% lidocaine) is often used to obtain rapid, yet prolonged anesthesia. Sterile saline containing 75 units/ml hyaluronidase was similarly introduced into the right orbit. All procedures involving animals conformed to the ARVO Resolution on the Use of Animals in Research.

Following survival times of 3–27 days, monkeys were anesthetized with pentobarbital sodium, heparinized, intubated and artificially respired with 95% oxygen-5% carbon dioxide prior to sacrifice. Monkeys were perfused transcardially through a cannula inserted into the ascending aorta. Physiological saline solution containing 1% sodium nitrite was introduced, followed by 3 l of fixative solution containing 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Following perfusions extraocular muscles were removed by dissection and immersed for 2 hr in cold fixative containing 4% glutaraldehyde in 0.1 M phosphate buffer. Transverse or longitudinal 500 µm sections of extraocular muscle were cut using a Smith-Mcllwain (Surrey, UK) tissue sectioner, and were processed for the histochemical demonstration of acetylcholinesterase (AChE) using a copper thiocolline method. Tetraisopropyl pyrophosphoramide (iso-OMPA, 10⁻⁴ M; Sigma, St. Louis, MO) was used as an inhibitor of non-specific cholinesterase. Muscle sections were then postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer, stained en block with 0.05% uranyl acetate, dehydrated in methanol and propylene oxide, and embedded in TAAB 812 resin. Muscle was examined in semithin (1 µm) sections cut on an ultramicrotome and stained with 1% para-phenylenediamine. Regions of interest were trimmed and ultrathin (80–90 nm) sections were cut with a diamond knife, collected on formvar-coated single-slot grids, and stained with uranyl acetate and lead nitrate. Sections were examined and photographed with a Zeiss 10C (Thornwood, NY) electron microscope.

In order to determine whether bupivacaine myotoxicity was specific, or if it represents a general property of aminoacyl anesthetics, two additional monkeys received retrobulbar injections of 1 ml of 2% lidocaine hydrochloride (Elkins-Sinn, Cherry Hill, NJ). Animals were sacrificed after 14 day survival and tissues were processed as described above.

In some experimental cases, brainstem blocks containing the oculomotor nucleus were removed following perfusions, postfixed, sectioned with a Vibratome, and processed for light and electron microscopy as described above.

Results

Retrobulbar application of bupivacaine hydrochloride produced rapid onset of pupillary mydriasis, ptosis and loss of the corneal blink reflex in all monkeys. Akinesia was present for the anesthetic treated orbit. Complete recovery from these effects required 8–10 hr. Anesthesia and akinesia of more limited duration was obtained from lidocaine hydrochloride injections. Lasting gross misalignment of the eyes and/or movement deficits were not observed. Control injections of sterile saline produced only a transient exophthalmos due to increased retrobulbar pressure.

Light Microscopic Observations

The extraocular muscles of the macaque monkey, like those of all mammalian species, exhibit two distinct regions: the orbital and global layers. Six muscle fiber types are differentially distributed in these layers. Fiber types are identified in semithin sections largely on the basis of the staining pattern of sarcoplasmic masses (Fig. 1). Extraocular muscles are unique among mammalian skeletal muscle in possessing not only singly-innervated fiber types, but also two types of multiply-innervated fiber. AChE reac-
Fig. 1. Phase contrast light photomicrographs of orbital (A) and global (B) layers of the monkey medial rectus muscle. (A) Orbital singly-innervated fiber type (1) exhibits dense sarcoplasmic masses both centrally and subadjacent to the sarcolemma. Orbital multiply-innervated fiber (2) is small, with a fine staining pattern. Orbital layer exhibits dense capillary (c) network. Ax, axons (X510). (B) Global singly-innervated fiber types (3-5) exhibit variations in pattern of staining of sarcoplasmic masses. Global multiply-innervated fiber (6) has fine staining pattern. Dark staining material in muscle fibers of both layers largely reflects mitochondrial content (X510).

A qualitatively identical pattern of morphopathological changes was observed in those monkeys that received retrobulbar injections of lidocaine. While the characteristic pattern of myofilament breakdown was evident, there appeared to be smaller numbers of pathological fibers in these cases.

The time course of bupivacaine-induced alterations is depicted in Table 1. Three days subsequent to injections relatively few fibers exhibited changes obvious at the light microscopic level. Pathological

![Image of muscle fibers](http://tvst.arvojournals.org/)

Fig. 2. Charting depicting distribution of pathological fibers in transverse section of monkey medial rectus muscle 14 days after retrobulbar application of bupivacaine hydrochloride. Each filled circle indicates position of one affected fiber. See text and Figure 3 for definition of lightly and heavily affected fibers.
changes in primate extraocular muscles were maximal at 14 days subsequent to retrobulbar injection, at which time 4.3% of fibers in the medial rectus muscle were affected. Alterations were largely resolved by 27 days after bupivacaine injections (Fig. 3D).

Electron Microscopic Observations

At the ultrastructural level the differential staining pattern of sarcoplasmic masses that was used to distinguish extraocular muscle fiber types with the light microscope was found to largely correspond to fiber mitochondrial content. Three global types and one orbital type of singly-innervated fiber could be distinguished on the basis of the number, size and distribution of mitochondria. Orbital and global multiply-innervated fibers were identified by their small size, poor delineation of myofibrils and occurrence of motor nerve terminals separated by 1 mm or more along the longitudinal extent of the fiber.

Retrobulbar injections of bupivacaine or lidocaine produced dissolution of myofilaments located around the periphery of the singly-innervated muscle fiber type with low mitochondrial content and extensive development of the internal membrane systems (ie, sarcoplasmic reticulum and t-tubules; Fig. 4). Pathogenic events followed a time course which was similar in all experimental animals. Mitochondria, which normally are small and homogenously distributed in this fiber type, initially migrated to a subsarcolemmal location where they increased dramatically in size (Figs. 4, 5B). The few mitochondria remaining in the central region of affected fibers retained normal characteristics. Swelling of subsarcolemmal elements of the sarcoplasmic reticulum also was noted in short-term survival cases (Fig. 5A). While some fibers never progressed further in the sequence of events following bupivacaine injections, many muscle fibers subsequently exhibited an outside-in pattern of myofilament breakdown. Muscle fibers at an early stage of myofilament dissolution frequently presented peripherally placed myofibrils which were oriented at a right angle to the longitudinal axis of the fiber (Ringbinden or ring fibers; Fig. 4A). Such aberrant myofibrils had prominent Z lines, but due to contraction the A and I bands were not readily discerned. Fibers in later stages of myofibril dissolution exhibited severe swelling of those portions of the t-tubule system that were subadjacent to the sarcolemma (Fig. 4B). In addition, subsarcolemmal tubular aggregates frequently occurred in heavily affected fibers (Fig. 5C). In rare instances a fiber exhibited total breakdown of myofilaments, ultimately containing only granular sarcoplasm and scattered mitochondria.

A clear relationship existed between the number and distribution of mitochondria present in a particular singly-innervated muscle fiber type and the degree to which that fiber type was affected by bupivacaine. The most severely affected fibers were those that normally exhibited few, small mitochondria. While pathological changes occurred in some fibers with high mitochondrial content (Fig. 6), alterations in these fibers were typically milder and much less frequent. In general, the most severe changes in such fibers were limited to swelling of peripheral mitochondria and vacuolization, probably the result of mitochondrial distension and the accumulation of lipid. Fibers with intermediate to high mitochondrial content did not undergo severe myofilament breakdown. Lysosomes, usually associated with peripheral mitochondrial aggregates, also were observed in this fiber type. Rarely a fiber with high mitochondrial content exhibited severe vacuolization and presumably would not have survived. However, even in these instances the patterned outside-in breakdown of myofilaments was

<table>
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<tr>
<th>Monkey</th>
<th>Survival (days)</th>
<th>Light effect</th>
<th>Heavy effect</th>
<th>Total</th>
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<td>06-27</td>
<td>3</td>
<td>0.08</td>
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<tr>
<td>06-04</td>
<td>5</td>
<td>0.22</td>
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<td>03-24</td>
<td>10</td>
<td>2.15</td>
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<td>06-09</td>
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<td>3.33</td>
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<tr>
<td>07-15</td>
<td>27</td>
<td>1.82</td>
<td>0.26</td>
<td>2.08</td>
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</table>

* Lightly affected fiber is defined as minimal breakdown of myofibrils around periphery of fiber, while heavy effect denotes extensive myofilament breakdown (see Fig. 3).
not noted. Multiply-innervated fiber types generally were not affected by bupivacaine treatment (Fig. 4B).

Activated satellite cells (Fig. 4B) and premyocytes (see Chou and Nonaka) often were associated with pathological fibers consistent with observations that many fibers apparently recover from bupivacaine treatment. Small peripheral clusters of myofilaments, oriented along the longitudinal axis of the regenerating fiber, and scattered aggregations of internal membrane system constituents were noted in premyocytes of 14 and 27 day survival cases. Other fibers, however, did not survive and were fragmented and engulfed by macrophages which increased in abundance within 10–14 days of bupivacaine injection.

Oculomotor motoneurons, intramuscular nerves, and neuromuscular junctions (Fig. 7) appeared normal in experimental material. Motoneurons had the characteristic complement of organelles and distribution of axosomatic synaptic contacts. Neuromuscular junctions retained the close apposition of axon terminal with sarcolemma, AChE reaction product in intercellular cleft and postjunctional folds, and postjunctional mitochondrial accumulations that characterize those of normal extraocular muscle. There was no specific association of bupivacaine-induced morphopathological changes with sites of neuromuscular junctions. In addition, there was no evidence that bupivacaine produced morphological changes in the vascular network of the extraocular muscles, since capillary distribution and morphology appeared normal in experimental material.

Discussion

These studies have demonstrated that retrobulbar application of bupivacaine hydrochloride produces limited structural alterations in primate extraocular muscles. Morphopathological changes correlated with fiber type mitochondrial content, internal membrane system development and innervation pattern. Among singly-innervated fiber types, those exhibiting the lowest mitochondrial content and highest sarcoplasmic reticulum development were the most sensitive to bupivacaine. Multiply-innervated fiber types were unaffected by these treatments. The primarily myopathic nature of bupivacaine toxicity is evident since neither motor system components nor muscle vasculature exhibited alterations in these or other studies. Interruption of the motor innervation of the extraocular muscles produces disease-related changes in those muscle fiber types that are heavily dependent upon oxidative energy mechanisms (ie, singly-innervated fibers with high mitochondrial content), and does not lead to changes like those observed in the present study. Taken together, many of the alterations observed in this study may be explained as resulting from a direct perturbation of fiber calcium metabolism by bupivacaine. Limited data which demonstrate similar myotoxicity for lidocaine suggest that this is a property of all aminoacyl local anesthetics.

Skeletal muscle calcium stores include both that which is segregated into membrane-bound compartments, represented by the sarcoplasmic reticulum and mitochondria, and that fraction which is free in the cytosol. Excitation/contraction coupling is dependent upon fine control of intracellular free calcium concentration largely via the sarcolemmal Ca²⁺-ATPase, which actively extrudes calcium, and the calcium pump of the sarcoplasmic reticulum, which sequesters calcium during muscle relaxation. In normal muscle, the calcium flux which regulates contraction, a transient increase in free levels that facilitates interaction of actin and myosin myofilaments, occurs across the sarcoplasmic reticulum. Several experimental interventions, as well as some naturally occurring myopathies, are associated with alterations in calcium homeostasis—specifically, chronic elevations in intracellular cytosolic calcium that may trigger a cascade of events culminating in fiber degeneration.

Bupivacaine hydrochloride functions as a long-acting local anesthetic as a result of both its lipophilic properties, which confer good mobility through biological membranes, and its high protein binding capacity. In addition to serving as a sodium channel blocker, bupivacaine displaces calcium from membrane binding sites and increases the fluidity of, and thus the ionic flux across, the sarcolemma. The
myotoxic properties of the aminoacyl anesthetics probably stem from their primary effect of increasing intracellular free calcium levels by these mechanisms. Since retrobulbar injections of lidocaine also produced similar morphopathological changes in monkey extraocular muscle, such limited extraocular myotoxicity most likely is characteristic of the entire family of aminoacyl anesthetics used in orbital surgery.

The role played by the sarcoplasmic reticulum and mitochondria in calcium homeostasis is central to the understanding of aminoacyl anesthetic myotoxicity. In normal muscle, the sarcoplasmic reticulum is the principal site of calcium release and uptake. This is reflected in the present finding that the initial morphological changes in extraocular muscle subsequent to bupivacaine application include swelling of subsarcolemmal elements of the sarcoplasmic reticulum. These changes may represent an adaptive response to an increased flux of calcium across the surface membrane. While the affinity of mitochondrial transport mechanisms for calcium is low, both the maximum rate and capacity of calcium accumulation by mitochondria far exceed that of the sarcoplasmic reticulum. Present observations of mitochondrial movement to the periphery and their subsequent increase in size may then indicate that mitochondria become important as a calcium sink primarily at high intracellular free calcium levels. Corollaries of excess calcium accumulation by mitochondria (which requires energy) are the shunting of ATP away from other cellular processes and the uncoupling of oxidative phosphorylation, which together further reduce the ability of the muscle fiber to handle elevated free calcium levels.
calcium levels.\textsuperscript{32-36} Taken together, mitochondrial abnormalities represent a central element in many extraocular muscle pathologies.\textsuperscript{25-27} Furthermore, the formation of subsarcolemmal tubular aggregates also may represent an adaptive response to elevated calcium levels since these structures frequently occur in myopathic disorders.\textsuperscript{26-37,39}

Bupivacaine elicits a myotoxic response in extraocular muscle that is considerably milder than that seen for other skeletal muscles.\textsuperscript{6-9} These intermuscle differences in toxicity, as well as the fiber type specificity this anesthetic exhibits for the extraocular muscles, are directly related to fiber mitochondrial and internal membrane system content. Because of their capacity for calcium uptake, mitochondria may confer relative degrees of protection upon individual singly-innervated muscle fiber types. Consistent with the hypothesis that mitochondria serve as a calcium sink, the fiber type affected in the present study presents low mitochondrial content. It is likely that the protection provided by the generally high mitochondrial content of most extraocular muscle singly-innervated fiber types has limits, and excessive application of bupivacaine may produce a more generalized pathological response in these muscles. Likewise, the high sarcoplasmic reticulum content of the affected fiber type may contribute to its sensitivity to bupivacaine by providing a significant intracellular source of calcium. The absence of pathological alterations in the multiply-innervated fiber types, which does not correlate with mitochondrial content, must be mediated by some other mechanism.

The muscle fiber toxicity of bupivacaine has implications for eye movement control subsequent to its use in orbital surgery in humans. Rainin and Carlson\textsuperscript{17} hypothesize that local anesthetic myotoxicity is responsible for the occasional transient ptosis and diplopia following ophthalmic procedures conducted with retrobulbar anesthesia. To support this hypothesis, the authors cite data indicating that several aminoacyl local anesthetics (lidocaine, mepivacaine, and bupivacaine) produce a severe pathological response, which lacks fiber type specificity, in rat extraocular muscles.\textsuperscript{18} The gross atrophy observed following retrobulbar injections in the rat is similar to those findings obtained for other skeletal muscles.\textsuperscript{6,4} Inter-species variations in muscle fiber types, as well as differences in histological techniques employed, create difficulties in comparing present findings with those of Carlson and Rainin.\textsuperscript{18} The more generalized response seen by these authors may result from either: (1) potentially higher bupivacaine concentration

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Electron photomicrograph of neuromuscular junction of pathological fiber 14 days after bupivacaine treatment. Note normal appearance of axon terminals and characteristic localization of AChE reaction product in junctional cleft and postjunctional folds (arrowheads). While sarcoplasm deficient in myofilaments is normal at the neuromuscular junction, this fiber exhibits excessive clear sarcoplasm. As this micrograph is from a transversely sectioned fiber, the appearance of abnormally oriented myofibrils (ring fiber) is noted (x8500).}
\end{figure}
at the muscle surface in their study; or (2) actual lower mitochondrial content of rat extracocular muscle fiber types. The present study, by contrast, reports a mild fiber type-specific effect. Morphopathological changes produced by the retrobulbar injection of bupivacaine, at the dose used in the present study, most likely would not produce eye and lid position deficits (eg, diplopia and ptosis). However, given the low incidence of such postoperative deficits, these data do not exclude the possibility that severe idiosyncratic reactions to bupivacaine anesthesia may be responsible for occasional eye movement disorders.

For most skeletal musculature, it is well established that the structural characteristics of an individual muscle fiber type correlate with its functional properties. Speed of contraction is largely determined by the degree of development of internal membrane systems, and fatigability is a correlate of mitochondrial content. While the existence of a tight structure/function correlation has yet to be established for the extracocular muscles, the morphological properties of the affected muscle fiber type (ie, high development of internal membrane systems and low mitochondrial content) suggest that it is phasically active in rapid eye movements and fatigues easily. Thus, one would then expect that retrobulbar application of local anesthetics would produce transient reductions in saccadic velocity rather than fixation disorders. The precise functional effects of compromising particular extracocular muscle motor unit types, however, remain to be determined.

Key words: extracocular muscle, myotoxicity, bupivacaine hydrochloride, lidocaine hydrochloride, muscle calcium, monkey

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