Atrophy, but not necrosis, in rabbit skeletal muscle denervated for periods up to one year

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Much of our knowledge of the effects of long-term denervation of mammalian skeletal muscle comes from experimental studies of total sciatic section in the rat (7, 32, 54). The mass of the affected muscles falls rapidly within 5–7 days of axotomy (11, 18, 22, 25, 55, 57, 58) and declines further to 30–50% of control weight in succeeding weeks (1, 5, 11, 14, 23, 41, 60). After several months, muscle weight stabilizes at ~5–20% of control (1, 13, 45).

Within the muscles, individual fibers show a reduction of ~70% in cross-sectional area (CSA) over a period of months (5, 14, 21, 37), and over 90% (1, 21, 45) in the longer term. Initially, fast (type II) fibers are more susceptible to atrophy than slow (type I) fibers (5, 7, 32, 38, 48, 54, 59), but over more prolonged periods the fiber types atrophy to a similar extent (6, 54). Corresponding to this reduction in fiber CSA, there is a striking and progressive increase in interstitial collagen and fat (32). At the ultrastructural level, atrophic muscle fibers show evidence of disorganization, including loss or misalignment of sarcomeres, dissociation of the T system and sarcoplasmic reticulum (SR), and changes in the sarcomeric location of mitochondria (32, 51, 52).

The major physiological correlate of these morphological changes is a loss of force-generating capacity. Long-term denervated rat muscles develop only ~10% of the tetanic force of a normally innervated muscle. Since this loss is usually accompanied by a reduction in the specific force (force per unit area), it presumably includes a component arising from ultrastructural disorder within the muscle fibers.

In humans, chronic denervation of muscles can be the result of injuries to spinal roots, plexuses, or peripheral nerves. Although there is considerable variation among patients, the affected muscle invariably shows a marked loss of mass and CSA and an increase in overlying fat. Examination of biopsies reveals atrophy of muscle fibers and interstitial fibrosis (19). There is a partial or complete loss of the ability to generate force, even with electrical stimulation of high intensity. Although these features appear to have their counterparts in denervated muscles of the rat, there is a marked difference in timescale: changes in human muscle that take place over a period of years can be observed within a few months in the rat. Furthermore, there is little evidence in humans of the extensive necrosis and regeneration that have been reported in a number of studies of denervated muscles in the rat (13, 45).

This raises the question as to whether findings based on rat muscle are an adequate guide to the phenomena that occur after denervation in humans. The present study is based on a model of selective motor denervation in the rabbit. We examined physiological, histological, histochemical, immunohistochemical, and ultrastructural changes in muscles denervated for up to 1 yr. The picture that emerges from the rabbit over this extended experimental period is not unlike that of humans and shows both quantitative and qualitative departures from that of the rat.

Methods

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accordance with the Animals (Scientific Procedures) Act 1986. Fifteen New Zealand White rabbits were used in the study; five were denervated in one hindlimb for 10 wk, five for 36 wk, and two for 51 wk; three unoperated rabbits served as weight-matched controls.

**Denervation protocol.** Twelve rabbits (2.5–3 kg) were premediated with subcutaneous injections of atropine sulfate (3 mg/kg; Sigma) and intramuscular ketamine hydrochloride (Ketalar, 10 mg/kg; Pfizer). Broad-spectrum prophylactic antibiotic cover was provided by enrofloxacin (Baytril, 5 mg/kg; Bayer). Surgical anesthesia was induced and maintained with inhalation of 1.5–3% isoflurane (Forene; Abbott, Wiesbaden, Germany) in 2 l/min oxygen. Postoperative analgesia was provided with intramuscular buprenorphine hydrochloride (Temgesic, 0.1 ml/kg; Schering-Plough).

Under full aseptic precautions the common peroneal nerve was exposed through an incision in the biceps femoris muscle. In the initial experiments, a ligature was secured around the nerve and the nerve was cut distal to the tie. The proximal stump was inserted into a 10-mm length of silicone rubber tube, and two silk ties were placed around the tube, compressing the nerve inside. The tube was then reflected through 90° and sutured in place to prevent reinnervation. The first five animals tended to harm the denervated foot and therefore required daily attention and regular changes of a protective dressing. The denervation protocol was therefore modified: in all subsequent experiments, the common peroneal nerve was viewed under a dissecting microscope, and only the nerve branches supplying the tibialis anterior (TA) and the extensor digitorum longus (EDL) muscles were separated, ligated, and diverted as before, leaving the sensory branches intact. With this modification the problem of self-harm was avoided. No differences were observed in the response of the muscles whether they were subjected to full or selective motor denervation, and we have not, therefore, excluded the first five animals from analysis.

Implantable electrical stimulators (29) were inserted into the peritoneal cavity and secured in place. The wires and electrodes were routed subcutaneously from the abdomen to the left hindlimb. The electrodes were positioned and secured under the distal portion and over the proximal portion of the TA muscle. All incisions were closed, the animal was allowed to recover from general anesthesia, and it was then returned to its home cage.

In the study the implanted stimulators were included only for measurement of chronaxie and rheobase at weekly intervals by palpation of the muscles in the conscious animals; the results have been reported elsewhere (2). The technique did enable us to recognize a sudden decrease in chronaxie in one rabbit; this was attributed to reinnervation, and the animal was excluded from the study. In all other respects the findings in the present article are based solely on terminal measurements (see below).

**Physiological assessment.** After a predetermined period, physiological assessment was carried out on the denervated and contralateral TA muscles. Animals were premedicated with subcutaneous atropine sulfate (3 mg/kg) and fentanyl citrate (10 mg/kg; Auden McKenzie Pharma Division). Surgical anesthesia was induced and maintained by inhalation of isoflurane (2–3%) in 3 l/min O2, and analgesia was provided with continuous intravenous administration of fentanyl citrate (50 μg·kg⁻¹·h⁻¹). Fluid balance was maintained throughout by intravenous infusion of saline (30 ml/h), and body temperature was maintained by placement on a heat pad. Animals were ventilated mechanically via a tracheocannula at a frequency of 20 breaths/min throughout the procedure.

The TA muscles were exposed and partially freed from connective tissue attachments to the EDL. The distal tendon was cut and attached via a miniature clamp to a servomotor that incorporated both length and force transducers (Dual-Mode Servo system, model 310B; Cambridge Technology, Cambridge, MA). The distal tendons of all other muscles of the anterior compartment were cut to minimize any influence of these muscles on the force recordings. The lower limb was fixed with clamps at the knee and ankle. The denervated muscles were stimulated by a custom-designed external stimulator that delivered the constant-current stimulus pulses through electrodes placed directly on the muscle. In the case of the denervated muscle the electrodes used were the ones that had been implanted, although they were first detached so that subjacent connective tissue could be removed. Stimulating pulses were set at a supramaximal level, with 0.2-ms and 10-ms pulse widths for innervated controls and denervated muscles, respectively, and the optimal length of the muscle (the length for maximum active twitch tension) was determined.

The amplitude (twitch force, P1) time to peak (CT), and half-relaxation time (0.5R) of the isometric twitch were measured. A strength-duration curve was constructed to determine excitability. The minimum amplitude to elicit a twitch (15–20% of maximum force) was determined at a range of bipolar pulse widths (0.1–100 ms). The rheobase (the minimum amplitude needed to elicit a response with 100-ms bipolar pulses) and the chronaxie (the minimum pulse duration needed to elicit a response with an amplitude of twice the rheobase) were calculated from these curves.

Force-frequency data were obtained by stimulating with 400-ms trains of pulses at frequencies from 5 to 50 Hz (denervated) and 5 to 200 Hz (innervated controls). In the denervated muscles the frequency had to be limited to 50 Hz because of the long pulse durations (10 ms) required to elicit a response.

The maximum recorded tetanic force was chosen as the highest load in a series of isometric contractions from which the force-velocity relationship could be determined. The maximum velocity of shortening (Vmax) was calculated from this curve.

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At the conclusion of the physiological measurements, the muscles were stimulated to contract isometrically with 40-Hz impulse trains of 330-ms duration delivered once a second for 10 min. The fatigue resistance of the muscle was assessed by calculating the time taken for the force to decline to 60% of the initial value (T60).

After the fatigue test, thin strips (2 × 10 mm) of denervated and contralateral control muscles were taken for electron microscopy. The muscles were held at optimal length, and the strips were fixed to small labeled splints of balsa wood. The samples were stored in 2.5% glutaraldehyde in sodium cacodylate buffer at 4°C pending further processing.

The animals were humanely killed by an intravenous overdose of pentobarbital sodium (Euthatal, 200 mg/kg; Rhone-Merieux) without recovery from general anesthesia. The denervated and contralateral TA and EDL muscles were removed and weighed. A full-thickness sample, 10 mm wide, was removed from the muscle belly, orientated on a cork disk for transverse section of the fibers, frozen in melting isopentane, and stored at −70°C until required.

**Histochemistry and immunohistochemistry.** Serial 10-μm frozen sections were cut from the cork-mounted specimens. A section from denervated muscle was paired with one from the contralateral muscle on each slide, and the two were thus processed in parallel. General morphological assessment was based on the regressive hematoxylin and eosin stain. The three fiber types (types I, IIa, and IIx) were distinguished by a modification of the myofibrillar ATPase procedure of Tunell and Han (53) in which the formaldehyde incubation was extended to 2.5 min and acidification was fixed at 50 min. Typing was confirmed by the use of indirect immunohistochemistry with mouse anti-rabbit monoclonal antibodies, WB-MHCr and WB-MHC, specific to fast and slow myosin heavy chain (MHC), respectively. Expression of developmental MHCs was demonstrated immunohistochemically with a mouse anti-rat developmental (embryonic) myosin (NCL-MHCα; Novocastra Laboratories, Newcastle-upon-Tyne, UK) and the antibody WB-MHCneo specific to neonatal MHC. The latter and antibodies WB-MHCr and WB-MHC, were first raised in this research group by Dr. W. E. Brown (16, 17). For all the antibodies, binding was detected with biotinylated horse anti-mouse IgG (Vector, Peterborough, UK) as the secondary antibody, amplified by the avidin-biotin horseradish peroxidase technique (ABC Elite, Vector), and visualized by incubation with 3,3′-diaminobenzidine tetraazolium hydrochloride.

**Expression of developmental MHCs was demonstrated immunohistochemically with a mouse anti-rat developmental (embryonic) myosin (NCL-MHCα; Novocastra Laboratories, Newcastle-upon-Tyne, UK) and the antibody WB-MHCneo specific to neonatal MHC.**

The latter and antibodies WB-MHCr and WB-MHC, were first raised in this research group by Dr. W. E. Brown (16, 17). For all the antibodies, binding was detected with biotinylated horse anti-mouse IgG (Vector, Peterborough, UK) as the secondary antibody, amplified by the avidin-biotin horseradish peroxidase technique (ABC Elite, Vector), and visualized by incubation with 3,3′-diaminobenzidine tetraazolium hydrochloride.
LONG-TERM DENERVATION IN RABBIT SKELETAL MUSCLE

NADH tetrazolium reductase (NADH-TR) was used as a mitochondrial marker. Sections were incubated for 30 min at 37°C in 0.1% nitro blue tetrazolium in 0.2 M Tris buffer (pH 7.4) to which NADH was added at a ratio of 1:1,000 (wt/vol). All stained sections were dehydrated through graded concentrations of alcohol to xylene and mounted permanently in DPX.

Muscle fibers were typed according to the following criteria. Type I fibers were unstained with myofibrillar ATPase, darkly stained with NADH-TR, negative for anti-fast MHC, and positive for anti-slow MHC. Type IIa fibers were stained at an intermediate intensity with myofibrillar ATPase and NADH-TR and were positive for anti-fast but negative for anti-slow MHC. Type IIx fibers were darkly stained with myofibrillar ATPase, unstained for NADH-TR, and positive for anti-fast MHC but negative for anti-slow MHC.

Morphometry. Specimens were examined on a Leica Diaplan microscope. A transparent grid of 1-mm squares was overlaid on the slide, and the entire area of the cross section was measured. The same grid was used to select 10 fields for analysis, distributed at equal intervals across the section. Fibers falling on the upper and left boundaries of each of these 1-mm² fields were included, and those falling on the lower and right boundaries were excluded. The number of fiber profiles present in the entire section (total fiber number) was estimated as the product of the mean number of fibers per square millimeter and the total CSA of the muscle. Point-counting morphometry was used to estimate the area occupied by different components by identifying the structure (muscle fibers, fibrous connective tissue, fat, or other tissue) present at each intersection of a 10 × 10 grid within each 1-mm² field.

The minimum diameters of the muscle fibers were determined with the public domain Image J program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ihimage/). Digital photomicrographs of sections stained for myofibrillar ATPase were taken with an adapted Nikon Coolpix camera and displayed on a computer screen. A digital grid was overlaid on the section to select fibers for measurement. The outline of each chosen fiber was traced, and the minimum diameter was then determined by the software. A minimum of 350 fibers were measured, and these were taken from five separate 1-mm² fields of view distributed over the section.

Electron microscopy. Specimens were fixed at room temperature in 3.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 for 2 h and stored at 4°C in the same solution. Small bundles were postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated through a graded ethanol series followed by acetone, and embedded in epoxy resin. Ultrathin sections (30–40 nm) were cut on a Leica Ultracut R microtome (Leica Microsystem) with a Diatome knife (Diatome, Biel, Switzerland) and stained in 4% uranyl acetate and lead citrate. Sections were examined with a FP 505 Morgagni Series 268D electron microscope (Philips) equipped with Megaview III digital camera and Soft Imaging System (Megaview).

Statistical analysis. The physiological and morphological properties of muscles after different durations of denervation were compared by ANOVA with the Tukey-Kramer posttest (GraphPad Instat software); unoperated and contralateral muscles served as controls. The criterion of statistical significance was P < 0.05.

RESULTS

Control muscles. The animals in which the ankle dorsiflexors had been denervated on one side showed no behavioral signs of favoring the opposite limb. Furthermore, the contralateral muscles were not significantly different in either morphological or physiological properties from the corresponding muscles in unoperated weight-matched animals. Contralateral muscles were therefore regarded as a valid source of control data for this study, particularly as its main focus was the effect of different durations of denervation.

Muscle weight, CSA, and total fiber number. Denervation of the TA muscle resulted in a significant 50–60% loss in wet weight and a corresponding 50–60% reduction in midbell CSA (Table 1). No significant differences in weight, CSA, or total fiber number were found between the groups denervated for 10, 36, or 51 wk.

Excitability. Strength-curves for the denervated muscles all showed the expected shift toward higher amplitudes, especially at short pulse durations (0.2–2 ms). Evidence of this reduced excitability is presented in Table 2, where it is expressed in terms of chronaxie. There were no significant differences in chronaxie between muscles denervated for different periods.

Contractile properties. The denervated muscles showed a marked reduction in contractile speed. This was evident both in the kinetics of isometric twitch contractions (CT, 0.5R) and in Vmax (Table 2).

Maximum isometric tetanic tension (P0) was approximately fourfold lower in the denervated muscles than in the innervated contralateral controls. This difference became progressively less marked when P0 was expressed per gram of wet weight, per square millimeter of overall CSA, and per square millimeter of CSA occupied by muscle fibers (N/g, N/mm, N/mm² muscle fibers, respectively, in Table 2).

Isometric twitch tension (Pt in Table 2) did not decline in parallel with tetanic tension but showed, if anything, a tendency to increase after denervation. As a result, the twitch-to-tetanus ratio (Pt/P0 in Table 2) was higher by a factor of 4 or more in the denervated muscles.

The denervated muscles were not more resistant to fatigue than innervated controls; if anything, they gave the impression of being more susceptible (Fig. 1). The fatigue indexes (T50 in Table 2) showed significant departures (P < 0.03, ANOVA),
but posttest comparisons between groups did not achieve significance. The posttetanic potentiation that is a typical feature of innervated fast muscles was not present in the force envelope recorded during fatigue testing of the denervated muscles (Fig. 1). For none of the physiological variables was there any significant difference between groups denervated for 10, 36, and 51 wk.

Changes in muscle composition. Gross accumulation of superficial connective tissue was removed during the preparation of the TA muscles. Thickening of the epimysium was nevertheless evident on microscopic examination of the denervated muscles, especially on their lateral aspect. There was an accompanying increase in the thickness of both the perimysium and the endomysium. Point-counting morphometry showed that a mean of 35% of the section area was occupied by fibrous connective tissue, significantly greater than the 11% for their innervated contralateral counterparts (P < 0.001; Fig. 2). There was also an increase in the area occupied by adipose tissue, from 0.03% in the innervated muscles to 4% after 10 wk of denervation and to ~9% in the muscles denervated for 36 and 51 wk (Fig. 2). Much of this was due to accumulation of fat around the larger neurovascular bundles and within the perimysium, particularly in the lateral part of the muscle. This increased fibrous and adipose connective tissue occupied space relinquished by muscle tissue, whose area had decreased from 88% to 50% (P < 0.001; Fig. 1). Although Fig. 1 appears to suggest some slight progression of these changes in composition with the period of denervation, the trend was not significant.

Changes in fiber size. Representative photomicrographs are shown in Fig. 3. The obvious way in which the denervated muscles differed from innervated controls was the generalized muscle fiber atrophy and fibrosis. Other abnormal features, such as the presence of hypereosinophilia, internal nuclei, and vacuolation, were found distributed throughout the section, although there were also some focally abnormal areas. The changes were, however, heterogeneous both within a given section and between individual animals. For this reason it was important to base any morphological conclusions on quantitative data obtained by systematic sampling.

The distribution of fiber diameters is presented in the size spectra of Fig. 4. In the innervated control muscles, fiber size

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Table 2. Physiological properties of control innervated and 10-, 36-, or 51-wk denervated muscles

<table>
<thead>
<tr>
<th></th>
<th>Contralateral (n = 15)</th>
<th>10 wk (n = 5)</th>
<th>36 wk (n = 5)</th>
<th>51 wk (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch, ms</td>
<td>3.5 ± 1.9</td>
<td>12.3 ± 1.6*</td>
<td>8.0 ± 1.6</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>RB, mA</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0.03</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>P₀, N</td>
<td>3.42 ± 0.27</td>
<td>5.00 ± 0.64</td>
<td>5.62 ± 1.31</td>
<td>6.16 ± 2.87</td>
</tr>
<tr>
<td>CT, ms</td>
<td>20.7 ± 1.0</td>
<td>48.4 ± 4.3%</td>
<td>34.8 ± 2.2%</td>
<td>46.5 ± 4.5%</td>
</tr>
<tr>
<td>0.5R, ms</td>
<td>17.3 ± 1.6</td>
<td>32.4 ± 3.9%</td>
<td>26.0 ± 1.8</td>
<td>35.0 ± 11.0</td>
</tr>
<tr>
<td>Pₚ, N</td>
<td>31.23 ± 1.64</td>
<td>7.96 ± 1.46%</td>
<td>7.86 ± 1.41%</td>
<td>7.20 ± 2.17%</td>
</tr>
<tr>
<td>Pₚ, N/mm</td>
<td>7.23 ± 0.29</td>
<td>4.91 ± 0.59%</td>
<td>4.08 ± 0.67%</td>
<td>3.34 ± 1.83%</td>
</tr>
<tr>
<td>Pₑ, of muscle fibers, N/mm</td>
<td>0.35 ± 0.05</td>
<td>0.22 ± 0.03</td>
<td>0.20 ± 0.03</td>
<td>0.18 ± 0.11</td>
</tr>
<tr>
<td>Pₑ/Pₚ</td>
<td>0.38 ± 0.07</td>
<td>0.36 ± 0.05</td>
<td>0.37 ± 0.04</td>
<td>0.35 ± 0.18</td>
</tr>
<tr>
<td>Vmax, mm/s</td>
<td>438.7 ± 13.2</td>
<td>138.6 ± 18.3%</td>
<td>206.4 ± 18.4%</td>
<td>146.0 ± 6.0%</td>
</tr>
<tr>
<td>T₆₀, s</td>
<td>96.6 ± 8.8</td>
<td>105.2 ± 23.4</td>
<td>48.4 ± 10.5</td>
<td>47.0 ± 28.0</td>
</tr>
</tbody>
</table>

Values are physiological variables measured in TA muscles denervated for 10, 36, and 51 wk and in their innervated contralateral controls; values are means ± SE (means ± SD for 51 wk). Ch, chronaxie; CT, time to peak twitch contraction; P₀, isometric twitch tension; 0.5R, time to half-relaxation; Pₚ, maximum isometric tetanic tension; Vmax, maximum velocity of shortening; T₆₀, time for force to decline to 60% of initial value; RB, rheobase. Significant differences from contralateral controls: *P < 0.05, †P < 0.01, ‡P < 0.001. No significant differences were observed between the three denervated groups in any variable.
Fig. 3. Fiber type and morphology in denervated muscles of the rabbit: photomicrographs of serial sections from an innervated contralateral control TA muscle (A–E) and from a 10 (F–J), a 36 (K–O), and a 51 (P–T)-wk denervated TA muscle. Sections were stained for general morphology (hematoxylin and eosin) (A, F, K, P), NADH tetrazolium reductase (NADH-TR; B, G, L, Q), myofibrillar ATPase (C, H, M, R), anti-fast myosin (D, I, N, S), and anti-slow myosin (E, J, O, T). Arrowheads identify exemplars of the different fiber types: black, type I fibers; yellow, type IIA fibers; blue, type IIX fibers. Note the presence of extremely large, as well as atrophied, fibers in the sections from 36-wk and 51-wk denervated muscles. Bar, 100 μm.
was distributed normally, with a mean diameter of 56.9 ± 0.7 μm. The denervated muscles showed a shift to smaller fiber sizes, with mean diameters of 34.3 ± 2.3, 40.8 ± 8, and 36.4 ± 1.3 μm for 10-, 36- and 51-wk denervated muscles, respectively. The distribution of fiber sizes was skewed, with more fibers of large diameter than would be predicted from a normal distribution. Although the majority of fibers were small (some very small), there were also fibers in the normal size range; these were often systematically arranged, with fibers of normal size surrounded by groups of much smaller fibers.

In the muscles that had been denervated for 36 or 51 wk, extremely large fibers could be seen. These were as much as three times normal size (150 μm) and were found both in groups and as isolated fibers distributed through the section. The presence of these very large fibers further skewed the size distribution toward higher diameters (Fig. 4). It should be pointed out that the fibers of larger diameter may be slightly overrepresented in these spectra, since they would be more likely to be selected for measurement by the systematic sampling technique used.

Changes in fiber type composition. The innervated contralateral muscles showed a typical mosaic pattern of fiber types (Fig. 3, A–E). The denervated muscles differed markedly from these controls in having very few type IIx fibers. This appeared to be the result of transformation of the type IIx fiber population to type IIa. The fibers noted above as being in the normal range of size appeared to be of type I; the mean minimum diameter of type I fibers in contralateral control muscles was 58.6 ± 2.3 μm, and the corresponding figure for type I fibers in all the denervated muscles was 51.6 ± 4.3 μm. The atrophic fibers, on the other hand, were usually of type IIa; the mean minimum diameter of type IIa fibers in contralateral control muscles was 54.1 ± 1.4 μm, and the corresponding figure for all the denervated muscles was 33.3 ± 1.7 μm. This agrees with previous observations that type I fibers are more resistant to atrophy than type IIa fibers. In most cases, however, the extremely large fibers were not type I fibers; their staining characteristics were more typical of type IIa (Fig. 3, K–O).

Changes in the proportions of the different fiber types are illustrated in the stacked column chart of Fig. 5. The contralat-

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**Fig. 4.** Distribution of fiber diameters for innervated and denervated TA muscles: minimum fiber diameters were measured for innervated unoperated control (A), innervated contralateral control (B), and 10 (C)-, 36 (D)-, and 51 (E)-wk denervated muscles. The diameters from at least 350 fibers were assigned to 10-μm bins, and the number in each bin is expressed as % of the total number of fibers measured.

**Fig. 5.** Percentage fiber type composition of contralateral innervated control and denervated TA muscles. Data show the % fiber type composition (means ± SE, except means ± SD for 51 wk) in innervated muscles (n = 15) and in muscles denervated for 10 (n = 5), 36 (n = 5), and 51 (n = 2) wk. Black, type I fibers; gray, type IIa; white, type IIx.
eral innervated muscles (1st column, Fig. 5) had the following composition: type I 2.4 ± 0.6%, type IIa 30.7 ± 3.8%, type IIx 66.9 ± 3.4%. Other columns in Fig. 5 show the almost total replacement of type IIx fibers by type IIa and the stable size of the type I fiber population. This distribution of fiber types was already established after 10 wk of denervation and did not change significantly thereafter.

**Degenerative and regenerative events.** Although the denervated muscles showed a range of structural abnormalities, there was little or no evidence of necrosis. There was, for example, no sign of infiltration of fibers by mononuclear cells, which is a hallmark of necrosis. However, the response to denervation takes place over a considerable period; necrotic events could therefore be missed if they occurred only infrequently. We therefore amplified our observations with measures of necrosis that were less direct but more cumulative in nature.

Muscle necrosis is normally accompanied by regeneration, and internal myonuclei, which were present in the sections, have been interpreted as evidence of such events. A more sensitive marker of regeneration is the presence of embryonic and neonatal MHCs, since these isoforms are expressed sequentially during development and during the formation of new muscle fibers from satellite cells.

As Fig. 6 illustrates, fibers in the innervated control TA muscles had no affinity for either anti-embryonic MHC or anti-neonatal MHC (Fig. 6, A and E). In the denervated muscles, 0.5–1% of muscle fibers stained positively for anti-embryonic MHC, although not always strongly (Fig. 6B); this limited expression was nonetheless at a significant level. To guard against the possibility that these low levels of embryonic MHC were due to insensitivity of the detection technique, some serial sections were stained with a fluorescein-conjugated second antibody and examined in conventional fluorescence and confocal microscopes; the result was the same (not shown). All of the denervated muscles showed a highly significant increase in the expres-

![Fig. 6. Expression of embryonic and neonatal myosin heavy chain (MHC) isoforms in denervated muscles of the rabbit. Serial sections were stained immunohistochemically for embryonic MHC (A–D) and neonatal MHC (E–H) for an innervated contralateral control TA muscle (A, E) and a 10 (B, F)-, a 36 (C, G)-, and a 51 (D, H)-wk denervated TA muscle. Arrows in B, D, and F indicate the only fibers within the field of view that were positive for anti-embryonic MHC. Arrows in F show a higher incidence of fibers that were positive for anti-neonatal MHC after 10 wk of denervation. After more extended periods (G, H) all of the small fibers were positive (arrows), and larger fibers were negative, for anti-neonatal MHC. Bar, 100 μm.](http://ajpcell.physiology.org/)

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The features described above were found in all specimens studied but were more pronounced and more widespread in the muscles denervated for longer periods. In those muscles there were larger accumulations of adipose connective tissue and a higher degree of disorganization of the contractile apparatus, and triads were more infrequent and more abnormal in orientation and appearance.

The denervated muscle fibers were therefore ultrastructurally abnormal. On the other hand, there was no evidence of necrotic degeneration, and recognizable myofibrillar structures were seen in every fiber examined.

**DISCUSSION**

Measurement of chronaxie during the terminal experiments confirmed that the experimental muscles had been denervated completely and had remained so throughout the study. The maximum period of denervation, 51 wk, represented something like one-seventh of the normal life span of a New Zealand White rabbit, and we anticipated that a full complement of changes due to denervation would develop during this time. We found, however, that the rabbit muscles in this study were much less severely affected by denervation than corresponding experiments in the rat would have led us to expect.

Rat muscle is said to respond to denervation in three phases (32). In the first phase (0–2 mo), muscle fibers undergo atrophy and there is mild fibrosis; these changes are accompanied by a substantial decline in muscle mass and force. In the second phase (2–7 mo), necrotic changes lead to an actual loss of fibers; fibrosis is also much more evident. In the third phase (7 mo onward), muscle mass and force stabilize at a low level. There is also some suggestion that during this final stage muscle fibers are replaced altogether by fibrous and adipose tissue. As a result of the changes during the first phase the muscle declines to 20–30% of its original mass by 3–4 wk (5, 8, 25, 44, 52, 60), and by 1–2 mo it develops only 25% of its original isometric tetanic tension (8, 47). In the denervated rat EDL muscle, tetanic force is reported to decline even further over this period, to 1–3% of control values (1, 14, 30); at this stage mean fiber area is only 20–30% of control (1, 46), falling to 2.5% of control over subsequent months (1, 13). In the rat TA muscle, we observed a decrease of ~50% in wet weight at 10 wk after denervation, with no further decline up to 51 wk. [In an early (1915) study on the rabbit gastrocnemius muscle, Langley and Kato (28) reported a similar degree of atrophy 5 wk after denervation.] Tetanic tension had declined to ~25% of control values after 10 wk and mean fiber diameter by ~30%, and neither change showed any evidence of progressing further up to 51 wk. Thus after nearly a year of denervation the muscles consisted mainly of small fibers that were not less than 10 μm in diameter, showed no signs of necrosis, and had a recognizable, albeit somewhat abnormal, ultrastructure. The total number of fibers within the cross sections was not less than that of control muscles. These muscles, although atrophied, were still capable of developing appreciable tension. This behavior is in stark contrast to that of rat muscle.

The direction of fiber type transformation was in some ways unexpected. An adaptive response to the loss of contractile activity (43) should have resulted in a transformation toward the fast, anaerobic glycolytic profile of type IIX fibers. Instead, there was a shift from type IIX to type IIA fibers, involving both...
the expression of the IIa MHC isoform and an increase in mitochondrial volume. The latter was indicated by the strong reaction for NADH-TR and confirmed by electron microscopy, which showed large mitochondria in rows between the myofibrils. A similar appearance has been seen in rabbit TA muscles subjected to chronic stimulation (42) and in heavily used muscles such as the pectoral muscles of the hummingbird (20); in both cases, it is associated with a very high resistance to fatigue. The denervated rabbit muscles were not, however, fatigue resistant—if anything, those denervated for longer periods appeared more susceptible to fatigue. Evidently loss of the motor nerve elicits a set of proteomic changes that are distinct from those produced by a mere loss of contractile activity and may not be coordinated in a functionally relevant way.

Fig. 8. Ultrastructural changes in denervated muscles of the rabbit: electron micrographs of an innervated contralateral control TA muscle and a 36-wk denervated TA muscle. Compare the registration of Z lines (large black arrows) and M lines (large white arrows) in the control muscle (A) with the loss of alignment in the denervated muscle (C). In the control muscle tubular elements of the sarcoplasmic reticulum (SR) are seen between myofibrils (small white arrows), and terminal cisternae and T tubules (TT) form triads, which are located close to the A-I junctions (small black arrows in A and at higher magnification in B). In the denervated muscle there is a paucity of sarcotubular profiles between the myofibrils, and junctions with T tubules, when present, are abnormal in morphology and alignment (shown at higher magnification in D and E). In the control muscle, mitochondria are infrequent, small, and located between the Z lines and triads (open arrows in A); in the denervated muscle mitochondria are more abundant, are much larger, and usually lie in rows between the myofibrils (open arrows in C). Bars: A and C, 1 μm; B, D, and E, 0.1 μm.

The fiber type transformation seen in rabbit muscles did not differ from that in rat muscles examined at an earlier stage of denervation (5, 23, 25, 34, 56, 59). In one of those studies, coexpression of the different isoforms showed that the switch occurred within individual fibers and not by replacement with fibers of another type (34).

Photomicrographs such as that of Fig. 3J clearly show that type I fibers were not affected by the atrophy to the same extent as type II fibers. This sparing of slow fibers has been observed before (5, 7, 26, 48). After prolonged periods of denervation, fast and slow fiber types are said to be equally affected (5, 6); this stage was not, however, reached in the present study.

An interesting feature of the rabbit muscles denervated for 36 or 51 wk was the appearance of extremely large fibers, between three and four times normal size. Similar fibers have
been observed by Bacou et al. (3) in rabbit muscles denervated for 2 mo and in the muscles of human patients with neurogenic atrophy (33). Maier and Bornemann (33) interpreted these as residual innervated or reinnervated fibers that had undergone compensatory hypertrophy. This cannot be the case here, where denervation was complete and where these extremely large fibers were often found in isolation with no evidence of type grouping. It is possible that the fibers were formed by fusion of smaller fibers, but we have no evidence for this, and we can offer no other explanation for them at this stage.

The denervated muscles had very slow contractile properties, not unlike those of the classic slow soleus muscle. An increase in series stiffness could account for the high twitch-to-tetanus ratio but would not explain the low $V_{\text{max}}$ and the prolonged CT and 0.5R. Neither is it likely that these were the result of the shift to the Ila MHC isoform, which under innervated conditions supports essentially fast kinetics. They would, however, be consistent with the structural disorganization that was observed at the electron microscopic level. The swollen and displaced sarcotubular systems and loss of normal triadic structures could have resulted in disruption of excitation-contraction coupling, resulting in longer diffusion distances for $\text{Ca}^{2+}$. At the same time, a reduced ability of the SR to sequester $\text{Ca}^{2+}$ would prolong activation, and this could account for the relatively high amplitude and slow kinetics of the isometric twitch.

Changes in the excitation-contraction coupling apparatus could result in a loss of force-generating capacity through inadequate or asynchronous activation of myofilaments. The reduction we observed in maximum tetanic tension was not present when we expressed it per unit area of muscle (corrected for occupation by connective tissue), even after denervation for 51 wk. Thus the loss of tetanic force was commensurate with the reduction in contractile tissue, indicating that the functional capacity of the remaining tissue was not significantly impaired.

It would seem that activation, although slow, was essentially complete in the atrophied denervated muscle fibers of the rabbit. In the rat EDL muscle, loss of force was greater than the loss of mass (10) and specific force decreased by 70–90% (1, 11). The EDL muscle was delivered once a week to assess chronaxie (see METHODS) had any major influence on the results. However, we cannot disregard the possible influence of other methodological differences. In this study the TA and EDL muscles were denervated by section of the corresponding motor branches in the common peroneal nerve. The sensory innervation of the hindlimb was otherwise intact, as was the motor supply to other agonistic and antagonistic muscles. Most studies of denervation in the rat involve section of the entire sciatic nerve, resulting in complete motor and sensory denervation of the hindlimb below the level of the lesion.

Vascular changes resulting from denervation have been considered in some detail by Sunderland (49). The loss of contractile activity produced by denervation includes the loss of the muscle vascular pump. This can result in venous congestion in the affected limb, with the development of vascular stasis and edema. At the same time, interruption of vasoconstrictor nerve fibers would produce a loss of vascular tone, which could exacerbate the problem in the initial stages. It is possible that at least some of the degenerative changes observed in response to sciatic section in the rat originate in this way. We do not know whether the selective motor denervation carried out in the present study produced a loss of vasoconstrictor tone in the affected muscles, but the presence of...
normally innervated muscles within the same limb would certainly have preserved much of the normal vascular pumping activity and therefore reduced the likelihood of degenerative effects resulting from venous congestion.

Recent developments in the clinical use of electrical stimulation to restore the mass and function of long-term denervated muscles (27) pose the need for a better understanding of denervation phenomena, such as may be gained through animal experiments. So which is the most appropriate animal model? Examination of the comparative literature suggests that in fact rat muscle is unusual in its susceptibility to denervation, compared not only to rabbit (3, 15, 24, 28, 35, 36) but also to mouse (12, 56), guinea pig (26, 31, 39), cat (40, 50), and turkey (4). The lack of necrotic degeneration in human quadriceps muscles in the first year following a denervating lesion bears certain clear similarities to the results reported here for the rabbit. Furthermore, lesions of the brachial plexus and cauda equina in humans are frequently incomplete, and our selective denervation model may therefore share some of the features of those conditions.

Should further studies reveal evidence of extensive necrosis in human muscles many years after the denervating injury, the associated phenomena would appear to be suitably modeled by sciatric section in the rat. In the first postlesion year, however, we conclude that the response to denervation of the TA muscle of the rabbit may be closer in nature and timescale to that of human muscles.

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REFERENCES

LONG-TERM DENERVATION IN RABBIT SKELETAL MUSCLE


