Cell Accumulation in the Junctional Region of Denervated Muscle

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Abstract. If skeletal muscles are denervated, the number of mononucleated cells in the connective tissue between muscle fibers increases. Since interstitial cells might remodel extracellular matrix, and since extracellular matrix in nerve and muscle plays a direct role in reinnervation of the sites of the original neuromuscular junctions, we sought to determine whether interstitial cell accumulation differs between junctional and extrajunctional regions of denervated muscle. We found in muscles from frog and rat that the increase in interstitial cell number was severalfold (14-fold for frog, sevenfold for rat) greater in the vicinity of junctional sites than in extrajunctional regions. Characteristics of the response at the junctional sites of frog muscles are as follows. During chronic denervation, the accumulation of interstitial cells begins within 1 wk and it is maximal by 3 wk. Reinnervation 1–2 wk after nerve damage prevents the maximal accumulation. Processes of the cells form a multilayered veil around muscle fibers but make little, if any, contact with the muscle cell or its basal lamina sheath. The results of additional experiments indicate that the accumulated cells do not originate from terminal Schwann cells or from muscle satellite cells. Most likely the cells are derived from fibroblasts that normally occupy the space between muscle fibers and are known to make and degrade extracellular matrix components.

DENERVATION of skeletal muscles results in a marked increase in the number of mononucleated cells in the connective tissue between muscle fibers, particularly in cells that look much like fibroblasts (29, 32, 33, 52). Since fibroblasts and other connective tissue cells make and degrade extracellular matrix constituents (6, 16), such changes raise the possibility that the cells that accumulate after denervation act to remodel the extracellular matrix of muscle. Indeed, several studies have shown that the concentrations of some extracellular matrix molecules are altered by denervation (4, 41, 45). This is of particular interest since there is evidence that extracellular matrix components play a direct role in regeneration of the neuromuscular junction. For example, in vivo studies of damaged motor nerves have revealed that regenerating axons preferentially grow through tubes of Schwann cell basal lamina to reinnervate muscle fibers at the original synaptic sites on the myofibers' basal lamina sheaths (17, 23, 27, 35), and in vitro studies have shown that neurites preferentially elongate on laminin, a major constituent of Schwann cell and muscle fiber basal lamina (10, 21, 26, 37, 43, 46). Moreover, the myofiber basal lamina is known to contain molecules that direct the formation of synaptic apparatus in regenerating axon terminals and myofibers (3, 7, 12, 30, 44).

In the study described here, we denervated frog and rat muscles and compared the change in number of mononucleated cells in junctional regions with that in extrajunctional regions. We report that for both species the increase in the number of mononucleated cells is severalfold greater in the junctional regions. As steps toward determining the function of the increase in mononucleated cells in the junctional region and identifying the signals that lead to it, we describe the time course of cell accumulation in frog muscle and the effect of reinnervation on it. Morphological and experimental evidence strongly supports the hypothesis that the cells that accumulate are fibroblasts. Brief accounts of portions of this study appeared elsewhere (8, 9).

Materials and Methods

Experiments were performed on male frogs (*Rana pipiens*, northern variety) and on male Sprague-Dawley rats (180–200 g). We used the paired cutaneous pectoris muscles in the chest of the frog and the paired platysma muscles in the scalp of the rat (13).

Operations

Frogs were anesthetized by immersion in 0.1% MS-222 (tricaine methanesulfonate) in water. Cutaneous pectoris muscles were denervated in one of two ways. To prevent reinnervation, at least a 1-cm segment of the second spinal nerve near the vertebral column was cut and removed. If reinnervation was to follow nerve damage, the nerve to the muscle was crushed with fine-tipped forceps within 2 mm of its point of entry into the muscle. To provide various lengths of denervation time before reinnervation, the nerve was recrushed at 2–3-d intervals until reinnervation was allowed to begin.

Rats were anesthetized by intraperitoneal injection of chloralhydrate (35 mg/100 g body wt) and nembutal (5 mg/100 g body wt). The platysma muscle was denervated by cutting the nerve within 2 mm of its point of entry into the muscle and removing a 1-cm length of the proximal stump.

General Procedures for Light and Electron Microscopy

Muscles were dissected in a Sylgard coated petri dish containing Ringer's solution (frog: 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $NaH_2PO_4H_2O$, pH 7.2; rat: 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 10 mM TrisCl, 10 mM glucose, pH 7.2). During the dissection they were illuminated from below using fiber optics. For most histological procedures, muscles were fixed with 1% glutaraldehyde in pH 7.2 phosphate buffer (0.09 M phosphate for frog muscle; 0.14 M phosphate for rat muscle) for 30 min, refixed with 1% osmium tetroxide in phosphate buffer, dehydrated in ethanol and embedded flat in a 0.1–0.5-mm thick wafer of Epon and Araldite (30). Sections for light microscopy were stained with toluidine blue. For electron microscopy, sections were stained with uranyl acetate and lead citrate.

Staining for Cholinesterase

In some preparations, the synaptic sites were marked by staining for cholinesterase (19) after glutaraldehyde and before osmium tetroxide fixation. The osmolarity of the cholinesterase stain for histological preparation of rat or frog muscles was adjusted by varying the sucrose concentration (frog: 5%; rat: 8.2%).

Staining Cell Nuclei for Observation in Whole Mounts

Pinned out muscles were fixed, dehydrated in increasing concentrations of ethanol, and stored in 100% ethanol overnight at 37°C. The muscles were then stained for 2 h in 0.1% cresyl violet in acetate buffer (100 mM sodium acetate, pH 3.5), rinsed in 95% ethanol, floated on xylene, and mounted whole on slides with Permount histological mounting medium (Fisher Scientific Co., Fair Lawn, NJ).

Determination of Interstitial Cell Density

We searched for changes in the density of interstitial cells in denervated muscles by comparing the number of interstitial cell nuclei to the number of myofibers. Counts on cross sections through the entire width of the cutaneous pectoris and platysma muscles showed that the myofiber number stays constant for both normal and denervated muscle fibers over the time course of our experiments. The mean number of muscle fibers per muscle in normal frog muscles was 480 ± 16 SEM, $n = 8$ muscles and in 10-wk denervated frog muscles was 520 ± 28, $n = 10$. The mean number of muscle fibers per muscle in normal rat muscles was 1,693 ± 162, $n = 4$ and in 10-d denervated rat muscles was 1,721 ± 138, $n = 4$. Quantitation of the number of interstitial cell nuclei per muscle fiber was carried out by light microscopy on 1-μm toluidine blue–stained cross sections of both junctional and extrajunctional regions of the muscle. The pattern of innervation in the frog and the rat muscles is such that the neuromuscular junctions are in the middle third of the muscle. Thus, sections of extrajunctional regions of the muscle contained no endplates while fields of junctional sections contained several muscle fibers with cholinesterase-stained sites. Counts were made of four fields from each section at ×1000; each field from frog muscles contained 10–18 muscles fibers and from rat muscles, 20–25 muscle fibers. An average value of interstitial cell nuclei per muscle fiber was obtained for each section. Data are presented as mean ± SEM.

Labeling of Nuclei of Mitotic Cells with Tritiated Thymidine

To identify cells undergoing mitosis after denervation, methyl-[^{3}H]thymidine (New England Nuclear, Boston, MA 81.7 Ci/mmol) was injected into the lymph sac of the frog (0.1 mCi in 200 μl of Ringer's solution) once a day for 21 d after denervation. We used the method of Heathcote and Sargent (14) to determine in other experiments that the tritiated thymidine was available to dividing cells for 2–5 h after injection. On the 22nd day, the muscles were removed, fixed, stained for cholinesterase, embedded, and sectioned. Slides containing cross sections of junctional regions of muscles were coated with liquid emulsion (Kodak NTB-2, Rochester, NY), exposed for 2 wk at 4°C, developed, and fixed. The sections were then washed, dried, stained with toluidine blue, and covered with coverslips mounted with glycerol. The percentage of nuclei that contained radiolabel was determined for each section for terminal Schwann cells and interstitial cells.

X-irradiation

For some experiments, frogs were irradiated for the first 3 d after denervation (irradiation procedure: Philips 250 kV 15 mA X-ray unit, 0.35 mm Cu filter, total dose/day: 2,300 rad). Lead shielding was placed over the frog to restrict radiation to the thorax.

Results

The cutaneous pectoris muscle of the frog and the platysma muscle of the rat are flat and thin. Nearly all of the neuromuscular junctions are situated in the middle third of the muscles, "the junctional region" (e.g., references 3 and 30). As illustrated in Fig. 1, within the junctional region there are several zones where the neuromuscular junctions of nearby muscle fibers are nearly in register.

When pinning out the isolated muscles over a light source, we noted that zones within the junctional regions of those that had been denervated for 1 wk or more were highly refractive when compared with the rest of the muscle (Fig. 2, *a* and *b*) and that the size, shape, and distribution of the refractive zones were similar to zones of clustered junctions in normal muscle. It was a search for the basis of this increased refractivity that led us to the observation that interstitial cells accumulate selectively in the vicinity of former synaptic sites in denervated muscles. We have no direct evidence that the increased refractivity of junctional zones is due

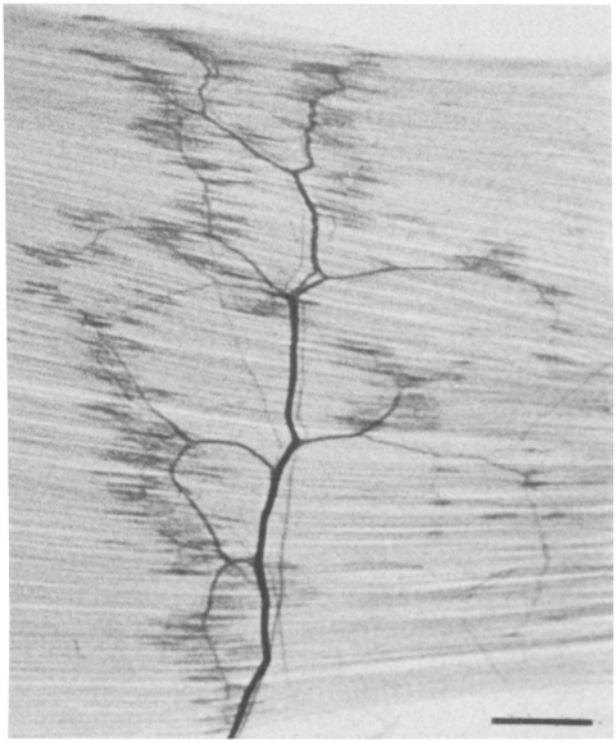


Figure 1. The neuromuscular junctions of the cutaneous pectoris muscle of the frog are situated in several zones within the middle third of the muscle, the junctional region. Elongate patches of cholinesterase stain on the myofibers mark the neuromuscular junctions, and myelin stain (osmium tetroxide) marks the nerve to the muscle and its intramuscular branches. The nerve enters the muscle from below, crossing the muscle's lateral edge. Most of the junctions lie along the nerve's secondary and tertiary branches. Within each zone, the junctions are nearly in register. Bar, 1 mm.

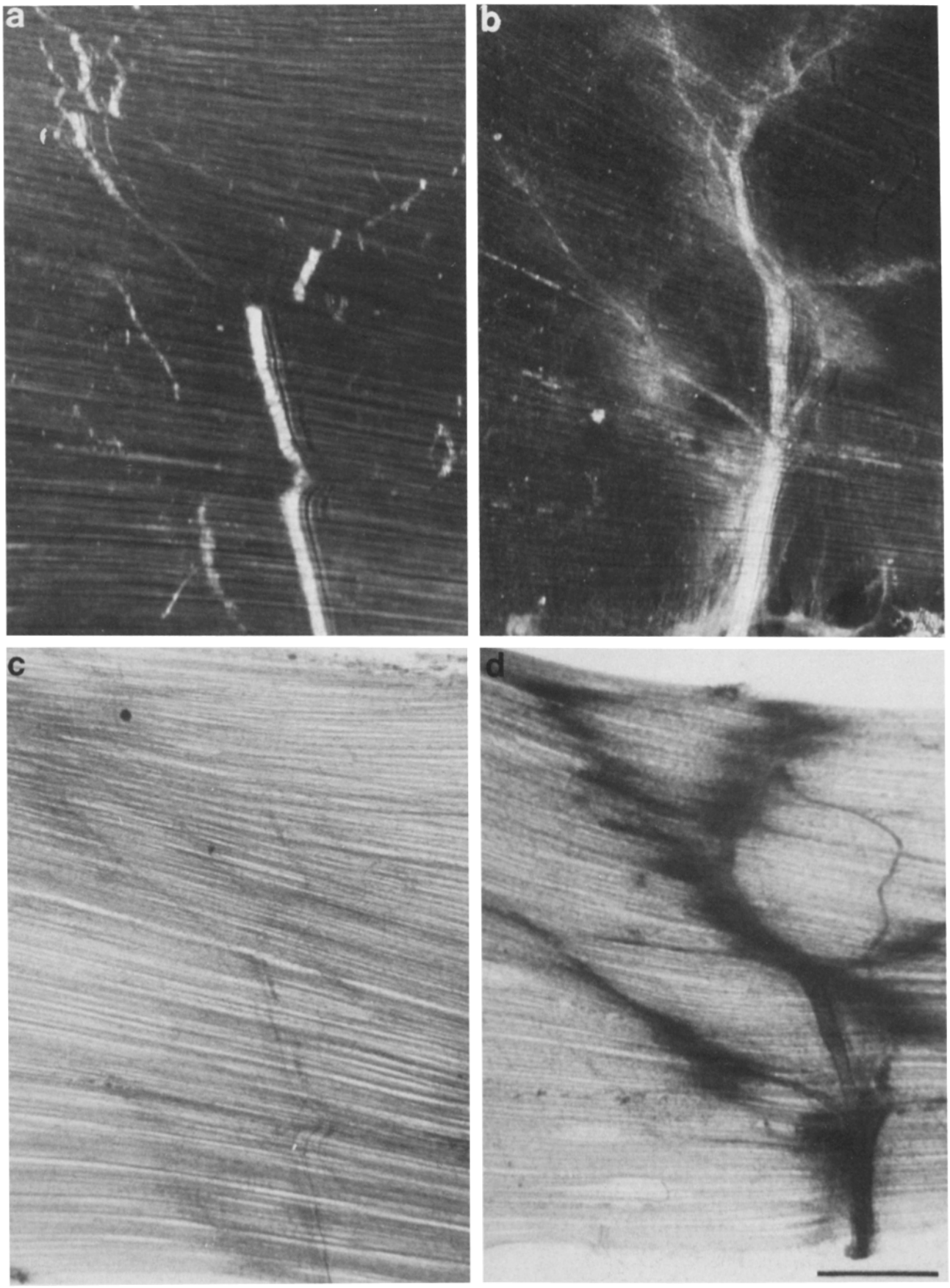


Figure 2. After denervation, zones in the junctional region of the cutaneous pectoris muscles become highly refractive when viewed in fresh preparations and they stain with cresyl violet, a marker for cell nuclei, after fixation. These zones have the same size, shape, and distribution as junctional zones in Fig. 1. Fresh preparations of a normal muscle (*a*) and a 3-wk denervated muscle (*b*) photographed with dark field illumination. (*c* and *d*) Same muscles as in *a* and *b* but fixed and stained with cresyl violet. Bar, 1 mm.

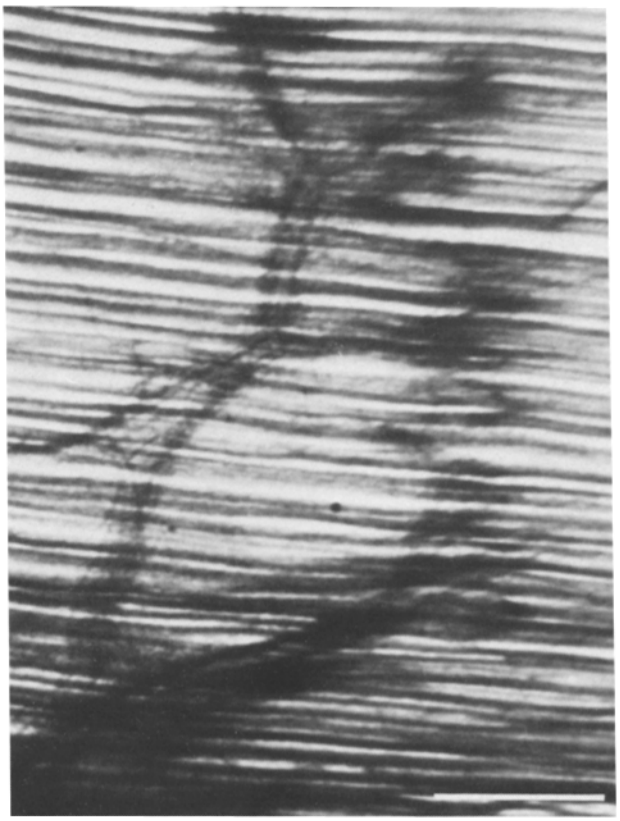


Figure 3. Nuclear staining associated with the intramuscular branches of a 6-d denervated nerve sheath in a rat platysma muscle. Compare with similar staining in denervated frog cutaneous pectoris muscle shown in Fig. 2 *d*. Bar, 1 mm.

solely to an increase in cell number; the molecular composition of the extracellular matrix near synaptic sites is also altered after denervation (45). That the cell accumulation contributes to the refractivity seems likely for the following reasons: (*a*) the increased cell number must alter the properties of the light path, (*b*) the highly refractive zones within the junctional region were coincident with zones of increased cell number (Fig. 2), and (*c*) the time courses, as presented in later sections, were similar both for the appearance of refractivity and the increase in cell number after denervation, and the disappearance of refractivity and decrease in cell number upon immediate reinnervation of the muscle.

Evidence for the Accumulation of Cells

We determined that cells selectively accumulate in the junctional region of denervated skeletal muscles by first examining the distribution of cell nuclei. Figs. 2, *c* and *d*, and 3 are from whole mounts of frog and rat muscles treated with the nuclear stain, cresyl violet. In normal muscles, the staining intensity is low and it is nearly uniform throughout. However, in junctional regions of denervated muscles there are zones of intense staining. As indicated above, these intensely stained zones are similar in size and shape to the zones of clustered neuromuscular junctions seen in normal muscles. The cresyl violet–stained zones are not associated with blood vessels nor with major intramuscular branches of the peripheral nerve stump, but rather are confined to the vicinity of short intramuscular nerve branches (now devoid of axons) that lead to the junctional sites on myofibers.

To document further that denervation caused an increase in number of cell nuclei selectively in the junctional region of muscles and to learn whether the nuclei belonged to muscle fibers or to cells in the connective tissue, we made cross sections through normal and denervated frog and rat muscles and examined them by light microscopy. The synaptic site of each muscle fiber was marked by cholinesterase stain. When we examined zones where synaptic sites were concentrated, we observed a striking increase in the number of nuclei of cells in the connective tissue of the denervated muscles. On the other hand there was no change in the number of myofibers in either rat or frog muscles throughout the course of our experiments (see Materials and Methods). In frog muscles, the increase in interstitial cell nuclei was easily discernible (Fig. 4) after 3 wk of denervation which, as described below, is the time when the response is maximal. When we compared the ratio of the number of interstitial cell nuclei to myofiber profiles in normal frog muscles to that of frog muscles denervated for 3 wk, we observed as much as a 400% increase in the concentration of interstitial cell nuclei in junctional zones of denervated muscles (see Fig. 8). In rat, there was a 200% increase in the junctional interstitial cell concentration by 10 d of denervation (normal muscle: 0.40 $\pm$ 0.02, $n = 6$ muscles; 10 d denervated muscle: 1.09 $\pm$ 0.06, $n = 4$).

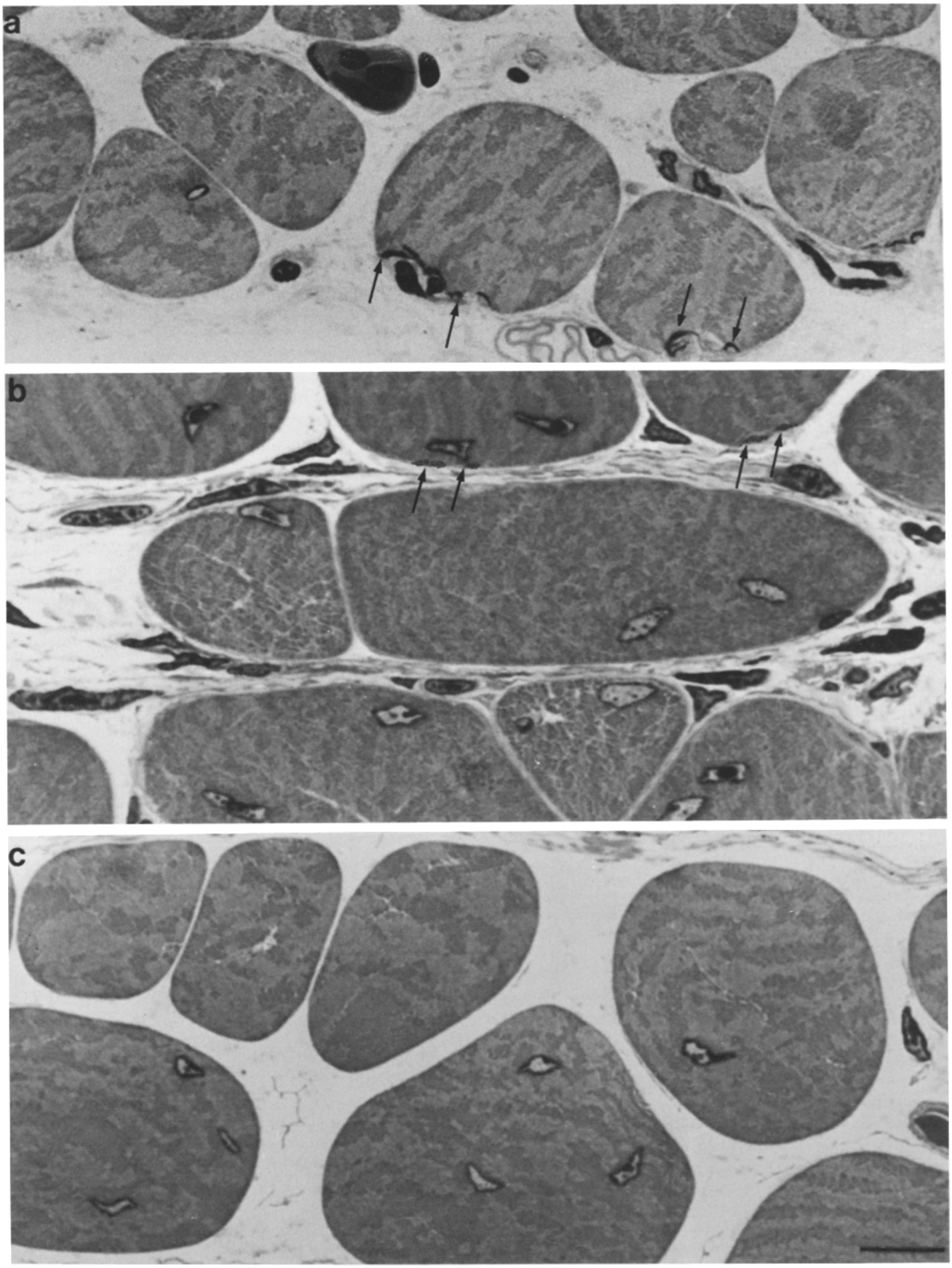
We measured only a 25% increase in the concentration of interstitial cell nuclei in extrajunctional regions of denervated frog muscles up to 10 wk after denervation (see Fig. 8), and a 24% increase in denervated rat muscles at 10 d (normal rat muscles: 0.25 $\pm$ 0.03, $n = 6$ muscles; 10 d denervated rat muscles: 0.31 $\pm$ 0.05, $n = 4$). Thus, while interstitial cell number increases throughout the muscle after denervation, the increase is far greater in junctional regions than in extrajunctional regions (14-fold in frog; sevenfold in rat).

There was no discernible difference in the ratio of myofiber nuclei per muscle fiber in junctional and extrajunctional regions of frog muscles up to 6 wk after denervation. Thus, redistribution of myofiber nuclei after denervation of frog muscle does not account for any of the increase in nuclear density in junctional zones of muscles viewed in whole mounts.

Structure of the Accumulated Cells and Relationship of the Cells to Synaptic Sites

A mixture of zinc iodide and osmium tetroxide is commonly used for staining nerve terminals in whole mounts of muscle (e.g., 18, 31, 51). In many such preparations the stain also darkens mononucleated cells. We denervated frog muscles

Figure 4. Cells accumulate in the connective tissue of denervated muscles selectively in the vicinity of the sites of the former neuromuscular junctions. (*a–c*) 1-μm-thick cross sections of frog cutaneous pectoris muscles stained with toluidine blue. Sites of neuromuscular junctions are marked with cholinesterase stain (*arrows*). (*a*) Innervated muscle, junctional region. (*b*) 4-wk denervated muscle, junctional region. Numerous elongate cells with flattened, chromatin-rich nuclei occupy the space between muscle fibers. (*c*) Same denervated muscle, extrajunctional region. Bar, 10 μm.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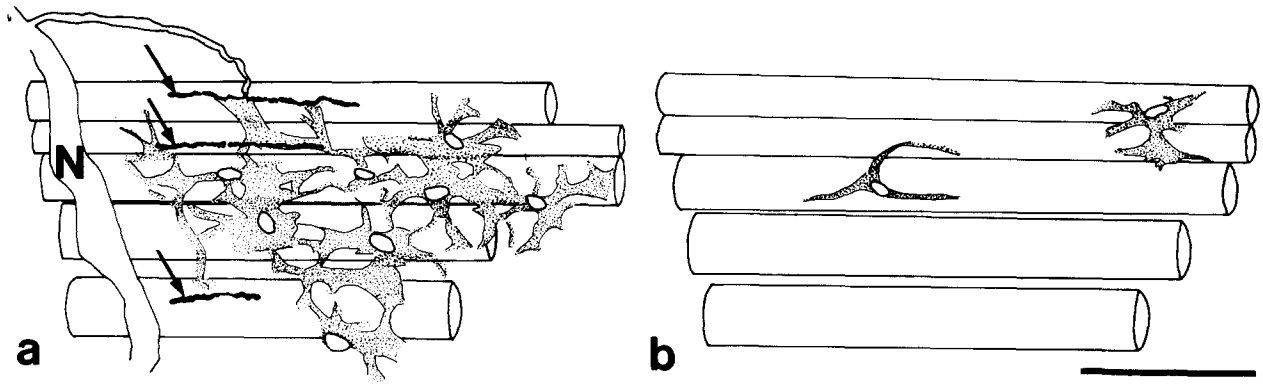

Figure 5. The cells that accumulate in the junctional region of denervated muscle are stellate-shaped. They are similar to cells in the extrajunctional region. Camera lucida tracings from a whole mount of a frog cutaneous pectoris muscle treated with zinc iodide and osmium tetroxide, which stains both axon terminals and interstitial cells. The muscle had been denervated for 2 wk and then reinnervated so that synaptic sites could be identified. (*a*) Junctional region. (*b*) Extrajunctional region. *N*, nerve; *arrows*, regenerated axon terminals. Bar, 50 μm.

for 2–3 wk and then allowed an additional 3–4 wk for the nerve to reinnervate them so that synaptic sites could be identified. In the extrajunctional regions of such muscles stained with zinc iodide and osmium tetroxide, we observed scattered stellate-shaped cells with round nuclei (Fig. 5). Similar cells were seen in the junctional region, but they were so densely packed that the boundaries between them often could not be resolved (Fig. 5). Cells having the same shape were observed at a low frequency in normal muscles; there was no obvious difference in frequency between junctional and extrajunctional regions.

Light and electron microscopy on sections of denervated frog muscle, prepared by conventional methods, revealed additional features of the accumulated cells (Figs. 4 and 6). The profile of the nucleus of each cell was elongate regardless of whether the sections were longitudinal or cross with regard to the long axis of the muscle fibers, indicating that the nuclei were discoidal in shape. The nuclei had abundant chromatin. The cytoplasmic ground substance was moderately osmiophilic. The granular endoplasmic reticulum was characterized by loosely arranged elongate cisterns distributed throughout the cell body and processes. Based on their shape and the appearance of their organelles and cytoplasmic ground substance, the accumulated cells resemble cells observed in the junctional and extrajunctional regions of normal muscles and share features common to fibroblasts (6).

Processes of the accumulated cells were situated within 2 μm of >80% of the synaptic sites in denervated frog muscles (Fig. 6; 83% ± 4 SEM, $n = 6$ muscles denervated for 3 wk, >50 synaptic sites were examined per muscle). Such processes extended tens of micrometers beyond the synaptic sites, enwrapping much of the junctional zone of individual muscle fibers (Fig. 7). We calculated from electron micrographs of innervated and denervated (3 wk) frog muscle fibers cross-sectioned through a cholinesterase-stained site that on average 53% (± 2 SEM; $n = 66$ muscle fibers from three muscles) of the surface area of the junctional region of denervated muscle fibers was apposed by at least one interstitial cell process within 2 μm of the myofiber membrane while only 16% (± 1, $n = 84$ muscle fibers from three muscles) of the surface area of innervated fibers was apposed by similar cells; the cholinesterase-stained spots occupied only 8% (± 0.4, $n = 77$ muscle fibers from three muscles) of the surface area in innervated muscle fibers. In addition, after denervation there were often processes from more than one interstitial cell wrapping the same muscle fiber, resulting in multiple layers of cell processes between fibers. Thus, the increase in cell number results in a diaphanous veil of cell

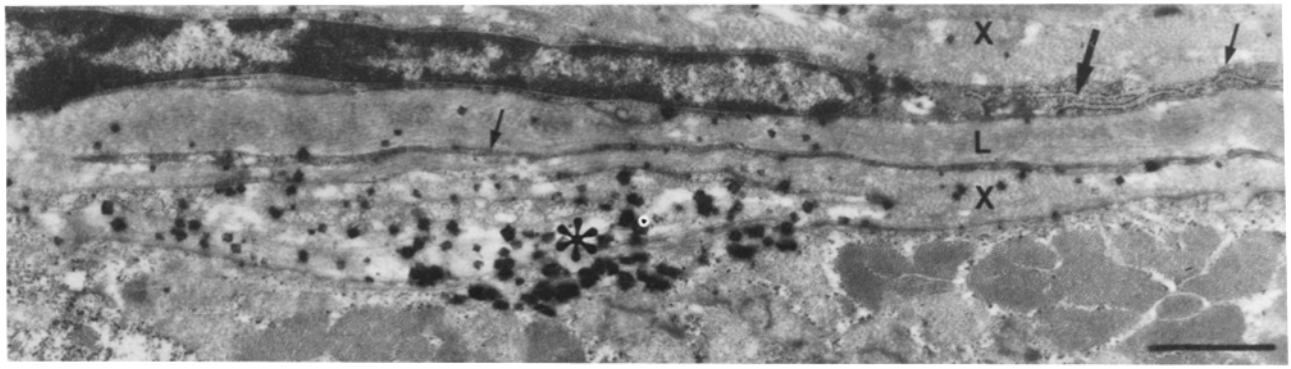

Figure 6. Cell body of an interstitial cell and interstitial cell processes (*small arrows*) appose a former synaptic site on a 4-wk denervated frog muscle fiber. The thin interstitial cell nucleus is rich in chromatin and one of the processes contains prominent endoplasmic reticulum (*large arrow*). The cell body and processes are surrounded by collagen fibrils (cross sectioned, *X*; longitudinal sectioned, *L*). The former synaptic site (*asterisk*) on a myofiber is marked with cholinesterase stain. Bar, 1 μm.

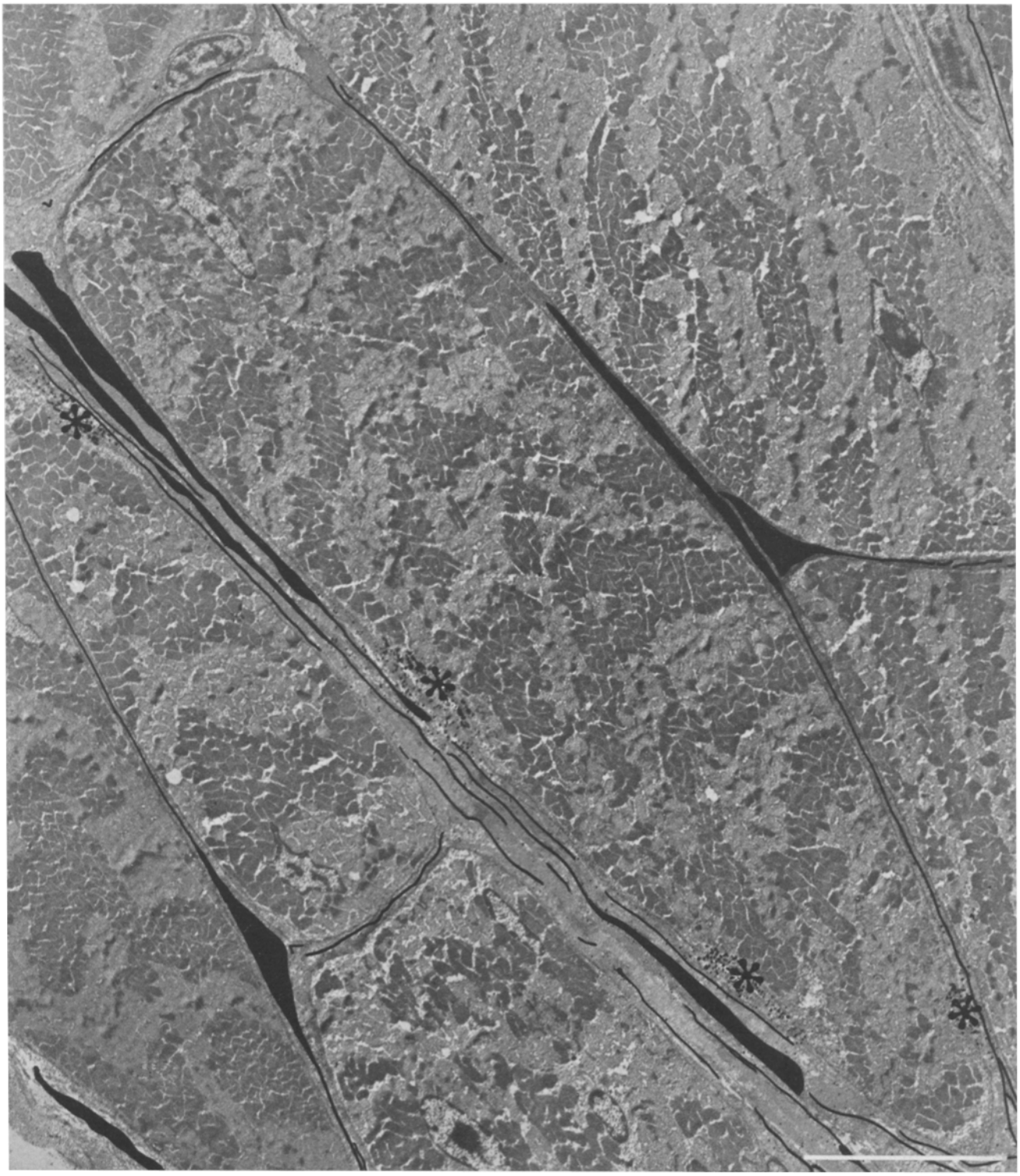

Figure 7. The processes of interstitial cells in the junctional region of denervated myofibers enwrap the fibers in a way that often results in multiple processes layered between adjacent fibers. In this photograph, which is from a 4-wk denervated frog muscle, the interstitial cells have been accentuated by ink. Synaptic sites (*asterisk*) are marked by cholinesterase stain. Bar, 10 μm.

processes selectively enclosing a region of each muscle fiber centered around the synaptic site.

We examined by electron microscopy cross sections of over 100 denervated myofibers at each of 3 d and 1, 2, and 3 wk after denervation. Each myofiber profile had at least one cholinesterase-stained synaptic site and was enwrapped by interstitial cell processes. In every case the interstitial cells were surrounded by a layer of collagen fibrils. Accordingly, the cell processes were not in direct contact with either the plasma membrane or basal lamina sheath of muscle fibers or Schwann cells.

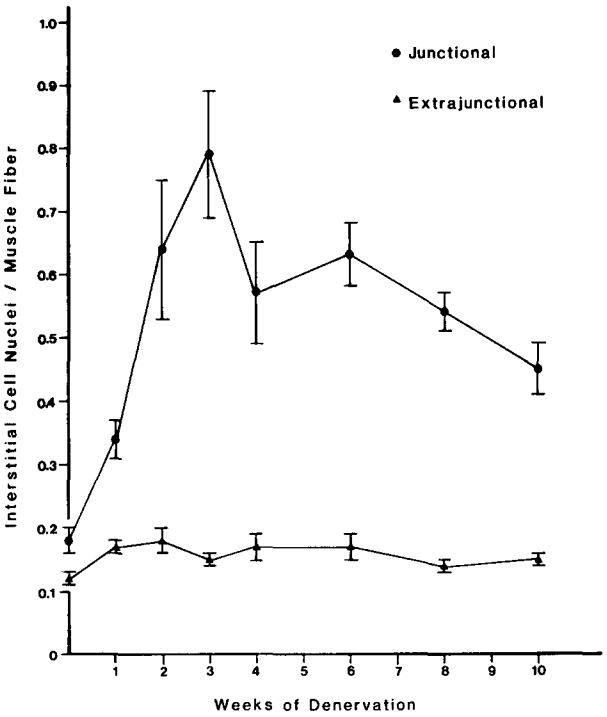

Figure 8. Time course of the selective accumulation of interstitial cells in the junctional region of denervated frog muscles. Data from innervated muscles are plotted at 0 wk of denervation. Counts of interstitial cell nuclei were made from cross sections through both junctional (*closed circles*) and extrajunctional (*closed triangles*) regions of muscle. Each value is the mean ± SEM of data from at least seven frog muscles. The accumulation peaks at 3 wk after denervation and by 10 wk there is a significant decline in cell density from the 3-wk maximum (Student's *t* test, $P < 0.01$).

Time Course of the Response and the Effect of Reinnervation

Fig. 8 illustrates the effect of denervation on the number of interstitial cells per myofiber in junctional zones of the cutaneous pectoris muscle at different times after nerve damage. A significant increase in the interstitial cell/myofiber ratio was evident as early as 1 wk after nerve damage. At this time axons and nerve terminals had degenerated and had been phagocytized (23). The response reached a peak at 3 wk after denervation, at which time there was a fourfold increase over normal. The maximum density of interstitial cells in junctional zones was not maintained for periods of denervation >3–4 wk. By 10 wk of denervation there was a significant decrease in the cell density to 57% of maximum.

Reinnervation of the muscle immediately after nerve damage interrupted the accumulation of interstitial cells. In these experiments the nerve to the cutaneous pectoris muscle was crushed a single time near its entry to the muscle, and its axons were then allowed to regrow, resulting in functional synaptic contacts by 2 wk after nerve damage (23). Muscles were examined at different times after the nerve crush. As illustrated in Fig. 9, the initial increase in interstitial cell density in junctional zones was the same in reinnervated and chronically denervated muscles. However, as the muscles were reinnervated, the density of interstitial cells failed to rise as in chronically denervated muscles; instead it gradually declined, returning to levels found in innervated muscle by 5 wk after nerve damage.

When reinnervation was delayed to allow for maximum accumulation of interstitial cells, the density of cells was unaffected by the return of the nerve. In this experiment, the nerve to the cutaneous pectoris muscle was crushed every third day for 3 wk, then allowed to regrow for 5 wk. At this time, 8 wk after the initial nerve crush, the muscle had become reinnervated and twitches could be elicited. Findings from previous studies (23) on repeated damage to the cutaneous pectoris nerve indicate that the muscles in our experiments had been reinnervated for at least 2 wk. When examined by light microscopy, there was no statistical difference between the junctional density of interstitial cells in the reinnervated muscles (0.43 ± 0.05 nuclei/muscle fiber, $n = 15$ muscles) and in muscles that had been chronically denervated for 8 wk (0.51 ± 0.04, $n = 9$; Student's *t* test, $P < 0.01$). Thus, while reinnervation of the muscle immediately after nerve damage interrupted the accumulation of interstitial cells in junctional zones, reinnervation had no discernible influence on interstitial cell number when delayed until the cell accumulation had been established.

On Identification of the Interstitial Cells

Muscle and nerve contain several types of mononucleated

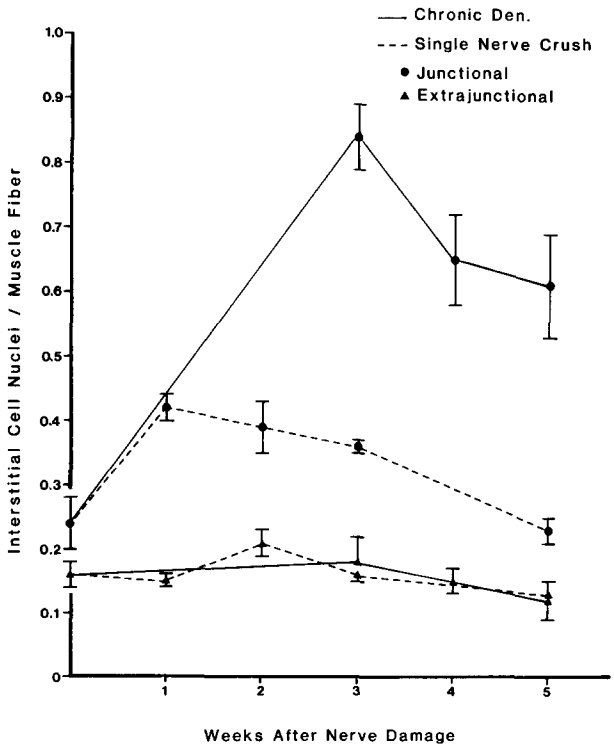

Figure 9. Reinnervation of frog muscle within 1 wk after nerve damage interrupts the accumulation of interstitial cells in the junctional region. The solid line indicates the density of interstitial cell nuclei in muscles that were chronically denervated. The dashed line is data from muscles that were denervated by a single crush to the nerve and subsequently reinnervated. Counts were made from sections of both junctional (*closed circles*) and extrajunctional (*closed triangles*) regions of muscle. Each point is the mean ± SEM from at least eight frog muscles.

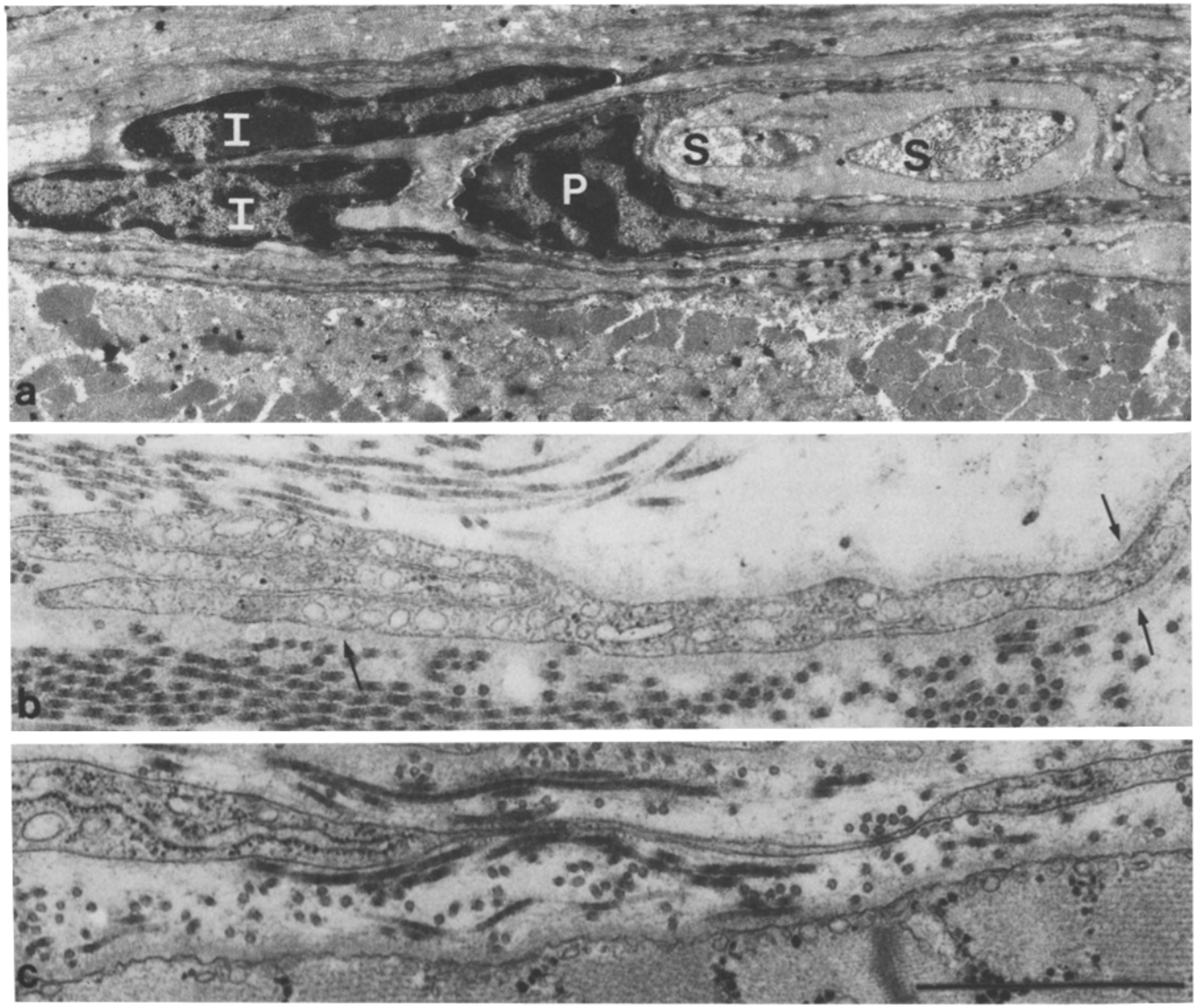

Figure 10. The interstitial cells that accumulate after denervation have structural features that distinguish them from Schwann cells and perineurial cells. (*a*) Cross-section of a 3-wk denervated cutaneous pectoris muscle showing interstitial cells (*I*) and the remnants of a nerve branch including a perineurial cell (*P*) that encloses two Schwann cell processes (*S*) formerly associated with axons. The cytoplasm of interstitial cells is similar in electron density to that of the perineurial cell but is distinctly more dense than that of Schwann cells (*b* and *c*). Characteristically a process of a perineurial cell (*b*) contains linear arrays of caveoli and is lined by basal lamina (*arrows*), but a process of an interstitial cell (*c*) does not have a series of caveoli and does not have a basal lamina. Bar: (*a*) 4 μm, (*b* and *c*) 1 μm.

cells, any or all of which could give rise to the increased number of interstitial cells at junctional sites. As noted above, the shapes, cytoplasm, and nucleus of the accumulated cells are similar to those of fibroblasts. Their cytoplasmic characteristics distinguish the accumulated cells from Schwann cells and perineurial cells in both normal and denervated muscle. Specifically, the cytoplasmic ground substance of Schwann cells is much less osmiophilic than that of the accumulated cells; and the processes of perineurial cells are occupied by linear arrays of caveoli and are lined by basal lamina (Fig. 10; see also reference 49). The following experiments provide further evidence that the accumulated cells are not derived from the Schwann cells that originally capped the axon terminal and make it unlikely that they originate from muscle satellite cells.

First we established that the accumulated interstitial cells arise from cells that undergo mitosis after denervation (see also reference 33). Once each day after permanently denervating the cutaneous pectoris muscles, we injected tritiated thymidine into the frog's lymph sac (see Materials and Methods). Analysis of cross sections through junctional regions of muscles denervated for 3 wk revealed that 63% ($\pm$ 9, mean $\pm$ SEM, n = 5 muscles) of the interstitial cell nuclei concentrated in junctional regions of the muscle contained radiolabel and thus were the product of cell division.

Evidence against Terminal Schwann Cells. After denervation, the Schwann cells that normally cap axon terminals phagocytize the nerve terminals and come to occupy the terminal's position directly opposite the muscle fiber. We found that unlike the Schwann cells that surround the parent axons (5, 40) or the cells that accumulate in the nearby connective tissue, the terminal Schwann cell nuclei did not label with

tritiated thymidine in denervated preparations; 64 Schwann cell nuclei were examined at cholinesterase-stained synaptic sites in seven muscles 3 wk after denervation and none was labeled. Moreover, the frequency of Schwann cell nuclei at synaptic sites did not change during the 3 wk after denervation (Table I). Since no significant number of terminal Schwann cells divide after denervation nor leave the synaptic site, they cannot be the source of the interstitial cells concentrated in the junctional region of denervated muscle.

Evidence against Muscle Satellite Cells. Muscle satellite cells are situated along myofibers, just inside the myofiber basal lamina sheath (28). They undergo division after denervation of muscle (15, 22, 34, 47) and under certain conditions they cross the myofiber basal lamina to take a position in the muscle's connective tissue (20, 25). As expected, we also found that after denervation and exposure to tritiated thymidine, the nuclei of muscle satellite cells contained radiolabel. To establish whether the cell accumulation in the junctional region of the muscle can be attributed to migration of satellite cells into the interstitial space, we x-irradiated frogs at the time of denervation; there is good evidence that x-irradiation kills dividing satellite cells in the cutaneous pectoris muscle under the conditions that we used (see Materials and Methods and references 3 and 44). We found that despite the x-ray treatment, cells accumulated in the connective tissue and their density was similar to that in denervated muscles of unirradiated frogs (Fig. 11). We conclude that the accumulation of cells after denervation does not arise from muscle satellite cells.

Discussion

Previous studies (29, 32, 34, 52) concerning the accumulation of interstitial cells in denervated skeletal muscle did not include methods that would permit identification of junctional and extrajunctional regions. Indeed, it is unlikely that we would have discovered the selective accumulation in the vicinity of junctional sites had we not examined muscles that had zones highly enriched in junctional sites and had we not marked the sites with cholinesterase stain. In cross sections from extrajunctional regions of denervated muscles from frog and rat, we observed a 25% increase in the number of cells. However, in sections from the junctional region we detected a 200% increase in rat muscle and a 400% increase in frog muscle. Thus the difference between junctional and extrajunctional regions was sevenfold in the rat muscles and 14-fold in the frog muscles. The percent increase in frog was maximal at 3 wk as determined by examining muscles at different times up to 10 wk after denervation. The percent increase in rat is based on only one time point, 10 d after denervation; thus the maximal increase in rat may be greater than that we observed. Even though we selected fields from the junctional region that contained several junctional sites, only a small fraction of the myofiber profiles in each field had such sites. Thus our method of analysis, which related the number of interstitial cells to the number of myofiber profiles within a field, may have led to an underestimate of the extent of cell accumulation at junctional sites on myofibers; it may be much greater than the 200% and 400% increase we detect in areas enriched for, but not containing exclusively, junctional sites.

Table I. The Percent of Cross-sectioned Synaptic Sites Apposed by a Schwann Cell Nucleus in Innervated and Denervated Frog Muscle*

Muscle	*n*	Percentage of synaptic sites apposed by Schwann cell nuclei
Innervated	12	10.0 ± 0.6‡
Denervated (1 wk)	10	11.2 ± 1.2
Denervated (3 wk)	12	9.5 ± 0.7

* At least 50 synaptic sites, stained for cholinesterase, were examined per muscle.
‡ Data presented as mean ± SEM.

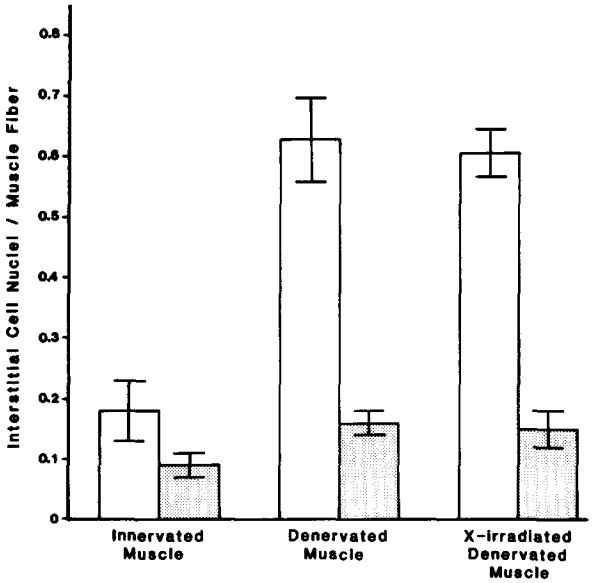

Figure 11. X-irradiation at the time of denervation did not alter the accumulation of interstitial cells. Muscles were denervated for 3 wk. Data are shown for junctional (*open bars*) and extrajunctional (*shaded bars*) regions of muscles. Each point is the mean ± SEM of at least five muscles.

The source of the cells that accumulate in the junctional region is currently unknown. Results of experiments conducted by Murray and Robbins (33) on denervated muscles after specifically labeling blood cells make it seem unlikely that the accumulated cells are derived from circulating leukocytes. Indeed, we observed few macrophages (characterized by microvilli, very electron dense cytoplasm, and phagocytic vacuoles; 33, 50) between muscle fibers. We show that the accumulated cells are derived from cells that undergo mitosis after denervation. Denervation results in mitosis of several cell types in nerve and muscle. These include fibroblasts, mast cells, Schwann cells, perineurial cells, and muscle satellite cells (33). We observed mast cells (characterized by conspicuous cytoplasmic granules) only rarely in the denervated muscles we examined, and thus they contributed little, if any, to the selective accumulation of cells in the vicinity of junctional sites. We rule out the possibility that muscle satellite cells make a significant contribution to the accumulation because x-irradiation of denervated muscles at a dosage that results in the death of dividing satellite cells had no discernible effect on cell accumulation. We also conclude that accumulated cells are not derived from Schwann cells because the cytoplasm of Schwann cells that

capped axon terminals and ensheathed axons in nerves is much less electron dense than that of accumulated cells. Moreover, we show that the terminal Schwann cells, unlike the Schwann cells that ensheath preterminal axons in nerves, do not divide in response to denervation. Remaining cells that could give rise to the cell accumulation in the junctional region are perineurial cells and fibroblasts (and/or capillary pericytes which are thought to give rise to fibroblasts; 38). The cytoplasm and nuclei of perineurial cells are similar in electron density to that of the accumulated cells. However, perineurial cells associated with nerves in both normal and denervated muscles have numerous caveoli in the plasma membrane and their surface is lined by basal lamina, neither of which are characteristics of the accumulated cells. Fibroblasts are the most likely source of the accumulated cells; the shape and fine structure of the accumulated cells is strikingly similar to that of fibroblasts in normal muscle and nerve. Fibroblasts in nerve, as in muscle, undergo mitosis when the nerve is damaged (1, 48). Accordingly, the accumulated cells could be derived from nerve fibroblasts that escape from the nerve near the junctional sites, or muscle fibroblasts that, after dividing, migrate to the junctional sites, or muscle fibroblasts in the vicinity of junctional sites that undergo mitosis at a greater rate than those in extrajunctional regions.

Degenerating axons may provide signals that lead to the cell accumulation; in vitro studies have shown that axons contain molecules that cause Schwann cell mitosis (40). An alternative explanation is that the signal is provided by remaining intact components of the neuromuscular junction—the Schwann cell or muscle fiber. In fact, we will document in an upcoming report that the muscle cell must be at the synaptic site in order for the cell accumulation to occur (for brief account see reference 9). We show here that reinnervation of frog muscles before maximal cell accumulation, but after degeneration and phagocytosis of axon terminals (23), inhibited the accumulation. Thus the signals that lead to the cell accumulation must be either attenuated or no longer effective in the presence of a regenerating axon. Reinnervation of muscle after the maximal cell accumulation did not reduce the cell number. This may mean that, once interstitial cells have accumulated at junctional sites, the maintenance of the increased population is not dependent on signals from cells of the neuromuscular junction, or that reinnervation of muscle after prolonged periods no longer influences the signaling. It is not likely that the signaling requires direct contact between interstitial cells and cells of the neuromuscular junction; we examined by electron microscopy junctional sites on over 100 myofibers at each of several different times before and during the cell accumulation, and in no case did we observe interstitial cells in direct contact with the plasma membrane or basal lamina of Schwann cells or myofibers.

The selective accumulation of interstitial cells in the junctional region of muscle naturally raises the question of what role the accumulation might play in degeneration and/or regeneration of the neuromuscular junction. It has long been known that there is an increase in the density of muscle connective tissue after long periods of denervation (4). In addition, denervation of rat muscle results in enhanced binding of antibodies against fibronectin and a heparin sulfate proteoglycan in the vicinity of synaptic sites (45). The accumulated cells may play a role in these or other alterations in the extracellular matrix around the junctional regions of muscle fibers. Fibroblasts produce several extracellular matrix constituents in vitro including Type I (fibrillar) collagen, fibronectin, and proteoglycans (6, 16). All are components of the connective tissue of muscles and nerves in vivo and all are associated with, or a part of, the basal lamina of muscle fibers and Schwann cells. It has been reported that fibroblasts promote the formation of basal lamina on myofibers during myogenesis in vitro (24, 42). An increase in extracellular constituents might stabilize or alter the basal lamina sheaths during the changes that take place in the cellular components of the neuromuscular junction after axon degeneration. Such an effect is of particular interest because the basal lamina sheaths of Schwann cells and muscle fibers contain information that influences axon elongation and directs the formation of synaptic apparatus in regenerating axons and myofibers (3, 7, 12, 21, 26, 30, 37, 44, 46). Moreover, fibronectin, like laminin, is known to be an excellent substrate for axon elongation in vitro (2, 37). N-CAM, a molecule that is associated with the surface of myofibers, axons, and Schwann cells and that can influence axon growth, appears in extracellular matrix after denervation (11, 36, 39). At least some interstitial cells in denervated muscles stain for N-CAM, suggesting these cells produce or bind the molecule (11). Studies aimed at identifying the interstitial cells that accumulate at the denervated neuromuscular junctions, determining the source and nature of the signals that cause them to accumulate, and defining their function, are underway.

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