Neonatal and Adult Myosin Heavy Chain Isoforms in a Nerve–Muscle Culture System

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Abstract. When adult mouse muscle fibers are co-cultured with embryonic mouse spinal cord, the muscle regenerates to form myotubes that develop cross-striations and contractions. We have investigated the myosin heavy chain (MHC) isoforms present in these cultures using polyclonal antibodies to the neonatal, adult fast, and slow MHC isoforms of rat (all of which were shown to react specifically with the analogous mouse isoforms) in an immunocytochemical assay. The adult fast MHC was absent in newly formed myotubes but was found at later times, although it was absent when the myotubes myotubes were cultured without spinal cord tissue. When nerve-induced muscle contractions were blocked by the continuous presence of α-bungarotoxin, there was no decrease in the proportion of fibers that contained adult fast MHC. Neonatal and slow MHC were found at all times in culture, even in the absence of the spinal cord, and so their expression was not thought to be nerve-dependent.

Thus, in this culture system, the expression of adult fast MHC required the presence of the spinal cord, but was probably not dependent upon nerve-induced contractile activity in the muscle fibers.

THE expression of the different myosin isoforms in skeletal muscle is developmentally regulated (41). In fast-contracting rat muscle, neonatal myosin replaces the embryonic isoform and is the predominant type by 7–11 d after birth; subsequently the neonatal myosin is replaced by the adult fast isoforms (9, 30, 45, 46). Development of slow muscle fibers can occur by several pathways, but similarly involves myosin isozyme transitions (8, 30). Analogous developmentally regulated myosin isoforms have also been found in rabbits (24), mice (18, 44, 47), humans (17), and chicks (1, 3, 29, 38).

In contrast to these transitions that occur in muscle tissue in vivo, chicken and rat muscle cells in culture contain either embryonic myosin (1–3, 32, 45) or embryonic and neonatal myosin, but the neonatal myosin was found only in long-term cultures (7, 42). The presence of adult fast myosin was not described in such cultures. However, we have previously demonstrated that the adult fast isoform of the myosin heavy chain (MHC)[1] is produced in some of the regenerated muscle fibers that develop in organotypic nerve–muscle cultures (16). In these cultures, explanted adult mouse muscle fibers degenerate and their satellite cells proliferate within the existing basal lamina tubes to form new myotubes, some of which become innervated by the embryonic mouse spinal cord neurons (13–15, 34, 36, 37). Previously, we found that adult fast MHC was present after prolonged culture (3–5 wk) in cultures that had been cross-striated and contracting for at least 10 d.

We have now investigated whether the production of adult fast MHC in organotypic cultures is associated with time of development in vitro, the extent of morphological differentiation, the diameter of the muscle fibers, or the presence of the spinal cord with its potentially neurotrophic (20, 21) or activity-induced effects (22, 23, 25, 28, 39) on the muscle fibers. The results indicate that the appearance of adult fast MHC in these cultures is indeed a nerve-dependent phenomenon, but that it is probably not the result of nerve-induced contractile activity in the regenerated muscle fibers.

Materials and Methods

Tissue Culture

Nerve–muscle cultures were established as described previously (14). Briefly, sections of 13-d embryonic CD-1 mouse spinal cord were cultured at 35°C on collagen-coated coverslips (31) in Maximow slide assemblies (6) for 4 d before the addition of a bundle of muscle fibers. The fibers were teased from adult CD-1 mouse semimembranosus muscles (a muscle in the thigh region that is composed of only type IIA and IIB fast fibers) and placed ~1 mm from the ventral aspect of the spinal cord. Cultures were washed and refed every 3 or 4 d with a drop of medium composed of 57% (vol/vol) Eagle's minimum essential medium, 33% (vol/vol) human placental cord serum, 10% (vol/vol) chick embryo extract, and 6 mg/ml glucose.

In one experiment, lung tissue from a 13-d mouse embryo was used instead of the spinal cord section because this would also induce regeneration of the explanted muscle bundle (14, 36). The lungs were removed, washed, and cut into 1-mm^3 pieces using scalpels. Two or three fragments were placed in a line on the collagen-coated coverslip, and 4 d later a bundle of muscle fibers was placed 1 mm away but parallel to the line of the fragments.

1. *Abbreviations used in this paper*: EDL, extensor digitorum longus; MHC, myosin heavy chain.

In other experiments, bundles of fibers were taken from semimembranosus muscles that had been denervated in vivo for 4 (one culture) or 28 (five cultures) d before explantation. Recent experiments (MSE-P, unpublished observations) have indicated that such denervated muscles will sometimes regenerate in culture without requiring interaction with embryonic tissues. Three muscle bundles were placed on each collagen-coated coverslip so that the volume of tissue was approximately that of the nerve-muscle and lung-muscle culture.

In a different series of experiments, α-bungarotoxin purified from the snake venom (Miami Serpentarium) at either 10^{-7} M or 10^{-8} M was added to the growth medium at the time of explantation of the muscle bundle, and the cultures were maintained in the continuous presence of the drug. A stock aqueous solution of α-bungarotoxin was added to the medium immediately before use, and Hanks' balanced salt solution containing the drug was used for washing the cultures between feeds. Used medium was collected from randomly chosen cultures at the time of feeding and applied to contracting nerve-muscle cultures; inhibitory levels of drug were still present.

Electron Microscopy

The amount of explanted muscle fibers remaining in a culture and the presence and number of new myotubes (and whether these contained organized striations or were contracting) was assessed by light microscopic observation of the living cultures immediately before fixation for electron microscopy. Cultures were then washed in Hanks' balanced salt solution, fixed for 1 h in 2.5% gluteraldehyde in 0.1 M phosphate buffer pH 7.4, washed in the same buffer, postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer, and then dehydrated and embedded in araldite. Thick sections (1 μm) were stained with 1% toluidine blue in 1% borax, and thin sections were stained with uranyl acetate/lead citrate before being viewed in a Zeiss 2A electron microscope.

Antibody Characterization

The rabbit antibodies against the different isoforms of rat MHC were prepared as previously described for the adult fast and neonatal antibody (8) and for the slow MHC antibody (44). The neonatal antibody had previously been shown to react with mouse neonatal heavy chain but not with mouse adult fast MHC or light chains (47), and this specificity is confirmed here by immunoblotting (Fig. 1). We cannot exclude that this neonatal antibody reacts slightly with the embryonic heavy chain, because a pure source of this mouse isoform is not currently available. The adult fast antibody binds strongly to the myosin heavy chain in adult mouse muscle extracts (Fig. 1 *C*, lanes *a–c*) but not in newborn muscle extracts (lane *d*). The slow antibody binds most strongly to extracts of soleus muscle (Fig. 1 *E*, lane *b*) which contains ~50% slow and 50% fast fibers. There is also a slight binding to MHC of adult semimembranosus muscles (lane *c*), as well as to that of mixed adult (lane *a*) and neonatal (lane *d*) muscles, both of which contain some slow myosin.

In immunocytochemistry, the slow antibody is negative on either adult semimembranosus (44) or the fast fibers of adult extensor digitorum longus (EDL) (Fig. 2 *B*). It reacts strongly with slow fibers in the soleus of either 3-wk mice (Fig. 2 *E*) or newborn mice (Fig. 2 *H*), but is negative on newborn gastrocnemius (Fig. 2 *K*) which contains embryonic and neonatal myosin. We have shown elsewhere (44) that this slow antibody stains ~10% of the fibers in the mouse semimembranosus muscle during the first month after birth. Because this muscle contains no slow fibers in the adult, we must conclude from these results that there is either a developmentally regulated disappearance of adult slow myosin, or that this antibody also recognizes a novel form of myosin that has not yet been characterized; we cannot exclude this latter possibility. The adult fast antibody stains the semimembranosus and EDL of adult mouse (Fig. 2 *C*) and fast fibers of 3-wk mouse soleus (Fig. 2 *F*) which also contain neonatal myosin (Fig. 2 *D*). It does

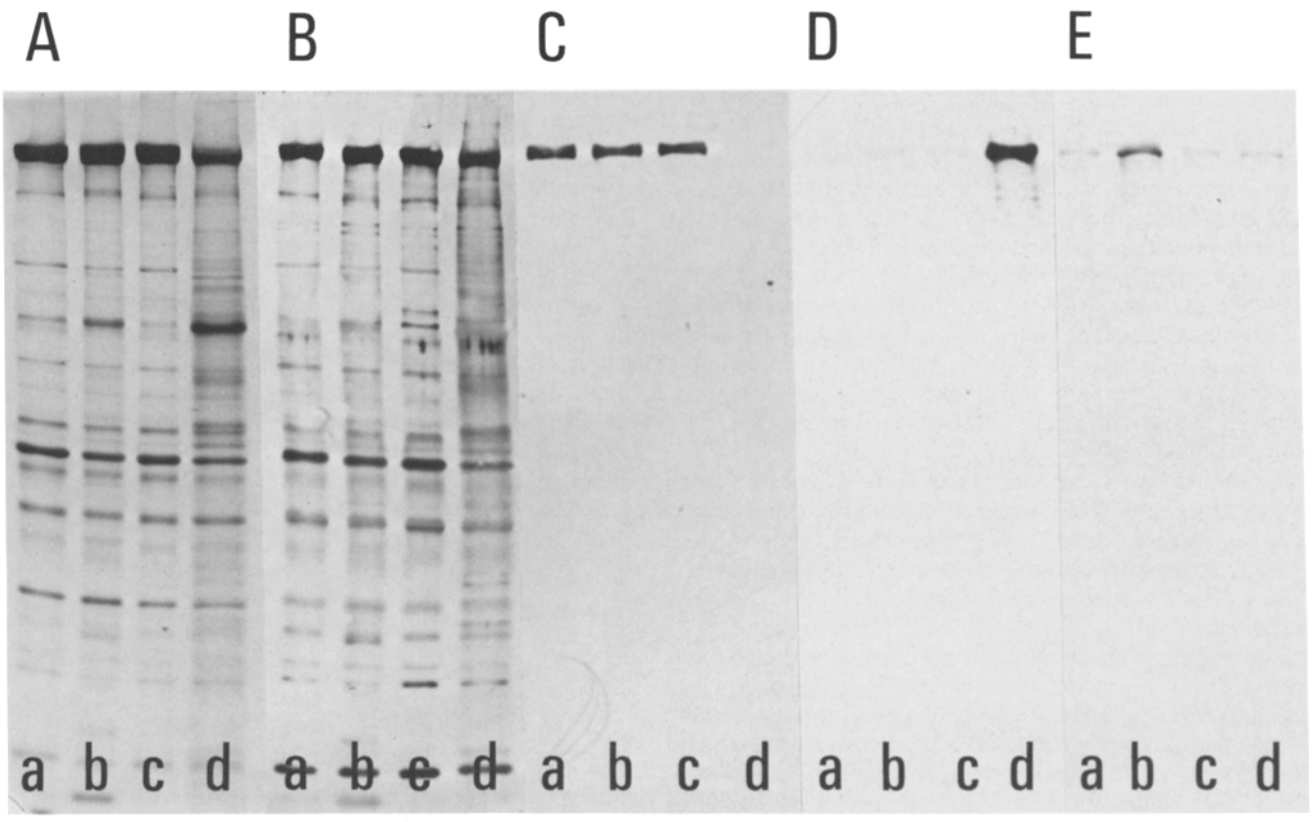

Figure 1. Immunoblot of antibodies to adult fast, neonatal, and adult slow myosin heavy chains using crude extracts of muscles from adult and 1–2-d-old mice. Muscle tissue was extracted as for electrophoresis of native myosin (8), denatured with SDS, and run on SDS polyacrylamide slab gels. One gel was stained with Coomassie Blue (*A*) and others were electrophoretically transferred to nitocellulose papers (47). One paper was stained with Indian ink (*B*) and others were reacted with each individual antibody, the binding of which was detected by the immunoperoxidase technique (8). *C*, *D*, and *E* correspond to adult fast, neonatal, and slow antibodies, respectively. In each panel the extracts were prepared from adult mixed hindlimb muscles (*a*), adult soleus (*b*), adult semimembranosus (*c*), and 1–2-d-old mixed hindlimb muscles (*d*).

not stain neonatal fast or slow muscles (Fig. 2, *L* and *I*, respectively) which contain embryonic, neonatal, and slow myosins. The neonatal antibody stains neither adult soleus or semimembranosus (results not shown) nor adult EDL (Fig. 2 *A*), but it does stain 3-wk soleus (Fig. 2 *D*) as well as newborn soleus (Fig. 2 *G*) and gastrocnemius (Fig. 2 *J*) mouse muscles.

Experimental Protocol

Each culture was assessed every 3–4 d by light microscopic observation at a magnification of 500, and at appropriate times the bundle of regenerated muscle fibers opposite the spinal cord was excised. The bundle was sandwiched between two blocks of liver together with bundles of fibers from a 3-wk-old mouse soleus and an adult mouse EDL. Frozen serial sections were reacted with each of the rabbit antibodies, followed by a rhodamine-conjugated goat anti-rabbit second antibody (Nordic Immunology, Tilburg, The Netherlands). The dilutions used for each antibody were determined in separate experiments on adult and newborn mouse muscles. The optimal dilution was that which strongly stained the appropriate muscle fibers without any nonspecific background on fibers containing heterologous myosins. These same dilutions were then applied to the frozen sections of blocks containing the cultures, the fibers of the soleus and EDL acting as both positive and negative internal controls for each of the antibodies on each section. The sections were each viewed and photographed using a Leitz Ortholux II fluorescent microscope and HP5 film. Each section was then stained with

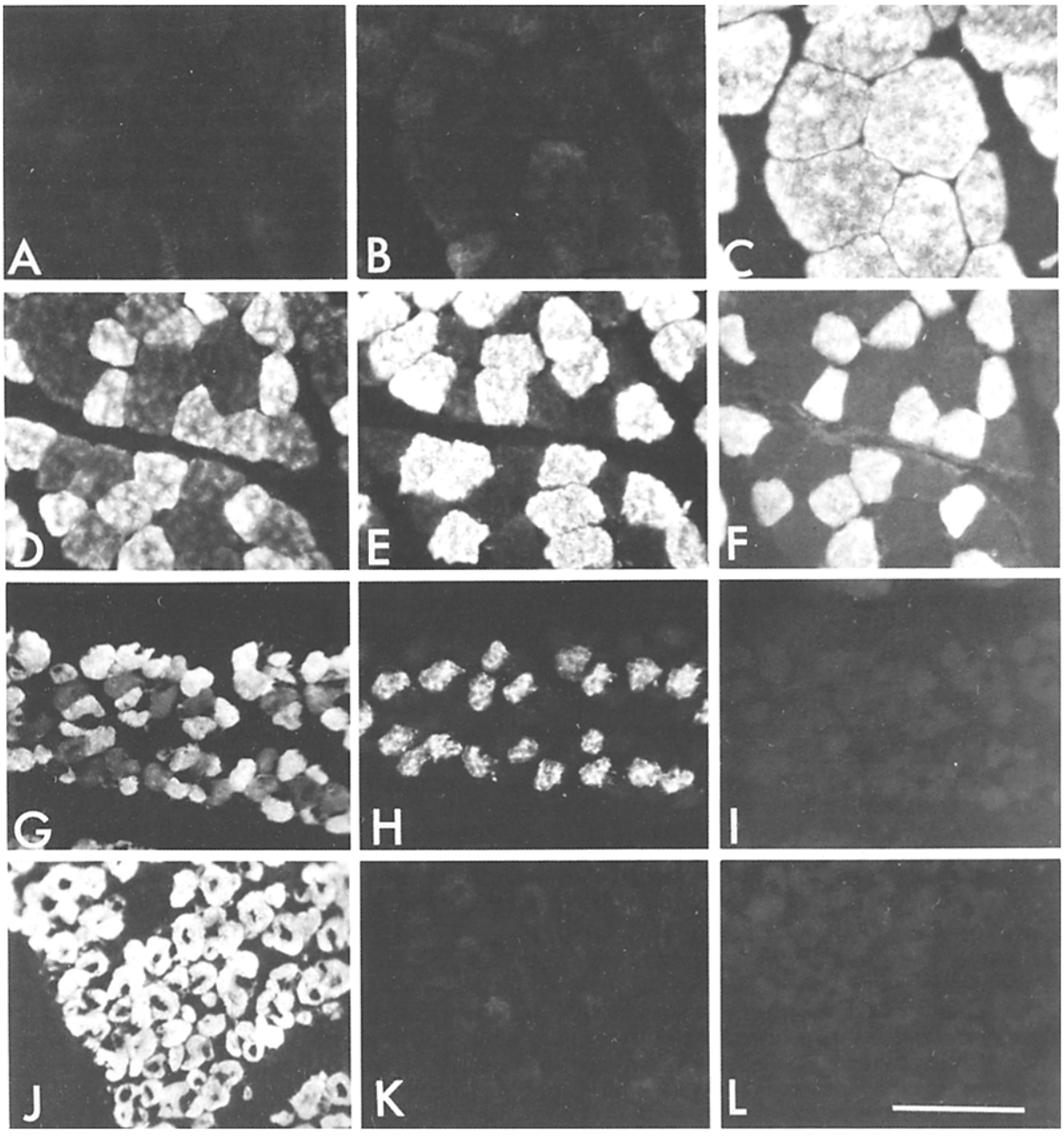

Figure 2. Staining of control mouse muscles with antibodies to the myosin heavy chain isoforms. Photomicrographs of frozen serial sections of adult mouse EDL (*A–C*), 3-wk-old mouse soleus (*D–F*), newborn mouse soleus (*G–I*), or newborn mouse gastrocnemius (*J–L*) stained with rabbit antibodies to neonatal MHC (*A*, *D*, *G*, and *J*), slow MHC (*B*, *E*, *H*, and *K*), or adult fast MHC (*C*, *F*, *I*, and *L*). The neonatal antibody does not stain the adult EDL but does stain some fibers in both the 3-wk and newborn soleus muscles and all the fibers in the newborn gastrocnemius. The slow antibody stains some fibers in both soleus muscles but none of the adult EDL or newborn gastrocnemius fibers. The fast antibody stains all the fibers of the adult EDL, some fibers of the 3-wk soleus (those that do not contain slow myosin but most of which contain neonatal myosin), and none of the fibers in the newborn mouse muscles. Bar, 30 μm.

hematoxylin and eosin to be certain that the culture had not been lost from the section. This was especially important when there was no fluorescence or the number of fluorescent fibers changed between sections. For example, in Fig. 3 *C*, there are five fibers marked with an asterisk because they contain adult fast MHC but not neonatal (Fig. 3 *A*) or slow (Fig. 3 *B*) MHC. Nevertheless, these fibers can be seen in the hematoxylin- and eosin-stained preparations of the sections seen in Fig. 3, *D* and *E*, respectively.

The number and size of the fibers that reacted with each antibody (to adult fast, slow, and neonatal MHC) was measured from the photographs of the fluorescence using a Videoplan (Kontron Elektronik GmbH, Zurich, Switzerland) semi-automatic image analyzer. The parameter used to estimate fiber size was the minimum diameter of an ellipse that has the same area as the fiber that has been drawn around on the photograph. This estimate makes some allowance for the error in measuring fiber diameter when the fibers are not round or are not cut in perfect transverse section, but will give lower values than those obtained previously (16) where fiber diameters were measured directly using a scale bar.

Results

The regeneration of adult mouse muscle fibers in the presence of embryonic spinal cord has been described previously (14, 36). Briefly, the explanted fibers degenerate and regenerate to form a bundle of new myotubes during the first 7–10 d in culture, and the fragments of degenerating parent fibers are removed by phagocytic cells; this latter phase is usually complete by 10–13 d in vitro. The regenerated myotubes subsequently develop cross-striations, and by 20 d at least 60% of the cultures show fibers with striations. Contractions usually develop after 13–16 d, and they are present in at least 40% of the cultures by 20–23 d in vitro.

We have now investigated, using indirect immunofluorescence, the presence of neonatal, adult fast, and slow MHC in these organotypic cultures in four different situations: (*a*) mature cultures, which had been contracting at least 11 d (25–48 d in vitro); (*b*) at different stages of development of the myotubes (7–23 d in vitro); (*c*) in 25–30-d-old myotubes, which had been cultured without spinal cord; and (*d*) in 20–31-d-old nerve–muscle cultures, which had been grown in the continuous presence of α-bungarotoxin to block nerve-induced muscle contractions.

In each case, the cultured muscle bundle was frozen in a liver block that also contained muscle fibers from a 3-wk-old mouse soleus and an adult mouse EDL. Individual serial transverse sections of the muscle bundles and control muscles were each reacted with one of the three antibodies to myosin heavy chain (neonatal, adult fast, and slow). The appearance of the control muscles stained with each of these antibodies is shown in Fig. 2. In the soleus control muscle, about half of the fibers contained both neonatal and adult fast MHC, whereas the remaining 50% of the fibers contained slow MHC on its own. In the adult EDL, the majority of fibers (95–100%) contained adult fast MHC, and staining for neonatal MHC was never found. In some EDL muscles in this mouse strain up to 5% of the fibers gave negative fluorescence with the antibody to adult fast MHC but were positive with the antibody to slow MHC (see also reference 44).

Using notes made at the time that the fluorescence was observed as well as photographs of the fluorescent fibers, MHC content of the regenerated fibers in the cultures was evaluated. It was not always possible to follow every fiber in the serial sections. This was especially true of the small diameter (<3 μm) fibers because their relative positions appeared to change in different sections, and some were not present in all sections. This is shown, for example, in culture 237.6 (Table II) where there were only four fibers in the section stained with the antibody to neonatal myosin (all of which were positive) but six fibers in those sections stained for slow and fast MHC. This is also true for culture 224.27 (Table IV). Simi-

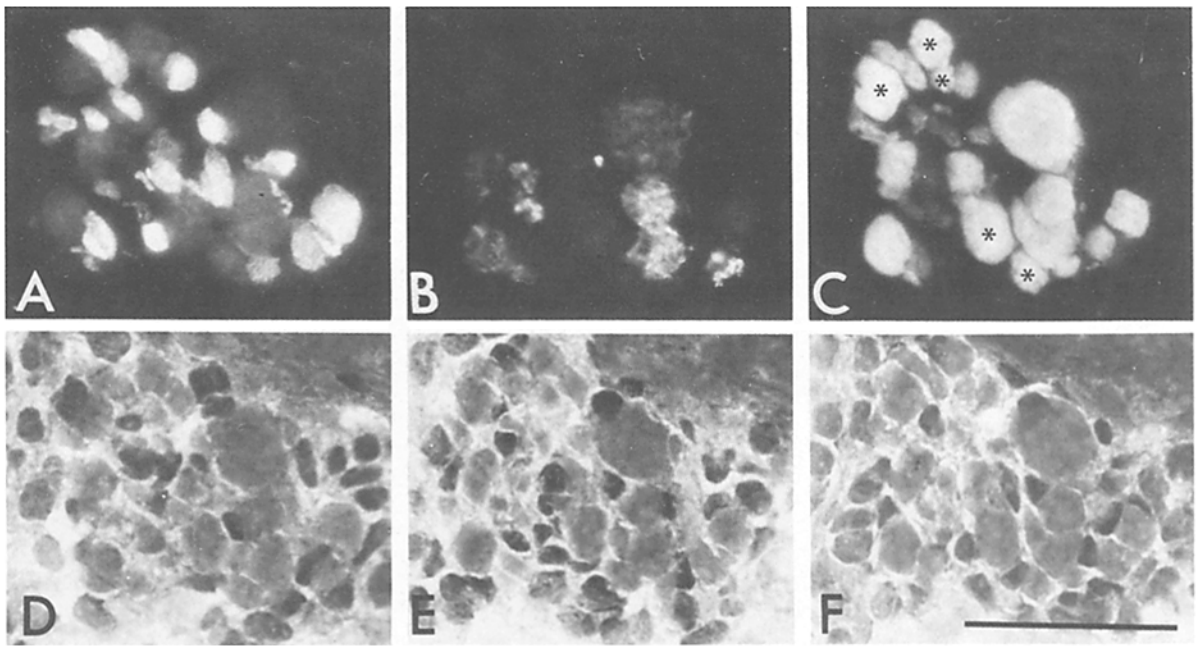

Figure 3. Staining of a mature nerve–muscle culture with antibodies to the MHC isoforms. The culture was 48-d-old and had been contracting for 21 d when the bundle of regenerated fibers was excised, frozen, sectioned, and stained with antibodies to neonatal (*A*), slow (*B*), and adult fast (*C*) MHC isoforms. The sections were then stained with hematoxylin and eosin (*E–G* respectively). It is then possible to check that the muscle fibers are still present when the fluorescence is negative, and that the fluorescence is only in regenerated myotubes. (The latter is more easily seen when the hematoxylin-and eosin-stained sections are viewed in color than in black and white photographs.) The fibers marked with an asterisk contain fast MHC in the absence of neonatal or slow isoforms. Bar, 20 μm.

Table I. Myosin Isoforms in Mature Nerve-Muscle Cultures

Culture No.	Days in vitro	Length of time (d) culture was		Number of fibers with MHC of type			Fast myosin alone	Total fiber No.
		Striated	Contracting	Neonatal	Slow	Fast		
184.17	25	11	11	41	17	8	3	44
186.20	30	21	14	65	14	5	5	70
181.6	27	17	17	7	1	5	2	9
181.16	30	17	17	18	0	6	4	22
181.1	27	13	13	7	4	3	3	10
176.5	27	17	14	22	7	3	1	23
166.11	27	14	14	5	4	5	1	6
229.5	26	15	15	3	3	1	1	4
229.3	26	14	14	57	31	12	3	60
238.5	34	21	14	11	10	2	2	13
241.24	48	21	21	18	8	21	5	22
243.11	28	15	15	11	4	11	1	12
243.10	28	18	18	10	10	7	1	11

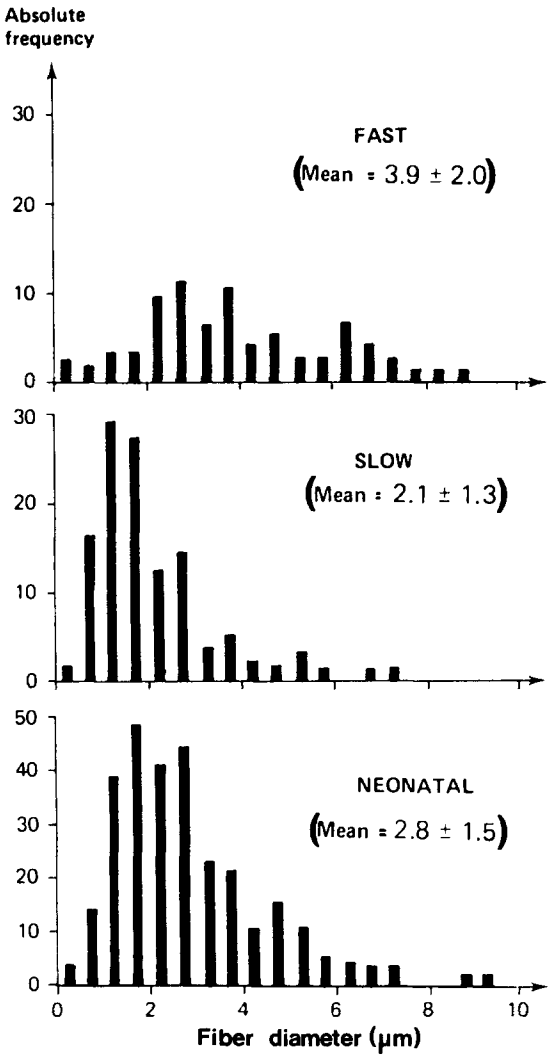

Figure 4. Three histograms showing the absolute frequency of regenerated muscle fibers of different diameters in mature nerve-muscle cultures (ones that have been contracting at least 11 d). The values represent the minimum diameter of an ellipse of the same area as the fiber that has been drawn around. This makes some allowance for fibers not being cut in a perfect transverse section. Each panel is the distribution of diameters of fibers containing either adult fast (*top*), slow (*middle*), or neonatal (*bottom*) myosin heavy chain. Fibers containing more than one MHC isoform appear in more than one histogram.

larly, in Table I, the number of fibers with neonatal plus the number with adult fast MHC alone add up to the total number, with the exception of 241.24 where there were 23 fibers in the neonatal-stained section but 22 in each of the sections stained with the slow or adult fast antibodies.

However, those larger diameter (>5 μm) fibers that could be followed in serial sections were seen to contain the MHCs in particular combinations. The only MHC distribution that was never found was that of slow MHC alone. Occasionally, regenerated fibers were cut in longitudinal section, and it was then possible to see that each of the antibodies stained only the striations in the fibers.

Myosin Isoforms in Mature Nerve-Muscle Cultures

We investigated the myosin isoforms in 13 nerve-muscle cultures (25–48 d in vitro), in which the muscle fibers had been cross-striated and contracting for 11–21 d (Table I). Of the 306 fibers present in these 13 cultures, ~90% contained neonatal MHC, 37% contained slow, and 29% contained adult fast MHC. Of the 89 fibers containing adult fast MHC, 32 (11% of total fiber number) contained fast in the absence of neonatal or slow MHC.

The appearance of antibody staining in one of these cultures is illustrated in Fig. 3. This is a mature muscle culture, 48-d-old, that has been contracting for 21 d. Serial frozen sections have been stained with antibodies to neonatal (3 *A*), slow (3 *B*), or adult fast (3 *C*) MHC, photographed, and then stained with hematoxylin and eosin (Fig. 3, *D–F*, respectively). Five fibers marked with asterisks in *C* contain adult fast MHC without neonatal or slow MHC. Most of the remaining fibers contain neonatal and adult fast MHCs, and a few contain slow and adult fast MHC together. The five fibers that are not stained with the neonatal or slow antibody can be seen in the histologically stained preparations, demonstrating that this is truly negative fluorescence and is not due to the loss of these fibers from the section.

The diameters of the fibers that contained each of the

MHC isoforms are presented in three histograms in Fig. 4. The diameters of fibers containing adult fast, slow, or neonatal MHC had similar ranges of ~0.5–9 μm. The majority of fibers containing slow and neonatal MHC tended to be of smaller diameter (1–3 μm), whereas fibers containing adult fast MHC had a wide range of diameters. Approximately 26% of fast MHC-containing fibers were ≥5 μm in diameter, whereas only 5% of slow- or 10% of neonatal-containing fibers were of this size. Of fibers that contained adult fast MHC without neonatal or slow MHC, 67% had diameters >5 μm, their mean diameter being 5.8 ± 1.6 μm.

It was important to be certain that the presence of adult fast MHC in these cultures did not reflect a persistence of the explanted parent fibers. Since these living cultures were viewed by bright field microscopy at regular intervals, the fate of the explanted fibers could be followed easily. They were seen to degenerate, to develop myotubes along their edges, and to be later phagocytosed, leaving only a bundle of regenerated myotubes. Photographs of this process in the living cultures have already been published in references 14 and 36. However, the possibility existed that the parent fibers were not completely removed, or that they atrophied and became so small that they were not easily observed by light microscopy (<3 μm) and so we studied cultures at the electron microscopic level.

At least three cultures were sectioned for electron micros-

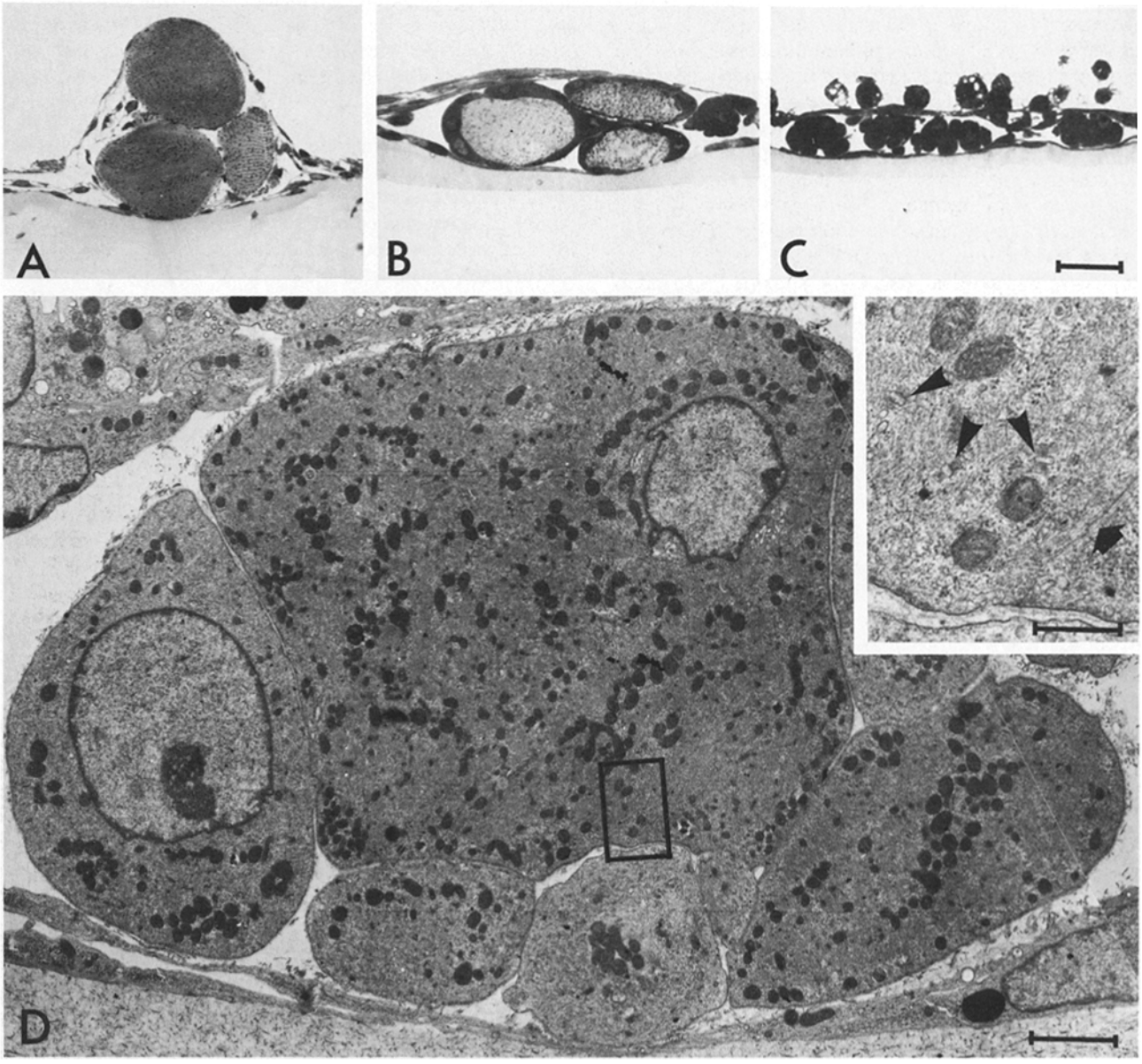

Figure 5. Degeneration and regeneration of the explanted muscle bundles. Araldite-embedded thick sections of bundles that have been in culture for 1 d (*A*), 6 d (*B*), or 12 d (*C*) were stained with toluidine blue. In *A*, three fibers 30–40 μm in diameter have pale sarcoplasm from which both Z lines and myonuclei are absent. In *B*, new darkly staining myotubes with large nuclei can be seen around the periphery of the degenerating explanted fibers. In *C*, no explanted fibers remain, but instead there are bundles of regenerating myotubes. One of these, on the left in *C*, is shown at higher magnification in *D*. There is one large myotube surrounded by five smaller myotubes. The inset shows the area inside the box at higher magnification. A continuous basal lamina surrounds the larger myotube which contains triads (*arrowheads*) and thick and thin filaments some of which can be seen in longitudinal section (*arrow*). Bars: (*A–C*) 30 μm; (*D*) 2 μm; (*inset*) 0.5 μm.

copy at 1-7, 10, and 14 d in vitro. The presence of persisting parent fibers, or pieces of fibers, and the number of new myotubes seen by light microscopic observation of the living cultures before fixation were noted. Thick sections (1 μm) were taken transversely through the explanted bundle and then stained with toluidine blue (Fig. 5). After 1 d in vitro (Fig. 5 *A*), the cellular outgrowth has just reached the explant and by 6 d (Fig. 5 *B*), new myotubes can be seen around the periphery of the degenerating parent fibers that now take up the stain less strongly. In contrast, the new myotubes are intensely stained with toluidine blue and contain large nuclei. The degenerating parent fibers were gradually cleared away by phagocytes leaving bundles of new myotubes, all intensely stained and often arranged in the groups in which they formed beneath the basal laminae (Fig. 5 *C*). The new myotubes were much smaller than the explanted fibers but the presence of organizing thick and thin filaments and of Z line formation in the electron microscope confirmed their myogenic character. One such bundle of myotubes, seen on the left in Fig. 5 *C*, is shown in a low-power electron micrograph in *D*. The inset is a higher magnification of the area in the box, showing a continuous basal lamina, triads, and myofilaments in one of the myotubes. The thick and thin filaments are still rather disorganized and most are cut obliquely although a few are seen in longitudinal section.

In no case was a parent fiber found at the electron microscopic level when it had not been found at the light level, and there was no evidence that the explanted fibers atrophied. Thus, the myosin isoforms identified by immunocytochemistry were present in regenerated myotubes and were not a reflection of persisting explanted fibers.

Myosin Isoforms in Regenerated Muscle Fibers at Different Stages of Development in Nerve-Muscle Cultures

15 nerve-muscle cultures were frozen when the bundle of regenerated myotubes was at different stages of development (7–23 d in vitro), and their myosin content was determined immunocytochemically. These results are summarized in Table II where the cultures have been ranked not according to their age in vitro but the status of their development at the time they were frozen. The status of development of individual living cultures was judged by light microscopic observation at 500 times magnification (this also enabled us to be sure that the muscle bundle that was subsequently excised contained no persisting explanted fibers). However, at a particular time in an individual culture, the myotubes were not all at the same stage of differentiation, i.e., some myotubes may have had cross-striations when the rest in that culture had none. Thus when a culture in Table II is designated as "without cross-striation," then no myotubes were seen to contain striations or to contract. When a culture was designated as "some cross-striations" then at least one of the myotubes had some striations. When a culture was designated to contain "cross-striations and contractions" then at least one myotube was clearly striated along its entire length. Some of these cultures also contained cross-striated fibers that were contracting. Precise correlation between, for example, the number of cross-striated fibers seen in the living culture and the number of cross-sections stained by antibody to fast myosin was not possible because small diameter fibers (<3 μm) were impossible to resolve by light microscopic observation of the cultures, especially when they were amid a bundle of larger myotubes. Thus, the number of fibers seen by light microscopy of the living culture was usually less than the number of profiles seen during immunofluorescence studies.

In this developmental study, all fibers in sections stained with the neonatal antibody were positive, and some cultures in each of the groups also contained slow MHC. However, only one of the five cultures in the group without cross-striations contained adult fast MHC, and these two fibers had only a pale stain. Two of the five cultures containing myotubes with some cross-striations had fibers staining for adult fast MHC, but all five of the cultures containing at least one fully cross-striated fiber contained adult fast MHC. There was, therefore, a suggestion that early myotubes contained neonatal myosins (with or without adult slow) but that as these matured morphologically, they also began to contain both adult fast and neonatal MHC. Because the development

Table II. Myosin Isoforms in Developing Nerve-Muscle Cultures

Culture No.	Days in vitro	Status*	Number of fibers with MHC of type			Total fiber No.
			Neonatal	Slow	Fast	
223.22	7	Without cross-striations	6	6	0	6
237.12	11		8	8	0	8
223.9	13		2	0	0	2
223.11	13		12	10	2(pale)	12
223.19	17		11	0	0	11
237.7	7	Some cross-striations	1	0	0	1
238.3	7		1	0	0	1
237.5	7		1	0	1(pale)	1
237.6	7		4	6	2	6
237.9	11		6	6	0	6
223.18	13	Cross-striations ± contractions	3	1	3	3
237.18	11		7	6	2	7
237.4	11		7	3	4	7
168.3	23		4	3	1	4
223.26	16		16	7	6	16

* Status judged by light microscopic observation of the living cultures.

of morphological differentiation was spinal cord dependent, and as the expression of fast myosin appeared to coincide with these events, the question arose that it too might be a spinal cord–dependent phenomenon. We therefore cultured myotubes without spinal cord tissue to test this hypothesis.

Myosin Isoforms in Muscle That Had Regenerated in the Absence of Spinal Cord

Unlike rat (4) or quail (27) muscle fibers, bundles of adult mouse muscle fibers do not spontaneously regenerate in the absence of nerve in this system. Regeneration was therefore induced in the absence of spinal cord by culturing adult muscle with embryonic lung tissue. Alternatively, the muscle was denervated for 4 or 28 d before being explanted without any embryonic tissue. Myotubes formed 7–10 d after explantation of the adult muscle. The cultures examined by immunocytochemistry were 20–35 d old (an age range similar to that of the mature nerve–muscle cultures). No cross-striations or contractions were observed in these cultures, which was in agreement with previous observations (14, 36). It can be seen from Table III that none of the seven cultures contained adult fast MHC; they all had fibers that all contained neonatal MHC, and four cultures (77% of the total fiber number) also contained slow myosin. Of the 60 fibers present in the seven cultures, diameter measurements made on the photographs of fluorescence with the neonatal antibody indicated that the mean diameter was 3.0 ± 1.9 μm with seven fibers (12%) having a diameter ≥5 μm. Thus these myotube diameters were in a range similar to those found in mature nerve–muscle cultures, but in the absence of the spinal cord there were neither cross-striations or contractions, nor the expression of adult fast MHC.

Myosin Isoforms in Muscle That Had Regenerated in Nerve–Muscle Cultures, But in the Continuous Presence of α-Bungarotoxin

Myosin isoforms were investigated in five cultures that had been established and maintained in the continuous presence of 10^{-7} M or 10^{-8} M α-bungarotoxin, to block nerve-induced muscle contractions, for 21–42 d in vitro. These levels of drug were not toxic to the spinal cord tissue as judged by the quality and quantity of central and peripheral myelin and of dorsal root ganglion neurons in the living cultures. When the muscle bundle had been removed, subsequent silver stains demonstrated that anterior horn cells were similarly healthy and in large numbers in the spinal cord explant. Although cultures were studied by light microscopy every 3–4 d, no cross-striations or contractions were seen in any of the cultures at any time.

Nevertheless, adult fast MHC was present in each of the five cultures (Table IV). They each also contained fibers that stained for neonatal myosin, and four of the five cultures contained slow myosin. Of the 67 fibers present, 100% contained neonatal MHC, 53% contained slow myosin, and 27% contained adult fast MHC. Thus the continuous presence of α-bungarotoxin did not prevent the expression of adult fast myosin heavy chain even though striations and contractions were not found by light microscopy.

Table III. Myosin Isoforms in Muscle That Has Regenerated in the Absence of Spinal Cord

Culture No.	Days in vitro	Status	Number of fibers with MHC of type			Total fiber No.
			Neonatal	Slow	Fast	
219.2	28	Myotubes without cross-striations or contractions	9	0	0	9
219.3	28		7	0	0	7
219.5	28		4	1	0	4
178.24	35		20	20	0	20
176.13	27		14	14	0	14
227.7	20		3	1	0	3
227.16	20		3	0	0	3

Discussion

We have investigated the presence of three MHC isoforms in regenerating adult mouse muscle in a nerve–muscle culture system. The presence of these isoforms was judged using antibodies to rat myosins in an immunocytochemical assay. A biochemical confirmation of the presence of these isoforms in the nerve–muscle cultures is not readily possible with current techniques because the volume of muscle is so small (e.g., 10 fibers, 5-mm long and 10-μm diam). Using the immunocytochemical approach we could determine the myosin compositions within individual fibers that would not have been possible with techniques that required the muscle bundle to be homogenized. We cannot exclude that these antibodies were reacting with myosins other than the known heavy chain isoforms, but they stained the cultured muscle fibers as brightly as they did the control muscles in the same section, at antibody dilutions chosen as appropriate for the controls. The antibodies were shown to react with the MHCs and their specificity to mouse myosins was also demonstrated using immunoblot techniques. We have also been able to demonstrate that the presence of adult fast MHC in regenerated fibers was not a reflection of the persistence of the adult muscle fibers that were explanted. These explanted fibers were 30–40 μm in diameter, whereas the myotubes containing fast myosin were not more than 10 μm. Using electron microscopy, we have documented that when we saw a loss of explanted fibers by light microscopy, they had indeed degenerated and had been removed by phagocytic cells, and that there was no evidence for atrophied explanted fibers. Only new myotubes were present.

Thus adult fast MHC was produced in these nerve–muscle cultures. It was not present in newly formed nonstriated myotubes, which all contained neonatal with or without slow MHC. At the time when myotubes with cross-striations and contractions were found in cultures, we also found fibers containing adult fast MHC in addition to the neonatal isoform. In the more mature cultures, which had been contracting for at least 11 d, just over one-quarter of the fibers contained adult fast MHC, and a third of these (32 of 89 fibers) contained adult fast MHC in the absence of neonatal or slow isoforms. Therefore there appeared to be a transition of myosin types from neonatal to neonatal-plus-fast to fast alone in some of the regenerated fibers as they matured in vitro.

The expression of adult fast MHC was coincident with the morphological development of the myotubes (cross-striations and contractions) which is known to be a spinal cord–dependent phenomenon in this system (14, 36). Moreover, adult fast MHC was not present in myotubes grown in the absence of the spinal cord, although they were of a similar age range

Table IV. Myosin Isoforms in Muscle That Has Regenerated in the Continuous Presence of α-Bungarotoxin

α-Bungarotoxin	Days in vitro	Culture No.	Status	Number of fibers with MHC of type			Total fiber No.
				Neonatal	Slow	Fast	
M							
10^{-7}	21	244–27		2	2	3	3
10^{-7}	42	244–24	Myotubes without	5	0	4	5
10^{-8}	28	245–8	striations or	20	10	3	20
10^{-8}	28	245–10	contractions	30	17	5	30
10^{-8}	28	245–13		10	8	4	10

and diameter distribution as in nerve–muscle cultures. Thus the expression of adult fast MHC appeared to be a spinal cord–dependent phenomenon in this system.

When nerve-induced muscle contraction was blocked in nerve–muscle cultures by the continuous presence of the drug α-bungarotoxin, adult fast MHC was still expressed in about one-quarter of the regenerated fibers, about the same as in controls. Thus nerve-induced activity was probably not responsible for the expression of adult fast MHC. The drug would not, of course, block any myogenic contractions that occurred and it may be that these were responsible for the expression of adult fast MHC. However, when these drug-treated cultures were observed by light microscopy every 3 or 4 d, spontaneous contractions were not seen, although this is no proof that they did not occur. If such contractions did occur in these bundles of mouse myotubes and they were responsible for the expression of adult fast MHC, then one might also expect them to occur in the cultures without spinal cord and to produce adult fast MHC there also. We did not find adult fast MHC in the absence of the spinal cord, and so feel justified in concluding that neither nerve-induced nor spontaneous contractions were responsible for the expression of adult fast MHC.

The spinal cord was therefore having some "trophic" effect on the regenerated muscle bundle. We do not know, as yet, either the nature of this trophic effect or whether it requires contact between, or simply close proximity of, the nerve and muscle. In any one regenerated bundle, the individual fibers could have different myosin contents. Thus a fiber that was in the same proximity to the spinal cord, had undergone identical culture conditions, and was subjected to the same mechanical activity and stretch, could have adult fast MHC when another did not. (When one fiber in a bundle contracts, the other members of the group are passively stretched.) There must, therefore, be a reason why some fibers contain adult fast MHC in nerve–muscle cultures when others do not.

We have shown here that diameter is not an important consideration, as adult fast MHC is present in fibers of 0.3–9.0-μm diam. It is known that nerve contact is required for the development of the cross-striations (14), and that the contractions are the result of synapse formation between the spinal cord neurons and regenerated muscle fibers (13, 34, 35). It has also been shown, using electrophysiological techniques, that all cross-striated fibers are innervated (37). However, not all the muscle fibers in a particular culture are cross-striated. This implies therefore that some fibers are innervated and that others are not, or that their innervation is compromised. Similarly, the particular association of nerve and muscle which allows the expression of the trophic effect of the cord may also not be available to all fibers equally, and hence this could explain heterogeneity of myosin isoforms in the fibers of a single culture. One might further speculate that this particular association of nerve and muscle is in fact innervation, because at the time that innervation causes fibers to become cross-striated, fast myosin is also produced. (The two events are obviously paraphenomena and are not themselves linked as cultures treated with α-bungarotoxin, which lack striations, do contain adult fast MHC.) Definitive proof of this awaits studies that identify innervated fibers by electrophysiology, and then investigate the myosin content of the identified fiber.

Nevertheless, current dogma would suggest that nerve is not required for the expression of adult fast MHC in vivo either in development (9, 19, 33), in regeneration (10, 26, 43), or after denervation of adult muscles (11, 33). In developing rat (9, 19) or chicken (33) muscle, which has been denervated at birth or 7 d postnatal, adult fast myosin is still expressed. However, since the developing muscles have been in contact with nerve before the denervation was done, it is still possible that the expression of adult fast myosin requires a nerve-dependent step to initiate the switching on of the gene (9). However, it is technically very difficult to prevent a developing mammalian muscle from ever coming into contact with nerve, and the interpretation of the result would also be difficult since the morphological development of the muscle similarly needs the nerve (5, 21–23).

In models of regeneration, where satellite cells form new myotubes within existing basal laminae, denervation can be carried out before new myotubes form. However, in these models nerve does not appear necessary for the production of adult fast MHC (10, 43). We have no explanation for this enigma between the in vivo denervation studies and our nerve–muscle culture system, but can only suggest that a trophic influence, supplied in this culture system by the presence of the spinal cord, is available from another source in vivo. The mouse semimembranosus muscle studied here in culture may of course be different in its myosin types and control from the rat and chicken muscles used in other studies, although we have no evidence to support this.

As well as considering why this nerve–muscle culture system differs from the situation in vivo, we should also consider why adult fast MHC has not been found in other culture systems (1–3, 7, 32, 42, 45). Although the medium in our nerve–muscle culture system differs from those used previously, it is not responsible for the expression of adult fast MHC because we found that myotubes cultured without the spinal cord tissue expressed only neonatal and slow myosin.

Thus, the spinal cord tissue is supplying this factor(s) that was absent in previous tissue culture studies. The influence is not due to the interaction of acetylcholine with its receptor, as α-bungarotoxin had no effect on the expression of adult fast MHC. We do not know whether homogenates of spinal cord can replace the presence of the tissue itself, but the factor need not just be a trophic substance produced by the cord that acts directly on gene expression. The turnover of protein, in particular of myosin, may be important in the control of myosin switches and/or accumulation of a particular myosin isoform. The nerve may change the muscle morphologically (e.g., the production of basal laminae) or induce the expression of receptors that allow the myotube to bind growth factors or hormones and thus cause myosin switches indirectly. Whatever the effect of the spinal cord in the nerve-muscle culture system, this effect is absent from other muscle culture systems to date and is probably available to denervated or regenerating muscle in vivo.

Neonatal MHC was present in myotubes that had regenerated in the absence of spinal cord tissue, and so its expression was clearly not nerve-dependent. This heavy chain isoform was present in nearly all of the muscle fibers and was present in newly formed myotubes (from 7 d in vitro). Neonatal MHC has been described only in long-term (14–21 d) cultures of primary rat myotubes (7, 42) being absent from short-term (<7 d) cultures of the same cells. The accumulation of neonatal MHC in the present study may reflect the different medium that was used, or the fact that the satellite cells here are activated in situ beneath the basal lamina to form new myotubes, rather than being enzymatically liberated. It is possible that the same "trophic" influence of the spinal cord, discussed earlier, leads to increased levels of neonatal MHC, although it is not required to switch on the gene.

The expression of slow MHC in this nerve-muscle system, was, like neonatal myosin, spinal cord-independent. It was also present at the earliest times (7 d) and in cultures treated with α-bungarotoxin. Cross-innervation and electrical stimulation experiments in adult animals have suggested that the expression of the slow phenotype is controlled by a specific activity pattern of the nerve (25, 39). Although spontaneous firing of the spinal cord neurons in these cultures causes contractions in the muscle bundle, the pattern of firing is not typical of adult neurons (12). As the expression of slow MHC was also nerve-independent and was unaltered in α-bungarotoxin-treated cultures, it is unlikely that the presence of slow MHC was the result of the nerve-induced activity. However, muscles of fetal and newborn mammals also have slow myosin-containing fibers (30, 40, 44) and it has been suggested that this process is independent of the nerve-induced pattern of activity on the muscle (44). There might therefore be two mechanisms for the expression of slow MHC. One would be nerve activity pattern dependent (in adults) and the other would be independent of a specific slow muscle pattern of nerve activity (in developing muscles). Thus, the expression of adult slow myosin in these cultures may be more related to the expression of slow MHC in fetal or neonatal muscle, and the continuous presence of "fetal" spinal cord may mean that this expression is not lost with time in culture. We cannot exclude that the antibody is recognizing not only adult slow myosin, but a fetal myosin form sharing common epitopes with the adult form, and that it is the latter that is expressed in the nerve-muscle cultures.

In summary, the expression of neonatal and slow MHC in these nerve-muscle cultures is nerve-independent, whereas the expression of adult fast MHC is a nerve-dependent process. It is not, however, a result of nerve-induced contractile activity, but is probably the result of some "trophic effect" of the spinal cord tissue. We are now in a position to investigate the nature of this trophic effect using this unique culture system which, although complex by tissue culture standards, may prove useful in understanding the control of fast myosin expression.

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