Expression of Muscle-Gene-specific Isozymes of Phosphorylase and Creatine Kinase in Innervated Cultured Human Muscle

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Abstract. Isozymes of creatine kinase and glycogen phosphorylase are excellent markers of skeletal muscle maturation. In adult innervated muscle only the muscle-gene-specific isozymes are present, whereas aneurally cultured human muscle has predominantly the fetal pattern of isozymes. We have studied the isozyme pattern of human muscle cultured in monolayer and innervated by rat embryo spinal cord explants for 20-42 d. In this culture system, large groups of innervated muscle fibers close to the ventral part of the spinal cord explant continuously contracted. The contractions were reversibly blocked by 1 mM *d*-tubo-curarine. In those innervated fibers, the total activity and the muscle-gene-specific isozymes of both enzymes increased significantly. The amount of muscle-gene-specific isozymes directly correlated with the duration of innervation. Control noninnervated muscle fibers from the same dishes as the innervated fibers remained biochemically immature. This study demonstrated that de novo innervation of human muscle cultured in monolayer exerts a time-related maturational influence that is not mediated by a diffusable neural factor.

Tissue culture of diseased human muscle provides an important model for manifesting abnormalities intrinsic to the muscle cell. Several abnormalities have been reproduced in aneurally cultured human muscle (3, 23). However, there are still presumably primary muscle diseases, e.g., Duchenne muscular dystrophy, myotonic atrophy (dystrophy), muscle carnitine deficiency, and muscle phosphorylase deficiency, in which aneural cultures of the patients' muscle have not permitted phenotypic expression of the disease (20, 22, 34). This could be due to either an insufficient maturation of aneurally cultured human muscle, a relatively short survival of aneural muscle in culture (6-7 wk), or a lack of innervation per se.

Recently we have developed a new system in which human adult muscle (AM)[1] cultured in monolayer from myoblasts can be innervated de novo by explants of spinal cord from 13-14-d-old rat embryos. Such innervated cultured human muscle fibers become entirely cross-striated, survive beyond 3 mo, and contract nearly continuously (4). Those contractions can be stopped by *d*-tubocurarine (4). Nicotinic acetylcholine receptors and acetylcholinesterase accumulate at the de novo formed nerve-muscle contacts, and the postsynaptic muscle membrane achieves a high degree of specialization (6,17). Our system is distinctly different from organ culture, which was previously the only system in which human AM fibers were innervated in vitro (8, 13, 24). In muscle organ culture, new myofibers form within the originally explanted basal lamina sheaths, which remain as undegenerated tubes (19). Their innervation occurs at the original synaptic sites on those original basal laminae (19). This is the same phenomenon known to occur during in vivo muscle regeneration (19). Therefore, the organ culture system more appropriately serves as a model of in vitro reinnervation and not de novo innervation. In contrast to organ cultures, new muscle fibers in our monolayer cultures are formed from outgrowing myoblasts, without remnants of the original muscle fibers and the original basal laminae surrounding them. Therefore our culture system seems much more appropriate than does organ muscle culture for studying postsynaptic membrane specialization and various molecular events associated with de novo innervation of cultured human muscle.

It is well established that electrophoretic patterns of isozymes of creatine kinase (CK)[1] and glycogen phosphorylase of adult innervated normal muscle are composed only of muscle-specific isozymes. During early embryogenesis, fetal isozymes predominate and muscle-specific isozymes are either absent or weakly expressed (21). The switch from the embryonic pattern of those isozymes to the adult pattern appears during muscle maturation, but the factors governing this process are not fully understood. In this study, we investigated the influence of de novo innervation of monolayer-cultured human muscle on the expression of muscle-gene-specific isozymes of creatine kinase (CK, EC 2.7.3.2) and glycogen phosphorylase (EC 2.4.1.1.). In aneurally cultured human muscle, fetal isozymes predominate; muscle-gene-specific isozymes are very weakly expressed (16, 21).

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1. *Abbreviations used in this paper:* AM, adult muscle; CK, creatine kinase; CK-BB, CK-brain form; CK-MB, CK-hybrid form; CK-MM, CK-muscle form.

Materials and Methods

Muscle Culture

Human muscle cultures were established from the diagnostic muscle biopsies of 12 patients. After all diagnostic studies were performed, these patients were considered free of intrinsic muscle disease.

Muscle cultures were established according to our explant–reexplant technique (4). Cultures were initiated from 1-mm³ muscle explants, five of which were placed in each 35-mm petri dish (Falcon Labware, Oxnard, CA) coated with gelatin–human plasma mixture. After an abundant growth of cells had emerged, the explants were removed and re-explanted into collagen-coated petri dishes. After abundant growth of cells had again emerged, the explants were removed and discarded. The muscle was cultured in F14 medium (Gibco, Grand Island, NY) (35), which we modified to contain 600 mg/dl of glucose, 10% fetal calf serum, 50 ng/ml fibroblast growth factor, 10 ng/ml epidermal growth factor, and 10 μg/ml insulin (5). Fetal calf serum was purchased from Hyclone Laboratories (Hogan, UT), fibroblast growth factor and epidermal growth factor from Collaborative Research, Inc. (Waltham, MA), and insulin from Sigma Chemical Co. (St. Louis, MO). Cultures were fed twice a week, and examined every day or every other day by phase-contrast inverted microscopy. About 9–15 d after myoblast fusion, explants of whole transverse slices of rat embryo spinal cord with dorsal root ganglia attached were placed on the muscle monolayer cultures (four fragments per petri dish). The muscle–spinal cord co-cultures in this study were maintained in the same medium as above, but without fibroblast growth factor and epidermal growth factor, for up to 42 d.

Regions of innervated muscle could be distinguished easily from regions of noninnervated muscle in the same culture dish by their characteristic appearance. Innervated muscle was located close to the ventral part of the spinal cord explant and consisted mainly of long contracting muscle fibers entirely cross-striated throughout their length. Most of these fibers were densely packed parallel to each other and were contacted by neurites. In innervated cultured muscles, acetylcholine receptors and acetylcholinesterase accumulated at the nerve–muscle contacts, and the postsynaptic muscle membrane was well organized (6, 17). Muscle fibers near the dorsal region of the spinal cord explants did not become innervated and began to degenerate after 6–7 wk of co-culture. (Fig. 1, a–c).

Biochemistry

Biochemical studies were performed on cultured muscle innervated for 20–42 d. The moment of innervation was arbitrarily considered the time when the spinal cord explant began to be co-cultured with the muscle.
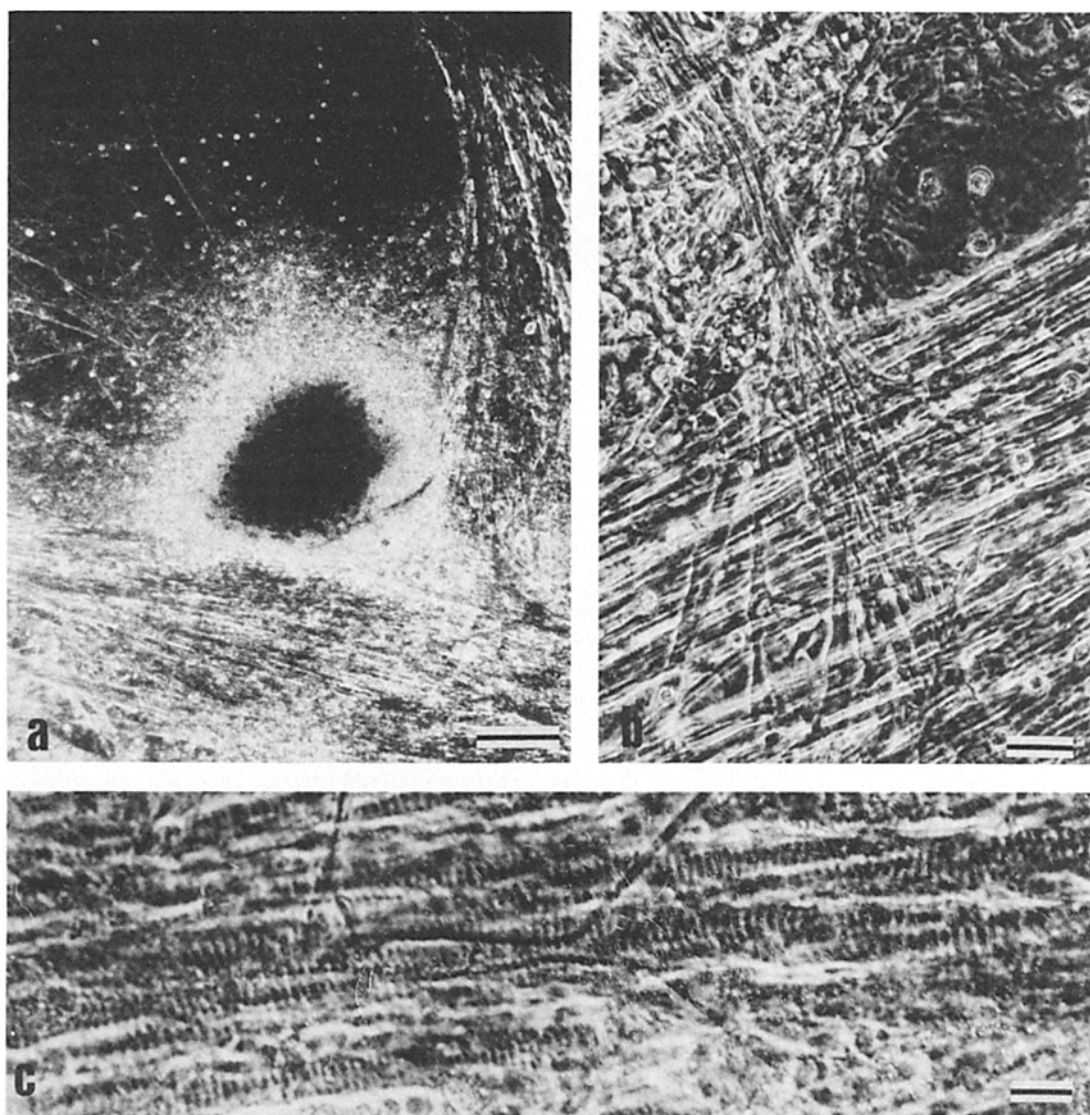

Figure 1. Phase-contrast microscopy of living cultured human muscle innervated for 7 wk. (*a*) Low power photomicrograph illustrating abundance of muscle fibers near the ventral part of the spinal cord explant. Neurites emerging from the dorsal part of the spinal cord explants are visible, but there are no muscle fibers present at the dorsal part. Bar, 100 μm. (*b*) Large "nerve trunk" emerging from the spinal cord explant and branching among muscle fibers that are densely packed and parallel to each other. Bar, 3 μm. (*c*) High power photomicrograph illustrating that the muscle fibers contacted by neurites are entirely cross-striated. Bar, 5 μm.

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Under the dissecting microscope, regions of innervated cultured muscle were carefully dissected out from 120 culture dishes (10 from each biopsy). Regions of noninnervated muscle from the same dishes were dissected out to serve as control tissue. The spinal cord explants were also dissected out and separately processed for isozyme studies.

Harvested tissues were rinsed with PBS and centrifuged at 400 g for 10 min. The pellet was kept frozen at −80°C. For the enzymatic studies, the pellet was thawed, resuspended in 30 μl of PBS, and sonicated. Protein content was measured according to the method of Lowry (18).

CK activity was measured as described by Hess (14) and modified by Desjarlais (11). CK activity was expressed in international units per milligram of protein (1 IU converts 1 μmol of creatine-phosphate to creatine/min at 37°C). The distribution of CK isozymes was quantified fluorometrically after electrophoresis in agarose gels (Corning Science Products, Corning, NY).

For phosphorylase studies, the sonicates were diluted 1:2 with extracting medium (containing 40 mM beta-glycerophosphate, 2 mM EDTA, 10 mM NaF, and 10 mM beta-mercaptoethanol), and centrifugated for 10 min at 23,000 g at 4°C. The activity of phosphorylase was measured according to the bioluminescent method developed especially for cultured cells (15). After 60 min of incubation at 25°C in a medium containing 75 mM Na phosphate (pH 7.0), 1.5 mM EGTA, 3 mg/ml glycogen, 0.75 dithiothreitol, 1.5 mg/ml BSA, and 1 mM AMP, the reaction was stopped by adding 0.05 ml of 2.5 N HCl. After 20 min, an equal amount of 2.5 N NaOH was added, followed by 0.5 ml of a mixture containing 120 mM Na phosphate (pH 7.0), 0.9 mM MgCl₂, 0.18 mM NADP, 2 μM glucose-1,6-diphosphate, 50 mU/ml glucose 6-phosphate dehydrogenase, and 300 mU/ml phosphoglucomutase. After another 20 min of incubation at 25°C, the newly formed NADPH was measured in a scintillation spectrophotometer by adding a solution containing 1 mg/ml bacterial luciferase and 0.25 mg/ml flavin mononucleotide in 0.1 Na phosphate at pH 7.0. Each sample was triplicated and matched with a phosphorylase standard curve. The activity was expressed in international units per milligram of protein (1 IU forms 1 μmol of D-glucose-1-phosphate from glycogen and P$_i$/min [pH 6.8] 30°C).

Polyacrylamide gel electrofocusing was performed on Ampholine Pagplate (1804-103; LKB Instruments, Inc., Bromma, Sweden) (pH 5.5–8.5) at 4°C, with a current of 1 mA/strip for the first 60 min and continuing overnight at a constant voltage of 500 V. The gels were then incubated for 24 h at room temperature in a solution containing 2% glycogen, 40 mM glucose-1-phosphate, 2 mM AMP with or without 0.7 M Na₂SO₄ (because liver and liver-like phosphorylase bands are revealed only in presence of high concentrations of sulfate, whereas muscle and brain type phosphorylase bands are only slightly increased by sulfate [1, 28, 29, 33]). At the end of the incubation, the gels were stained by immersion in a 1:30 diluted solution of 4% iodine, 6% KI, and 10% acetic acid, briefly rinsed in water, photographed, and scanned at 610 nm with a quick-scan Flurvis densitometer (Helena Laboratories, Beaumont, TX).

Immunological Studies

Rabbit antibodies against human muscle phosphorylase were obtained as described previously (12). Phosphorylase was purified from normal human pectoral muscle obtained during radical mastectomies by the method of Yunis and co-workers (36). The isolated enzyme was purified ~80 times compared with the starting homogenate. When the enzyme was stained for either protein or enzyme activity, one band appeared in acrylamide gel electrophoresis. The purified protein (3.0 mg) was injected into rabbits with complete Freund's adjuvant. A booster injection was given 20 d later. The animals were bled 1 wk after the booster injection, and the serum was stored
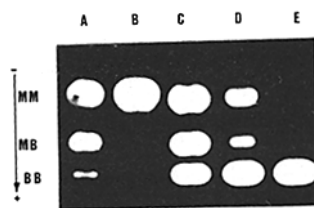

Figure 2. Agarose gel electrophoresis of CK isozymes of innervated and noninnervated cultured human muscle. (A) CK isozyme standard (47020; Corning Science Products) showing BB, MB, and MM isozyme. (B) Human AM. (C) Innervated cultured human muscle. (D) Noninnervated cultured human muscle. (E) Rat embryo spinal cord explants harvested from the same dishes as C and D. CK activity is detected by overlaying the gel with CK substrate containing ADP, creatine phosphate, Mg⁺⁺, hexokinase, glucose-6-phosphate dehydrogenase, and nicotinamide adenine dinucleotide. The same total CK activity was applied in each lane.
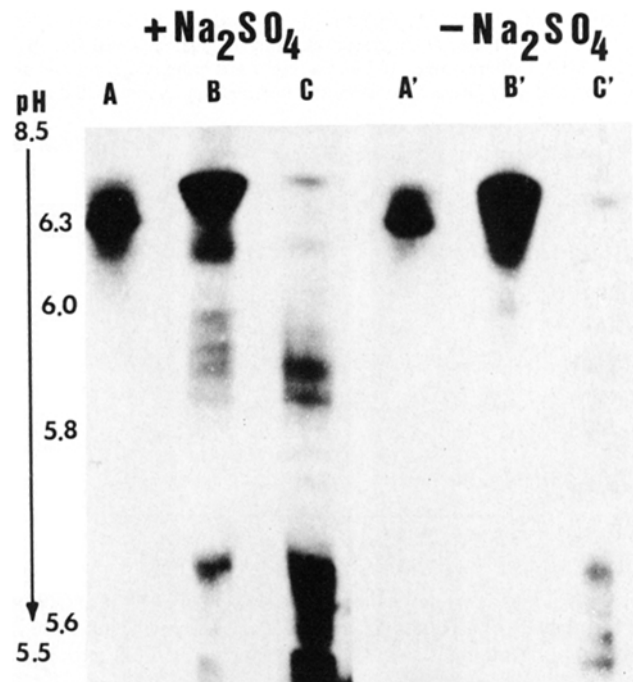

Figure 3. Phosphorylase isozymes of innervated and noninnervated cultured human muscle in the presence (lanes A, B, and C) or absence (lanes A', B', and C') of 0.7 M Na₂SO₄ in the phosphorylase substrate. Lanes A and A', human AM homogenate; lanes B and B', innervated cultured human muscle; lanes C and C', noninnervated cultured human muscle harvested from the same dishes as B and B'. Isoelectrofocusing on polyacrylamide slab gel (pH 8.5–5.5). Phosphorylase activity is detected by iodine staining after incubation with phosphorylase substrate as described in Material and Methods. The same total phosphorylase activity was applied in each lane.

Table I. Total Activity and Isozyme Pattern of CK and Phosphorylase in Innervated and Noninnervated Cultured Human Muscle

	n	CK mU/mg	Phosphorylase mU/mg	CK Isozymes BB %	MB	MM	Phosphorylase Isozymes Fetal %	Muscle
Innervated	12	3,427.1 ± 1,151	33.2 ± 13	28.7 ± 2.3	28.2 ± 3.5	43 ± 3.1	24.9 ± 7.4	35.1 ± 7.5
Noninnervated	12	2,039 ± 770	22.3 ± 10	42.2 ± 4.1	29.7 ± 3.7	28 ± 3.7	69.8 ± 4.8	2.7 ± 0.4
Innervated/noninnervated × 100		165	146	68	95	188	35	1,292
P		<0.05	<0.04	<0.005	NS	<0.005	<0.001	<0.004

Results are based on comparing values of each individual innervated cultured muscle with those of the noninnervated sister cultured muscle, and are expressed as the mean of the difference, ± SEM.
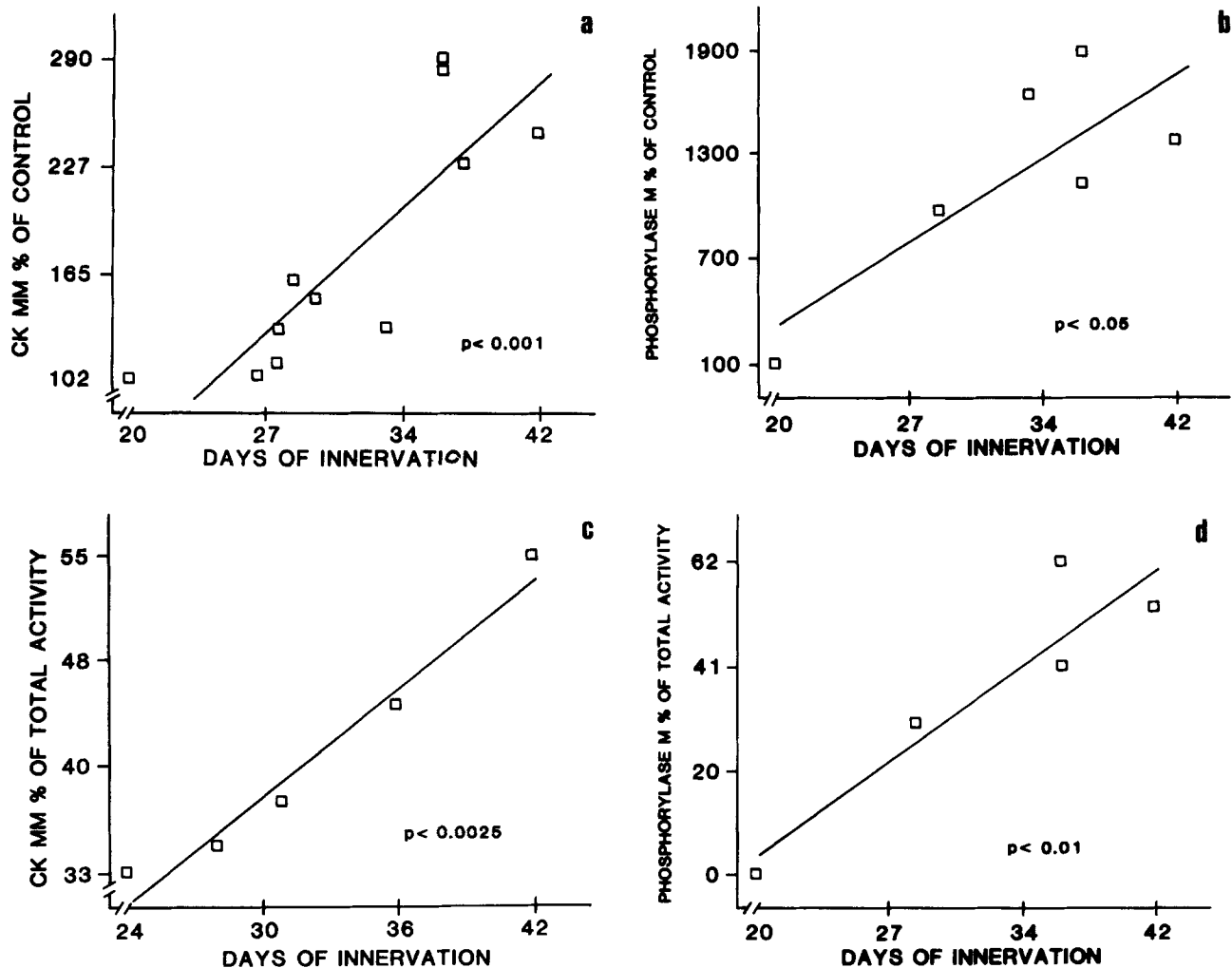NS, not significant.

Figure 4. Linear correlation of the muscle isozyme of CK and of phosphorylase with length of innervation in culture. (*a*) CK-MM isozyme of innervated cultured human muscle at various periods of innervation expressed as percent of CK–MM of noninnervated control; (*b*) as *a* but for phosphorylase. (*c*) CK-MM isozyme, and (*d*) muscle isozyme of phosphorylase at various periods of innervation expressed as percent of total activity.

frozen in small aliquots. Gel diffusion showed only one line of precipitation against normal muscle homogenate. Enzyme inhibition by antibodies was studied by incubating appropriate dilutions of tissue extracts at 37°C for 30 min with the increasing amounts of antiserum. Normal rabbit serum was used in control experiments. Total activity of phosphorylase and phosphorylase electrofocusing were studied after the tissue was incubated for 30 min at 30°C with various dilutions of antiserum; normal rabbit serum served as the control.

Data Analysis

Student's *t* test for pair samples was used in the evaluation of the innervated muscles vs. the noninnervated muscle controls. Student's *t* test was also used to test the significance of regression coefficients in linear correlations. Values of $P < 0.05$ were considered significant.

Results

Total activity of both CK and phosphorylase increased slightly but significantly in the innervated muscle. CK activity was 165% of the noninnervated control ($P < 0.05$) and phosphorylase activity was 146% of the noninnervated control ($P < 0.04$). Striking differences between innervated and noninnervated muscle were present in the pattern and rela-

tive amounts of the isozymes. The CK muscle-gene–specific isozyme MM of the innervated muscle was 188% of the noninnervated muscle ($P < 0.005$), whereas the CK BB band of the innervated muscle was only 68% of the noninnervated one ($P < 0.005$) (Fig. 2, Table I). This difference was even more striking for phosphorylase; activity of the AM band of the innervated muscle increased 1,292% ($P < 0.004$). The fetal isozyme was only 35% ($P < 0.001$) of the noninnervated muscle (Fig. 3). A highly significant linear correlation existed between (*a*) the ratio of muscle-gene–specific bands of CK and phosphorylase in innervated vs. noninnervated cultured muscle and the length of innervation, and (*b*) the percentage of muscle-gene–specific band of CK and phosphorylase and the length of innervation (Fig. 4, *a–d*).

Electrofocusing of normal human AM visualized one major phosphorylase band, and in close proximity to it, two to three weaker bands (Fig. 5). These thinner bands were not always easily detectable, but the application of 1:3 diluted homogenate of AM enabled more distinct visualization of the additional phosphorylase bands (Fig. 5). Innervated cultured muscle had a major "AM" phosphorylase band corresponding to the band of AM (Fig. 3). Also similar to AM, the in-
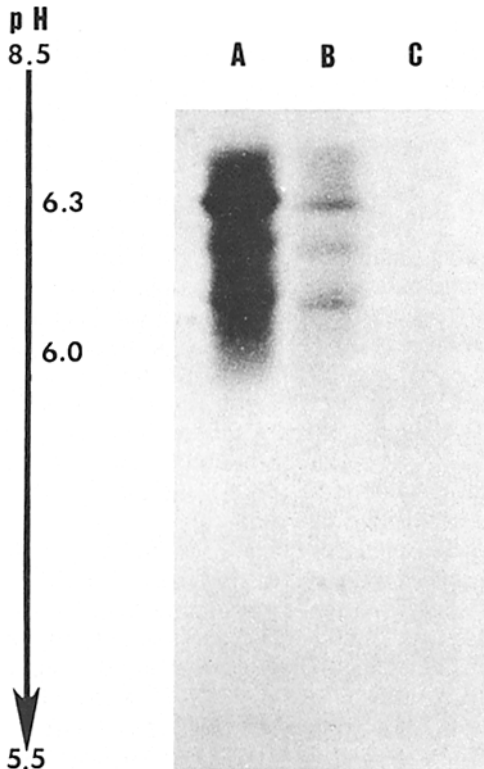
Figure 5. Phosphorylase isozymes of human AM applied at different dilutions and after preincubation with antiserum against human muscle phosphorylase. (*A*) Human AM; total phosphorylase activity, 0.1 mU. (*B*) Same as *A*; total phosphorylase activity, 0.033 mU. (*C*) Same as *A*; 0.1 mU total activity after preincubation for 30 min at 30°C with 1:20 anti-human muscle phosphorylase rabbit antiserum. Isoelectrofocusing on polyacrylamide slab gel pH 8.5–5.5 stained with iodine after incubation in phosphorylase substrate containing both 2 mM AMP and 0.7 M Na_2SO_4.

Figure 6. Influence of rabbit antiserum against human muscle phosphorylase on phosphorylase isozymes. (*A*) Human AM homogenate. (*B*) Same as *A* but preincubated for 30 min at 30°C in 1:20 antiserum. (*C*) Innervated human cultured muscle. (*D*) Same as *C* but preincubated in antiserum as in *B*. Isoelectrofocusing on polyacrylamide slab gel pH 8.5–5.5 staining and substrate as in Fig. 5. Equal total phosphorylase activity applied.

nervated cultured muscle had in close proximity to the major muscle phosphorylase band two to three weaker bands, which often fused to form one wider band (Figs. 3, 6, and 7). In contrast to AM, innervated cultured muscle had three to five phosphorylase bands between pI 6.0 and 5.8 and a weak band close to 5.6 (Fig. 3).

Neither major nor minor muscle phosphorylase bands in AM or innervated cultured muscle was significantly affected by the elimination of Na_2SO_4 from the incubation medium (Fig. 3). The phosphorylase bands between pI 6.0 and 5.8 virtually disappeared after the elimination of Na_2SO_4 from the medium (Fig. 3). Because dependence on Na_2SO_4 is typical of the adult liver isozymes (1), we considered those bands "liver-like." Electrofocusing of noninnervated cultured muscle visualized up to nine phosphorylase bands. There was a very weak group of two or three bands around pI 6.3 (the first of them was present only in 25% of the samples examined), a prominent group of four or five (of which the last two were stronger between pI 6.0 and 5.8), and a broad band (sometimes two thinner bands) ~pI 5.6 (Fig. 3).

Preincubation with antibodies against human muscle phosphorylase, diluted 1:1 or 1:20, completely inhibited in a dose-related fashion both major and minor muscle bands of phosphorylase of both innervated cultured human muscle and human AM (Fig. 5 and 6). Preincubation with the anti-
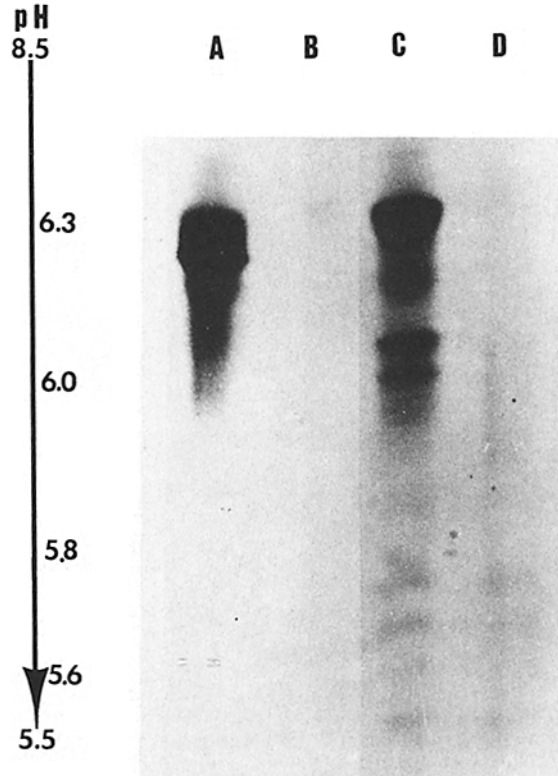
bodies diluted to 1:60 also caused disappearance of the minor muscle band, but caused only partial disappearance of the major muscle band (Fig. 7). The band of pI 5.6 did not react with the antibody (Fig. 6 and 7). The liver-like bands often appeared weaker after the preincubation with the antibody (Fig. 7). The reason for this occasional cross-reactivity is not clear at the moment.

Total phosphorylase activity was also inhibited by the antibody in a dose-related fashion. Antibody diluted 1:5 inhibited virtually all (95%) of phosphorylase activity in the AM, 60% in the innervated cultured muscle, and only 36% in the noninnervated cultured control muscle (Fig. 8). The similarities between AM and innervated cultured muscle became more apparent when we used a more diluted (1:80) antiserum. This antiserum inhibited the phosphorylase in AM 42%, in innervated cultured muscle 32%, and in noninnervated muscle only 6%.

Discussion

The present study provides evidence that biochemical maturation of human muscle cultured in monolayer is influenced by the innervation by fetal rat spinal cord. Muscle-gene-specific isozymes of both CK and phosphorylase were significantly increased in innervated cultured human muscle as compared with noninnervated cultured control muscle. The ratio of increase was related to the length of innervation. Of
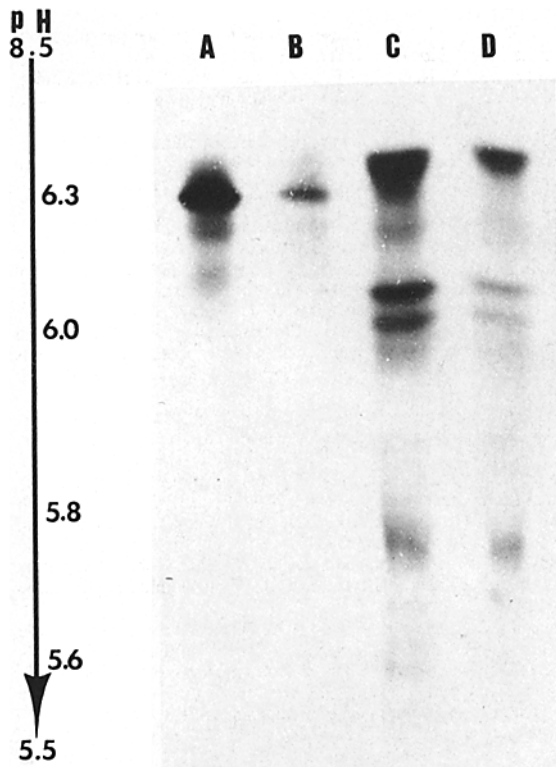
Figure 7. Effect of preincubation with diluted antiserum against human muscle phosphorylase on phosphorylase isozymes. (*A*) Human AM homogenate. (*B*) Same as lane *A* but preincubated in 1:60 antiserum for 30' at 30°C. (*C*) Innervated human cultured muscle. (*D*) Same as *C* but preincubated with antiserum as in *B*. Isoelectrofocusing on slab gel pH 8.5–5.5. Staining and substrate as in Fig. 5. Equal total phosphorylase activity applied.

particular interest is the presence of a distinct, large AM band of phosphorylase in our innervated cultured muscle. Existence of this isozyme in aneurally cultured human muscle was denied by some authors (12, 30). Other authors asserted it was a small band (20). This discrepancy could be due to the fact that cultured muscle was studied at different stages of development—from immediately after fusion (12) to 4 wk after fusion (20). In the present studies, a thin band of muscle-type of phosphorylase, not dependent on Na_2SO_4 and inhibited by antibodies against muscle phosphorylase, was present in noninnervated control cultured muscle (Table I).

Slab gel isoelectrofocusing allows a clear separation of phosphorylase bands that are not visualized as distinct in gel electrophoresis (32). The pattern of phosphorylase isoenzymes differs depending on the method used for their separa-
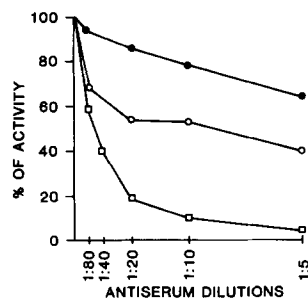

Figure 8. Inhibition of total phosphorylase activity by different dilutions of antiserum. (●) Noninnervated cultured muscle; (○) innervated cultured human muscle; (□) human AM.

tion. The pattern we describe is somewhat different from the one described by the authors who used disk and slab gel electrophoresis. In their studies, the liver band migrates in almost the same position as the muscle band (9, 10, 12, 26, 30, 31), whereas our pattern is similar to the pattern described by the authors who used electrofocusing (25, 28, 32). Meienhofer et al. (20), performing isoelectrofocusing on gel rods, did not describe any liver-like phosphorylase bands in human aneural muscle culture. In our cultures, electrofocused on polyacrylamide slab gel, liver-like phosphorylase bands were present in both innervated and noninnervated cultured muscle. The existence of liver-like bands could be either due to the presence of fibroblasts (which have distinct liver-like bands) in our muscle cultures, or to the immaturity of the cultured muscle, or both. Studies of cultured human muscle innervated for a longer period of time (3-5 mo), in which maturation should be more advanced, should generate a better explanation of this phenomenon.

In some diluted samples of human AM (Fig. 5) we observed three definite phosphorylase bands not affected by the omission of Na_2SO_4 from the incubation medium but that were inhibited by anti–muscle-phosphorylase antibody. A partial explanation of this complex pattern is that both phosphorylase *a* and *b* forms in our system were active in the presence of AMP. Muscle phosphorylase *b* has a lower pI than the *a* form, and this would give a double image on gel (25). Whether the third band reflects a molecular aggregate of *a* and/or *b*, or other phenomenon, awaits further studies.

Our studies provide the first evidence that: (*a*) de novo innervation of human muscle cultured in monolayer exerts a maturational influence on biochemical parameters of the muscle fibers; and (*b*) physical nerve–muscle contact but not diffusable neural factors is required to produce this biochemical maturation. We can cite evidence for the latter because control muscle fibers were biochemically immature even though they came from the same dish and were equally exposed to diffusable neural factors released into the medium from cells of the spinal cord explants.

Although the innervated cultured muscle fibers clearly were more advanced in their isozyme differentiation, as reflected by CK and phosphorylase, they still had more of the immature isozymes than biopsied adult normal human muscle. Future studies should be directed toward: (*a*) the pattern of isozymes in cultured human muscle innervated for a longer time (e.g., 3-5 mo); and (*b*) the pattern of isozymes in the inactive innervated cultured human muscle (paralyzed by *d*-tubocurarine or tetrodotoxin) and in electrically stimulated noninnervated cultured human muscle. This will distinguish between purely neuronal influences and those induced by activity. The increase of total phosphorylase in our cultured innervated muscle fibers is likely due to a neurogenic influence rather than simply the contractile activity, even though the innervated cultured human muscle contracted nearly continuously whereas the aneural cultured human muscle only occasionally contracts (4). Our preliminary studies indicate that repetitive electrical stimulation of aneurally cultured human muscle can be successfully performed, but 2 wk of electrical stimulation did not exert any influence on the expression of the muscle-gene–specific isozyme of creatine kinase, despite moderate increase of total CK activity (27).

This paper can serve as a basis for investigating the iso-

zyme maturation of innervated diseased human muscle. For example, it will be important to determine whether long-term innervated muscle cultures from patients with muscle phosphorylase deficiency will develop, either gradually or suddenly, the typical phosphorylase deficiency, and whether innervated control muscle continues to maintain or increase its AM-type of phosphorylase isozyme. If this can be determined, researchers will have reproduced the disease in culture and could then study in vitro the mechanism of that enzymatic failure.

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