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A combined histochemical and immunohistochemical study on the dynamics of fast-to-slow fiber transformation in chronically stimulated rabbit muscle

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Summary. Chronically stimulated fast-twitch muscles of the rabbit were histochemically and immunohistochemically analyzed in serial cross sections (1) for percentages of fiber types, and (2) for the presence of myosin heavy chain isoforms during fast-to-slow transformation. By four weeks of stimulation the number of type-I fibers had increased more than fourfold, while only about 6% of the original IIB fibers remained. Type-IC and -IIC fibers transiently rose to 20% of the total fiber population. After 16 weeks, the number of type-I fibers had increased to 42%. With prolonged stimulation fewer fibers reacted with antibodies against embryonic and neonatal myosins and more with the antibody against slow myosin. The reaction for embryonic myosin was most often detected in the C fibers (IC, IIC). Immunohistochemical subtypes were observed for each fiber type in the stimulated muscles. The greatest number was seen in type-IIC fibers, which, in addition to their reaction for fast/neonatal and slow myosins, might also react with the antibodies against neonatal/embryonic and embryonic myosins. These findings indicated that the transforming fibers temporarily expressed myosin heavy chain isoforms normally not detectable in adult skeletal muscle. Myotubes reacted strongly with the antibodies against fast/neonatal and embryonic myosins, and some of them also with the antibody against slow myosin. Thus, it appears that under the influence of the low frequency stimulus pattern some of the newly formed myotubes developed into type-I fibers.

Key words: Skeletal muscle – Chronic stimulation – Fiber transformation – Myosin heavy chain isoforms – Immunohistochemistry – Histochemistry – Rabbit

Chronic indirect stimulation of rabbit fast-twitch skeletal muscle with a pattern normally delivered to a slow-twitch muscle induces a progressive slowing of the muscle (Salmons and Vrbová 1969). At the molecular level this functional change corresponds to a progressive exchange of fast with slow myofibrillar protein isoforms. Long-term stimulation ultimately results in the predominant expression of slow myosin light and heavy chains (Sréter et al. 1973; Pette et al. 1976; Mabuchi et al. 1982; Brown et al. 1983; Seedorf et al. 1983; Staron and Pette 1987a; Staron et al. 1987). The fiber transformation includes stages in which fast and slow myosin light and heavy chain isoforms are coexpressed. Microchemical (Pette and Schnez 1977; Mabuchi et al. 1982; Staron et al. 1987; Staron and Pette 1987a) and immunohistochemical analyses (Rubinstein et al. 1978) indicate the coexistence of fast and slow myosins in transforming fibers. This suggests that differentiated muscle fibers have the capacity to change their myosin phenotype. In this context the question arises whether the exchange of fast with slow isomyosins during fiber transformation is a one-step process or involves several steps, such as a reversion to the embryonic stage with a sequential expression of the different isomyosins in the embryonic-neonatal-adult pathway (Whalen 1985) seen during normal muscle development (Hoh and Yeoh 1979; Whalen et al. 1979, 1981; Bandman et al. 1982; Lowey et al. 1983; Winkelmann et al. 1983; Lyons et al. 1983; Obinata 1985).

Until recently it was believed that fast-to-slow transformation is entirely due to a conversion of existing fast-twitch fibers. However, work from our laboratory has demonstrated that, in addition to fiber transformation, replacement of fast-glycolytic fibers makes an important contribution (Maier et al. 1986; Maier and Pette 1987). The fibers replacing degenerated fast-twitch fibers stem from newly formed myotubes that initially express embryonic myosin (Maier et al. 1986), similar to what has been observed in other studies on muscle fiber regeneration (Sartore et al. 1982; Matsuda et al. 1983). It has been suggested that the synthesis of adult slow myosin in normal developing, presumptively slow-twitch muscle fibers can occur at any stage of development without an obligatory prior appearance of neonatal or fast myosin isoforms (Butler-Browne and Whalen 1984). It would be of interest to inquire whether or not newly formed myotubes in the low-frequency stimulated muscle are also capable of switching directly from embryonic to adult slow myosin under the influence of an imposed foreign stimulus pattern. The present study was undertaken to follow the expression of myosin isoforms in transforming and in regenerating fibers.

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Materials and methods

Chronic stimulation

Changes in myosin expression and general morphology were examined in chronically stimulated extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of adult male and female White New Zealand rabbits ranging in body weight from 2.6 to 4.0 kg. Our technique for implanting electrodes, and the method of low frequency (10 Hz) telestimulation, have been described in detail previously (Pette et al. 1973; Schwarz et al. 1983). One week of recovery was allowed between the implantation of the electrodes and the onset of stimulation. Postoperatively, animals received for 3 days daily injections of 15 mg neomycin sulfate. The applied stimulus voltage ranged from 1–3 V and the pulse width was 0.15 ms. Stimulation was always carried out on the left hindlimb.

Cross sections of more than 4000 muscle fibers from stimulated and contralateral muscles of 32 animals were examined. Muscles were stimulated for 4, 6, 8, 10, 14, 21, 28, 30, 35, 60, 82, 91, or 117 days. In most animals muscles were stimulated alternately for 1 h and then rested for 1 h, resulting in 12 h of stimulation per day. The muscles of four animals were continuously stimulated for 24 h/d. In one rabbit electrodes were implanted and left in place for 35 d, but muscles were not stimulated. In addition, EDL muscles from two normal, untreated rabbits were analyzed. At the end of each experimental period, the rabbits were killed and the left and right EDL or TA muscles dissected. Tissue for histological preparations was routinely taken from the midsection of muscles. The specimens were divided into small pieces and frozen in melting isopentane (−159° C). Composite blocks consisting of the corresponding experimental and contralateral muscles were prepared. From these, 5-μm (immunohistochemistry) or 8-μm (histochemistry) thick serial cross sections were cut in a cryostat and transferred to coverslips for further processing.

Immunohistochemistry

Five monoclonal antibodies against myosin heavy chains were used: anti-fast/neonatal (BF-13), anti-slow (BF-F8), anti-neonatal/embryonic (BF-B6), and two anti-embryonic (BF-45 and 2B6) myosin antibodies. The anti-slow and anti-embryonic myosins are specific for only one isoform, while the other two recognize two isoforms, i.e., fast and neonatal, and neonatal and embryonic, respectively. The production and properties of the antibodies have been described elsewhere (Gambke and Rubinstein 1984; Gorza et al., in preparation; Schiaffino et al. 1988). The BF antibodies were from mouse ascites and stored as precipitates in ammonium sulfate. They were used in dilutions ranging from 1:1000 to 1:2000 in phosphate-buffered saline containing 1% crystalline bovine serum albumin (PBS). The 2B6 antibody was stored as lyophilisate and was reconstituted with PBS in a 1:2000 dilution. In each case, the primary antigen-antibody complex was reacted with a biotinylated anti-mouse IgG. The resulting complex, in turn was coupled to an avidin-peroxidase complex (Vector Laboratories Inc., Burlingame, CA 94010, USA). The final reaction product was visualized by the peroxidase reaction, using diaminobenzidine as a chromogen. Incubations with the primary antibody lasted 1 h and with the second antibody 40 min. The peroxidase reaction was terminated after 30 min. Prior to the first antibody incubation, endogenous binding of avidin and biotin was blocked by preincubating the sections for 10 min with avidin-biotin blocking solution (Vector Laboratories Inc.). Endogenous peroxidase activity was inhibited with 2% H_2O_2 in methanol after incubation with the secondary antibody. Following each incubation, sections were repeatedly washed with PBS. After completing all reactions, sections were dehydrated and mounted in Entellan (E. Merck, Darmstadt, FRG). Control sections were treated in the same manner, except that incubation with the primary antibody was omitted.

Histochemistry

Serial cross sections were stained for myofibrillar actomyosin ATPase (mATPase) using a modification (Staron et al. 1983) of the method of Brooke and Kaiser (1970). Classification of fiber types was performed according to the staining intensities after preincubation pH values of 4.3, 4.5 and 9.6. With this method, type-I fibers are dark after preincubation at pH 4.3 and 4.5, and light at pH 9.6. The reverse is true for type-IIA fibers. Type-IIB fibers appear the same as IIA fibers, except that they stain darker at pH 4.5. Although variable, the C fibers are stable at all three pH values. The IIC fibers are closest in appearance to IIA fibers, and IC fibers stain similar to type-I fibers (Staron and Pette 1986, 1987b). In series with the mATPase-stained sections, one consecutive section was stained with hematoxylin and eosin.

Evaluation of fiber phenotypes

Representative regions from each experimental and contralateral, or unstimulated muscle with implanted electrodes, were analyzed by the following procedure. Outlines of muscle fibers were traced at 280-fold magnification with the aid of a camera lucida attached to a Leitz Orthoplan microscope. Using these drawings as a guide, samples of 60–70 fibers each were traced through the 9 serial sections representing the various immunohistochemical and histochemical reactions. The immunohistochemical typing was based on assigning values of low, moderate or strong intensities to the reactions with the various antibodies. Low reaction was considered not to be significantly above background level.

Results

Changes in histochemically classified fiber types

As previously observed (Pette et al. 1976; Heilmann and Pette 1979; Maier et al. 1986), muscles stimulated for identical periods of time showed variable responses. Therefore, it seemed practical to combine animals into groups that were exposed to stimulation for shorter, intermediate, or longer periods of time. This procedure to some extent adjusted for interanimal variations and permitted following a rough time course of the stimulation-induced changes.

Analyses of fiber-type percentages were limited to the EDL. The first changes in the composition of the fiber population consisted of an increase in the number of IIC fibers. These fibers, which amount to less than 1% in nor-

Table 1. Percentage distribution of histochemically identified fiber types in rabbit EDL muscles stimulated during different time periods. Values represent means ±SEM (n)

Fiber type	0 d	4 d	6–10 d	14–21 d	28–35 d	60 d	82–117 d
I	7 (8)	7.2±0.6 (13)	5.4±0.5 (30)	9.0±1.6 (39)	29.5±8.3 (65)	23.2±4.9 (65)	42.0±14.8 (132)
IC	0	0.6 (1)	1.4±0.9 (8)	5.1±4.4 (22)	5.0±1.7 (11)	1.4±0.9 (4)	0.6 (2)
IIC	3 (3)	2.8±3.3 (5)	11.9±4.2 (66)	14.7±4.1 (63)	10.5±6.4 (23)	7.2±2.8 (20)	10.2± 5.7 (32)
IIA	30 (34)	33.1±7.1 (60)	30.5±6.3 (168)	41.0±9.3 (176)	46.3±11.1 (102)	57.9±7.1 (162)	30.6±16.9 (96)
IIB	60 (69)	55.8±3.1 (101)	49.7±8.2 (275)	26.7±9.4 (115)	5.5±2.5 (12)	9.6±2.0 (27)	15.3±11.7 (48)
Unclassified	0	0.5 (1)	1.1±1.5 (6)	3.5±2.1 (15)	3.2±2.3 (7)	0.7 (2)	1.3± 0.5 (4)
Number of muscles	1	3	9	7	4	4	5

Table 2. Percentage distribution of histochemically identified fiber types in rabbit EDL muscles contralateral to the stimulated muscles listed in Table 1. Values represent means ±SEM (n)

Fiber type	0 d	4 d	6–10 d	14–21 d	28–35 d	60 d	82–117 d
I	9 (10)	6.7±3.7 (12)	8.7±0.8 (40)	8.7±0.7 (33)	9.3±1.3 (21)	6.5±0.4 (15)	6.8±2.8 (19)
IC	0	1.3 (2)	0	0	0	0	0
IIC	3 (3)	0	1.1±0.8 (6)	0.9±0.4 (3)	3.3±2.4 (6)	0	0.4 (1)
IIA	24 (28)	29.0±2.9 (52)	27.4±1.8 (151)	36.5±3.5 (139)	32.3±2.0 (73)	26.0±2.3 (59)	28.6±4.1 (81)
IIB	64 (74)	63.0±5.8 (113)	62.8±1.8 (333)	53.9±8.6 (204)	55.1±3.3 (125)	67.5±2.2 (156)	64.2±4.6 (182)
Unclassified	0	0	0	0	0	0	0
Number of muscles	1	3	9	7	4	4	5

mal EDL, increased to 3% at 4 d, reached 15% by 14 d, and decreased to about 9% in the longer stimulated animals (Table 1). The initial increase in IIC fibers was accompanied by a moderate decrease in the percentage of IIB fibers, which in normal EDL account for approximately 65% of the total fiber population (Leberer and Pette 1986). This fiber type continued to decline to 5.5% in the EDL of animals stimulated for 28–35 d. Although there were pronounced interanimal variations, the type-IIB percentage tended to slightly increase again with prolonged stimulation. The proportion of type-IIA fibers, which amounts to approximately 30% in normal EDL (Leberer and Pette 1986), began to increase by 14 d and reached a maximum of about 58% at 60 d. Thereafter, it declined to relatively normal levels. The type-I fibers were a stable population of about 7% until 14 d, but then their numbers increased markedly and reached about 40% in long-term stimulated EDL. If IC fibers, which peaked earlier than the type-I fibers, were also to be included into the type-I category, the percentage of slow-twitch fibers would increase another 5% at 14–35 d (Table 1).

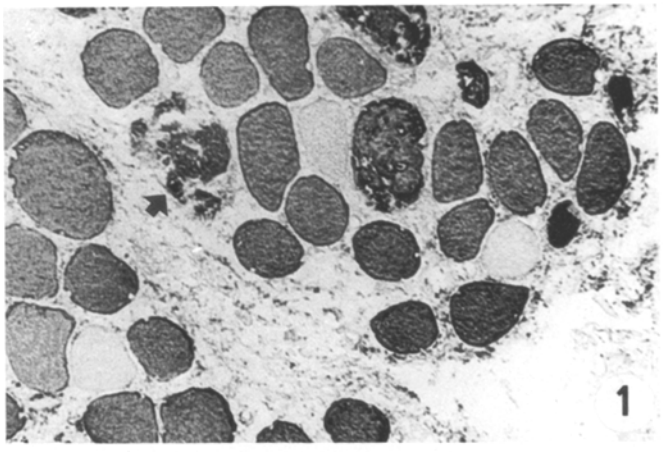
The histochemically defined fiber populations in the contralateral EDL muscles displayed no conspicuous deviations from normal percentages, although there was a transient decline in the number of IIB fibers accompanied by a transient increase in type-IIA, type-IIC and type-I fibers (Table 2). No deviations from normal were observed in the unstimulated muscle with implanted electrodes.

From 4 d on until 21 d a variable number of degenerating fibers was observed. These were identified according to morphological criteria (round shape, clumped myofibrillar pattern, invasion by foreign cells) and by uneven and atypical mATPase staining (Figs. 1–8). Degenerating fibers were only rarely seen in muscles stimulated for periods longer than four weeks. Concomitantly with degenerating fibers, and as much as two weeks later, appreciable numbers of myotubes appeared in the stimulated muscles (Figs. 3, 4). These myotubes stained moderately or strongly for mATPase after preincubations at pH 4.3 (not shown) and pH 4.5 (Fig. 8) and light after preincubation at pH 9.6 (not shown). Thus, the mATPase histochemistry of the myotubes indicated some heterogeneity already at this stage. Myotubes were rarely seen in long-term stimulated muscles. Small fibers, possibly stemming from myotubes, appeared as early as two weeks after the onset of stimulation.

Immunohistochemistry of myosin isoforms

In the untreated muscles, as well as in the unstimulated muscle with implanted electrodes, each of the three major histochemical fiber types coincided with one specific immunohistochemical type. Thus, type-I fibers strongly reacted with the anti-slow myosin, and type-IIA and -IIB fibers strongly reacted with the anti-fast/neonatal myosin. A slight reaction of the IIA and IIB fibers with the anti-embryonic myosins (2B6, BF-45) was interpreted as cross reaction of the two antibodies with adult-fast myosin.

Muscles contralateral to the stimulated leg did not show the clear-cut relationship between histochemical staining and immunohistochemical profiles seen in untreated muscles. In addition to their type-specific reactions (see above), each histochemical fiber type displayed several immunohistochemical subtypes. For example, in the 4–35 d stimulation period, 35% of type-I, 22% of type-IIA and 12% of type-IIB fibers of the contralateral muscles also reacted moderately with anti-neonatal/embryonic (BF-B6) myosin. A small portion of these type-IIA and -IIB fibers also strongly reacted with the two antibodies directed against embryonic myosin.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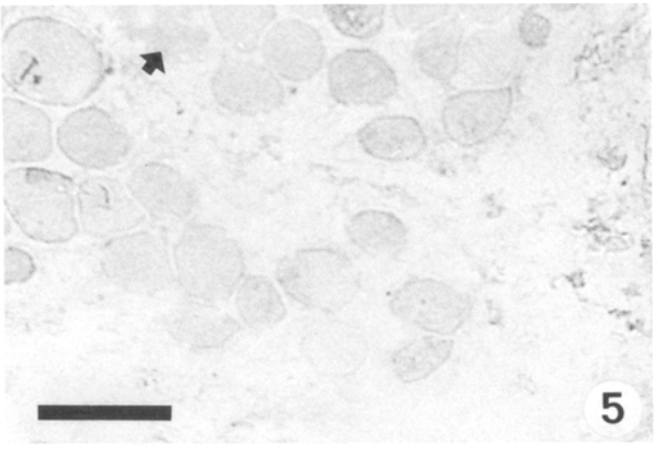
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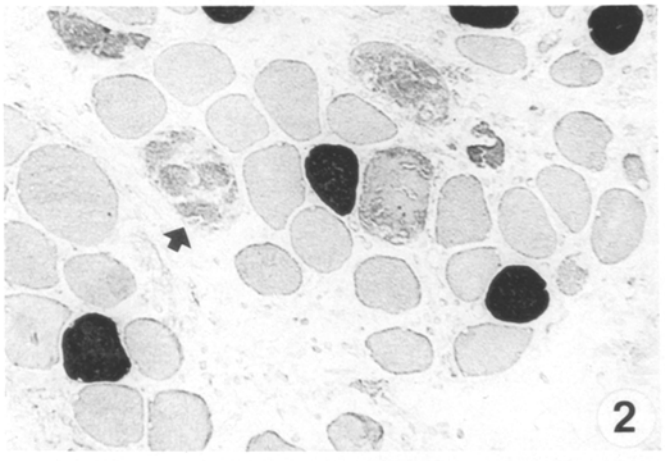
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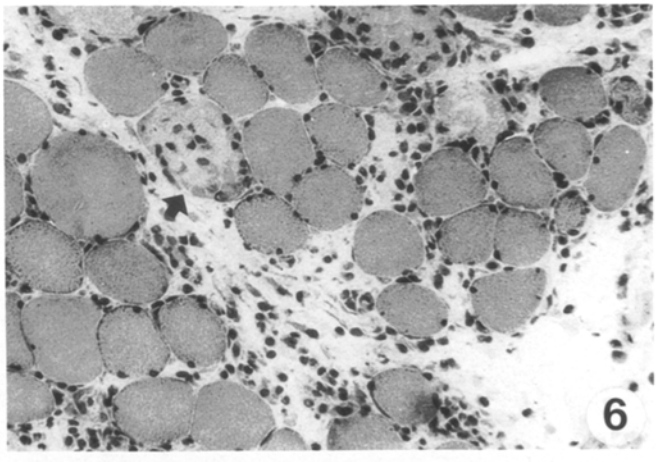
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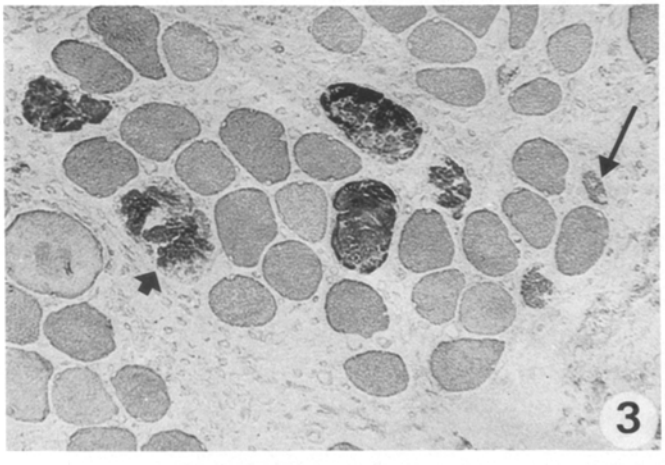
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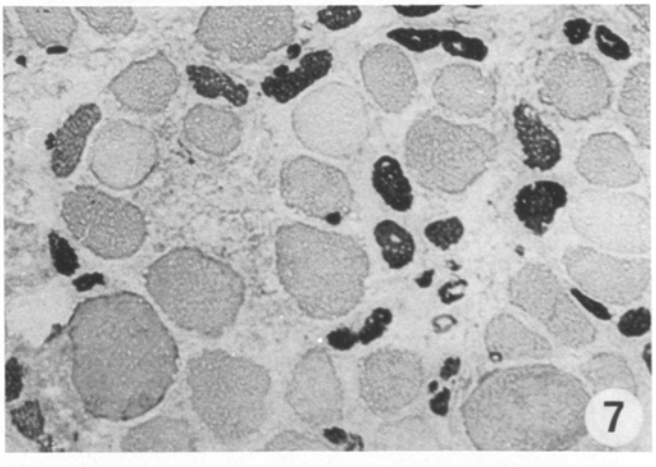
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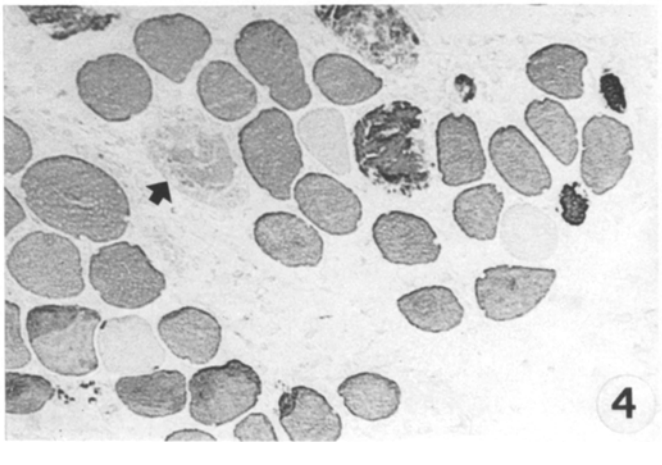
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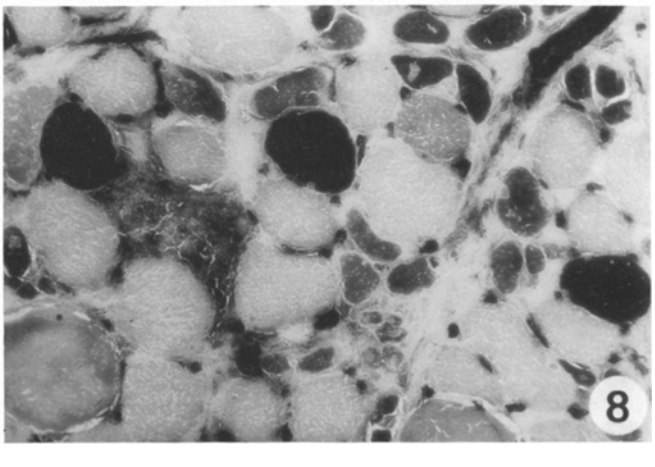
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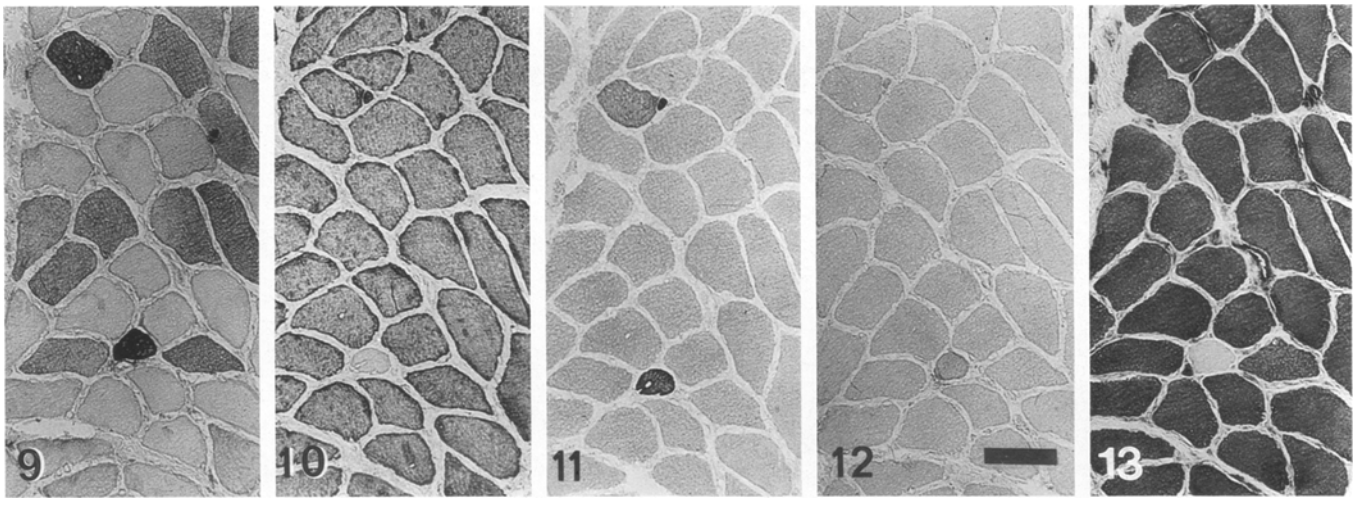


Figs. 9–13. Serial cross sections of a 60 d-continuously (24 h/d) stimulated rabbit TA. Sections were incubated with antibodies against fast/neonatal (**Fig. 9**), slow (**Fig. 10**), embryonic (**Fig. 11**) or neonatal/embryonic (**Fig. 12**) myosins, or stained for mATPase, pH 4.3 preincubation (**Fig. 13**). Almost all fibers stain uniformly (**Figs. 10–13**), except after incubation with anti-fast/neonatal myosin (**Fig. 9**), where considerable variation in staining intensities is recognized. Bar = 50 μm

In stimulated muscles each histochemically defined fiber type, as well as the myotubes, showed an entire spectrum of immunohistochemical subtypes. This spectrum changed with prolonged stimulation in such a way that reactions with anti-embryonic and anti-neonatal myosins decreased, while reactions with anti-slow myosin increased. The progressively more frequent anti-slow myosin reaction coincided with a greater presence of histochemical type-I fibers. However, type-I fibers in the longer stimulated muscles still showed immunohistochemical heterogeneity. This is convincingly demonstrated for a 60 d-stimulated TA in which uniform histochemical type-I fibers are a heterogeneous assembly after incubation with anti-fast/neonatal myosin (Figs. 9–13).

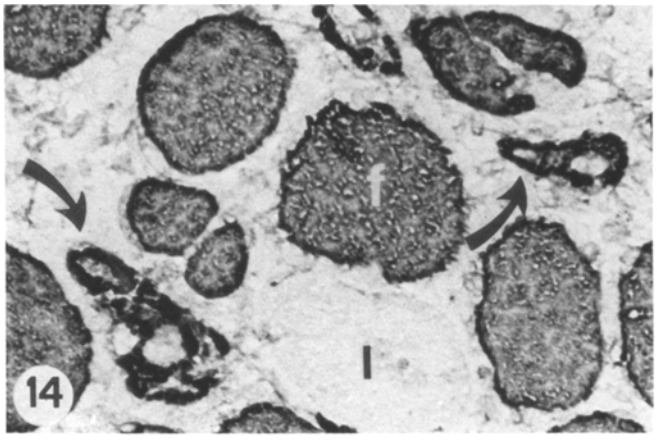
Myotubes in stimulated muscles reacted strongly with anti-fast/neonatal (BF-13) and anti-embryonic (BF-B45, 2B6) myosins. In addition, myotubes also might react with anti-neonatal/embryonic (BF-B6) and anti-slow (BF-F8) myosins (Figs. 14–21). An appreciable number of small fibers past the myotube stage in the longer stimulated muscles reacted with anti-fast/neonatal (Fig. 22), anti-neonatal/embryonic (not shown), anti-slow (Fig. 23), and anti-embryonic myosins (Fig. 24). The histochemical reactivity of the latter group resembled that of C fibers (Figs. 25–27).

The three most frequent combinations of reactions for different myosins in muscle fibers during the first five weeks of stimulation, the main period during which changes occurred, are given for each fiber type in Fig. 28. Surviving and/or transformed type-I fibers, and surviving IIB fibers (Maier et al. 1986), appeared to be less variable populations than the other fiber types because their predominant immunohistochemical subtypes (> 50% and > 70%, respectively) agreed with the immunohistochemical patterns of the respective normal fiber types. The next most numerous combinations in type-I fibers, each amounting to about 5%, displayed positive reactions with either anti-slow (BF-F8) and anti-neonatal/embryonic (BF-B6) or anti-slow (BF-F8) and anti-fast/neonatal (BF-13) myosins. In IIB fibers, the second and third most frequent combinations showed a stronger reaction than in the normal muscle with either anti-embryonic (10%) or anti-neonatal/embryonic (4%) myosins. Similar combinations also occurred in IIA fibers, but reactions with anti-neonatal/embryonic myosin were more frequent.

Although no distinction is made in Tables 1 and 2, type-I fibers reacting both with anti-slow and anti-fast/neonatal myosin could be distinguished in mATPase-stained sections. Their appearance was that of IC fibers, or they differed from normal type-I fibers in that they were slightly stained for mATPase after preincubation at pH 9.6 (Figs. 25–27). Thus, the latter resembled the type-I_t fibers described previously (Staron et al. 1987).

The greatest variability in immunohistochemical profile was recognized in the C fiber population. The majority of the IC and IIC fibers strongly reacted with anti-fast/neonatal myosin. Many type-IC (30%) and some IIC fibers (17%) reacted with both the anti-fast/neonatal and the anti-slow myosin (Figs. 22–24, 28). In addition, many IC fibers (45%) definitely displayed a positive anti-embryonic myosin reaction above cross-reaction level. There were also many type-IC fibers (32%) that did not react with anti-slow myosin, but instead reacted with both anti-embryonic and anti-fast/neonatal myosins (Figs. 22–24, 28). The major fraction (70%) of the IIC fibers showed many divergent

Figs. 1–8. Serial cross sections of 8 d-stimulated rabbit EDL incubated with antibodies against fast/neonatal (**Fig. 1**), slow (**Fig. 2**), neonatal/embryonic (**Fig. 3**) or embryonic (**Fig. 4**) myosins, without primary antibody (**Fig. 5**), or stained with hematoxylin and eosin (**Fig. 6**). A degenerating fiber is marked by a *short arrow*. Note the strong reaction with the antibody against neonatal/embryonic myosin (**Fig. 3**). A myotube is marked by a *long arrow* (**Fig. 3**). Two consecutive sections from a 14 d-stimulated EDL are shown in Figs. 7 and 8. Reaction with anti-embryonic myosin (**Fig. 7**) and staining for mATPase, preincubation at pH 4.5 (**Fig. 8**). Bar = 100 μm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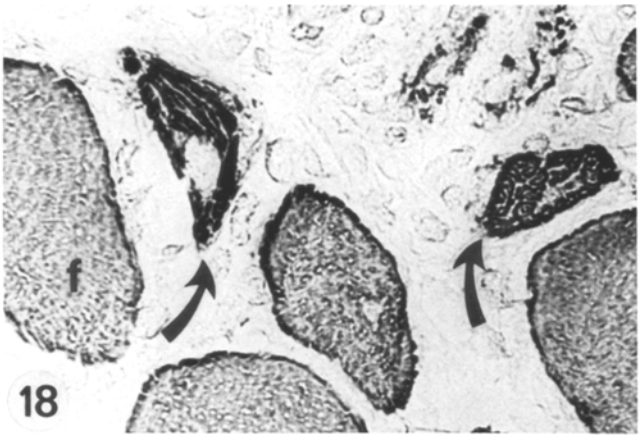
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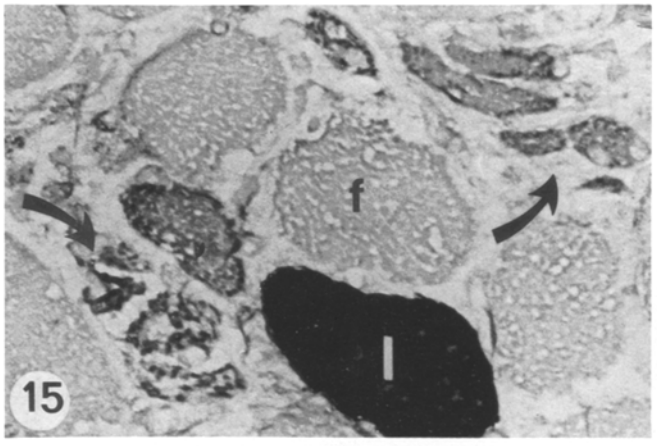
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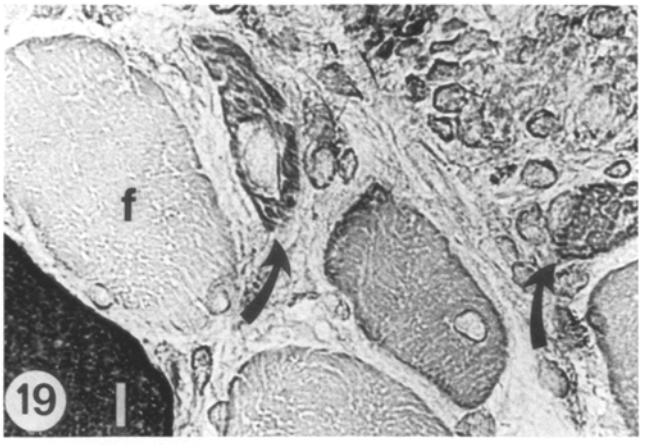
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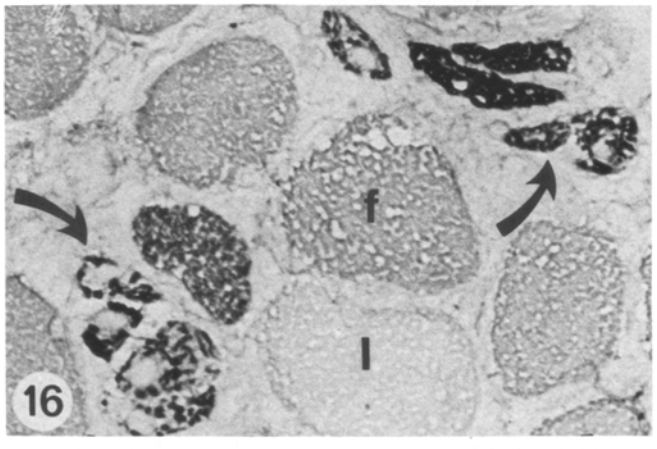
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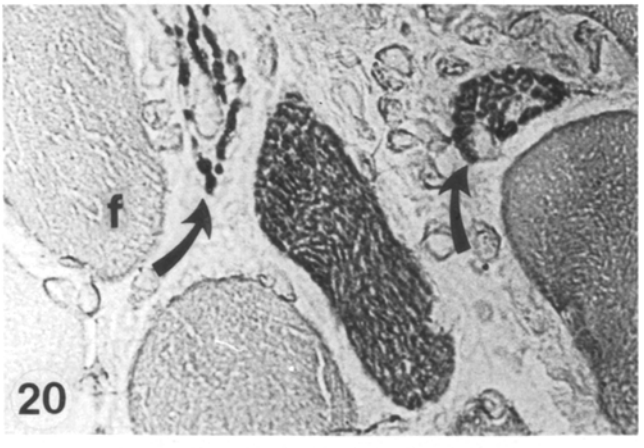
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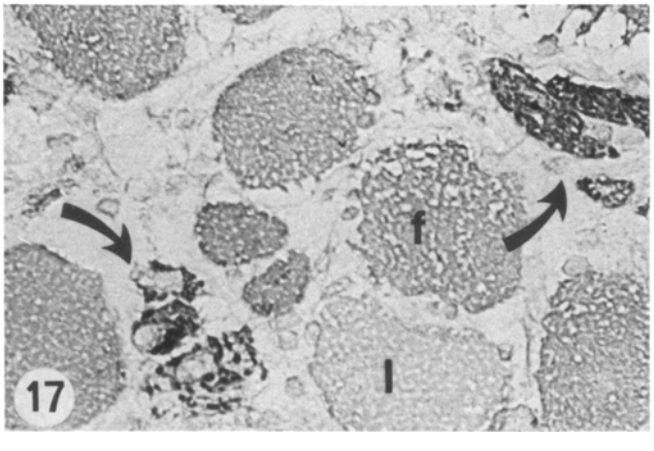
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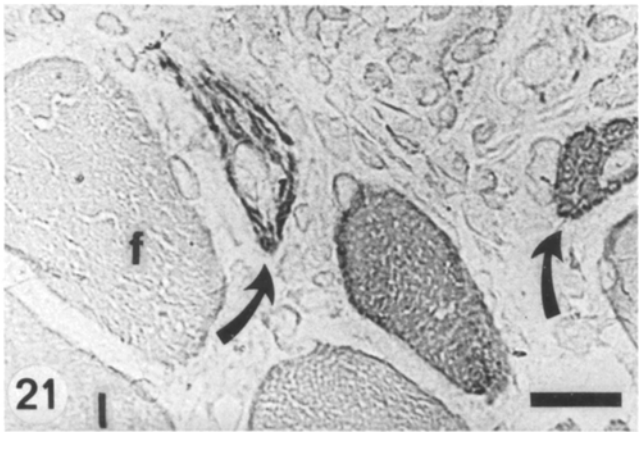
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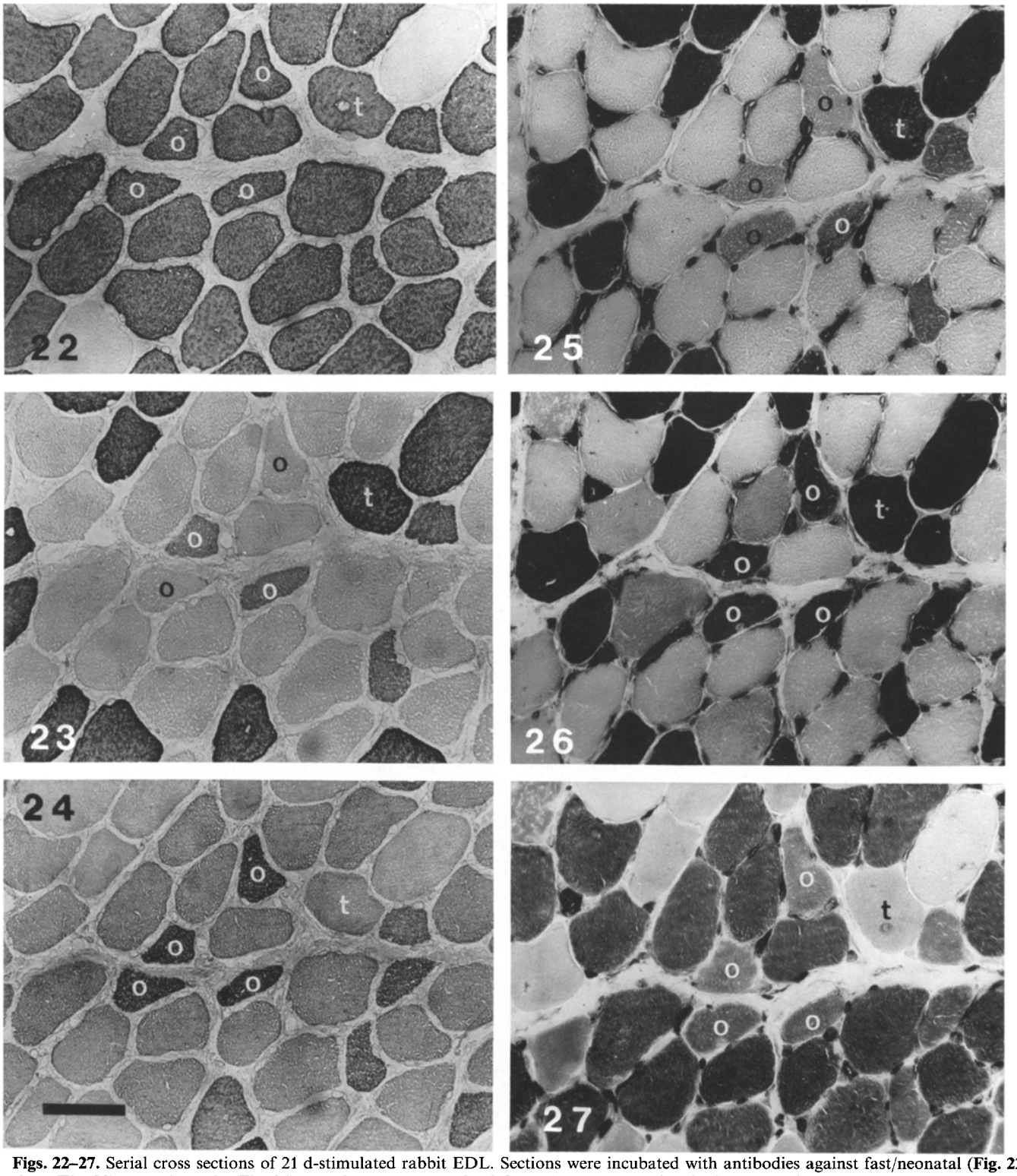

Figs. 22–27. Serial cross sections of 21 d-stimulated rabbit EDL. Sections were incubated with antibodies against fast/neonatal (**Fig. 22**), slow (**Fig. 23**) or embryonic (**Fig. 24**) myosins, or were stained for mATPase at preincubation pH values 4.3 (**Fig. 25**), 4.5 (**Fig. 26**), and 9.6 (**Fig. 27**). Note that small fibers (*circles*) react with antibodies against fast/neonatal (**Fig. 22**) and embryonic (**Fig. 24**) myosins, and may also react with anti-slow (**Fig. 23**) myosin. These fibers correspond to type-IC fibers. A fiber tentatively classified as type I_t (*t*) reacts with anti-fast/neonatal and anti-slow myosins. Bar = 50 μm

Figs. 14–21. Serial cross sections of rabbit EDL stimulated for 10 d (**Figs. 14–17**) or 14 d (**Figs. 18–21**) incubated with antibodies against fast/neonatal (**Figs. 14, 18**), slow (**Figs. 15, 19**), embryonic (**Figs. 16, 20**), or neonatal/embryonic (**Figs. 17, 21**) myosins. *Arrows* point to single or several myotubes. When incubated with anti-slow myosin, myotubes stain intermediate between type-I (*I*) and fast-twitch (*f*) fibers. Bar = 20 μm

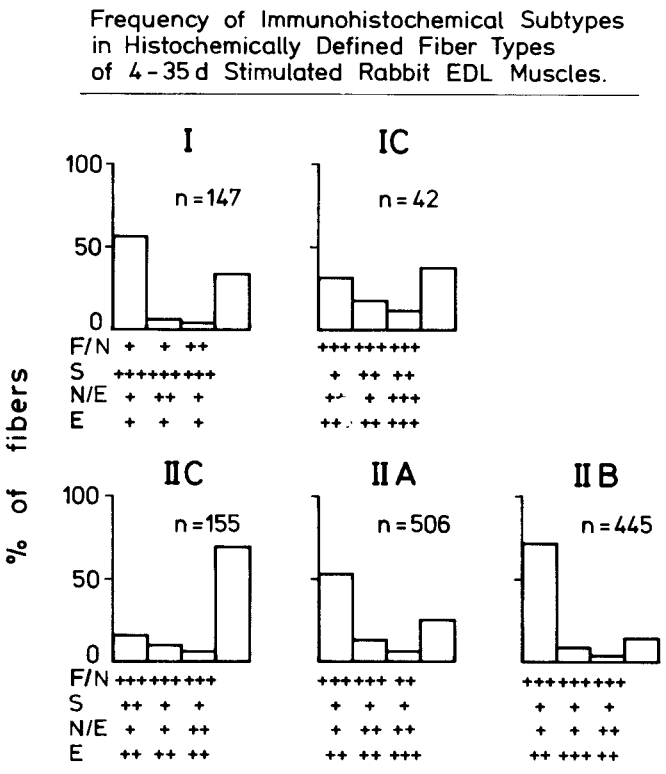

Fig. 28. Histograms of the three most frequent immunohistochemical profiles of five histochemically identified fiber types in EDL muscles stimulated from 4–35 d. Most of the immunohistochemical combinations occur during this time span. Because of the great number of infrequently occurring combinations, these were combined and are represented by the fourth bar. The greatest number (33) of combinations applied to the type-IIC fibers. Staining intensities as judged by visual inspection are indicated by + (low), + + (moderate), + + + (strong). *F/N* anti-fast/neonatal; *S* anti-slow; *N/E* anti-neonatal/embryonic; *E* anti-embryonic; *n* number of fibers

immunohistochemical profiles arising from variable staining intensities with the five antibodies. Similar minor subtypes were also observed for types I, IIA and IIB; however, their frequency was much less than in the IIC fibers.

Degenerating fibers, primarily in the earlier stages, strongly reacted with the anti-fast/neonatal and especially with the anti-neonatal/embryonic myosin (Figs. 1, 3). The strong reaction of the latter was impressive with regard to the lack of any reactivity of degenerating fibers in sections incubated without primary antibodies (Fig. 5). A moderate reactivity existed in degenerating fibers for the two antibodies directed against embryonic myosin (Fig. 4). However, there was no appreciable reaction with the anti-slow myosin (Fig. 2).

Discussion

Previous studies (Mabuchi et al. 1982; Staron et al. 1987; Sweeney et al. 1987) have suggested that stimulation-induced changes in the population of rabbit fast-twitch muscle fibers occur in the sequence of type IIB to type IIA to type I. The sequential increases in the number of type-IIA and type-I fibers, coupled with the initial decrease in type-IIB fibers observed in the present study, further support this scheme. However, the immunohistochemical data suggest that the process of fiber transformation is more complex than reflected by analyses that are solely based on mATPase histochemistry. One indicator of the complexity is the great number of immunohistochemical subtypes identified in the present study by five monoclonal antibodies. It must be kept in mind that the complexity may actually be greater because the analysis did not include antibodies specific for the IIA and IIb myosin heavy chains. Moreover, the distinction between fast and neonatal myosin heavy chains was not possible, except to suppose that in myotubes and in small, presumably regenerating fibers the neonatal myosin might be the dominant isoform. Fibers reacting with anti-neonatal/embryonic myosin, but not with anti-embryonic myosin, were assumed to contain neonatal myosin. Although separate specific antibodies would be preferable, a positive anti-neonatal/embryonic reaction is still a valuable observation because both neonatal and embryonic myosins are transitional isoforms that reflect similar temporal sequences of the same process, namely, a transition in myosin composition during development or during transformation in response to non-physiological stimuli.

Comparisons of data indicate that histochemically defined fiber types in normal muscle are not equivalent to the respective fiber types in transforming muscle. Thus, the stimulated fiber types I, IIA and IIB may display, in addition to their expected predominant heavy chain complement, also neonatal and/or embryonic heavy chains. Likewise, the C fibers not only coexpress fast and slow myosins, but in addition may contain embryonic and neonatal myosins. Approximately 30% of the type-IC fibers reacted stronger with anti-fast/neonatal, anti-neonatal/embryonic and anti-embryonic myosins than with anti-slow myosin. It is not surprising that to date the presence of other than fast and slow myosin heavy chains in the C fibers has escaped detection by electrophoretic analysis because currently available electrophoretic methods are probably not sufficiently discriminative to separate the neonatal from the IIb and the embryonic from the IIa heavy chains, respectively.

In our view, the data of the present study do not permit a reconstruction of a more detailed sequence of events than the one given above. One reason for this is that the numerous combinations of immunohistochemical reactions in a given fiber type make it difficult to establish a clear sequence of myosin heavy chain transitions. The histochemical and immunohistochemical appearance cannot indicate the stage of conversion. The presence of a given myosin-heavy-chain species in a fiber may be due to either ongoing or beginning synthesis, or, because of its slow turnover rate (Martin et al. 1977), to its persistence after syntheis has ceased. An example would be the coexistence of fast and slow myosins in fast (IIC) fibers transforming into slow ones. Another complication in the interpretation of the data is the possibility of nonuniform distribution of myosin isoforms along the length of transforming muscle fibers (Staron and Pette 1987a; Schiaffino et al. 1988). In addition, different fiber types may respond nonuniformly in time to the imposed impulse pattern (Pette and Tyler 1983; Maier et al. 1986; Maier and Pette 1987).

The presence of myosin reacting with antibodies against embryonic and/or neonatal heavy chains in any given fiber could indicate that it is newly formed or that the transformation process involves temporary activation of genes nor-

mally silent in adult skeletal muscle fibers. Izumo et al. (1986) have shown that the genes for embryonic and neonatal myosins are activated in some muscles of the adult rat under hypothyroid conditions. Messenger RNA for the neonatal myosin heavy chain was detected in the hypothyroid masseter, but not in diaphragm, soleus and EDL. Conversely, mRNA coding for the embryonic myosin heavy chain was detected in the soleus, but not in the other muscles. The observation that hypothyroidism enhances in fast-twitch skeletal muscles of the rat the expression of slower myosin isoforms and, in addition, induces the embryonic isoform in soleus muscle (Izumo et al. 1986), is of interest with regard to the present findings. Thus, it may be suggested that the expression of embryonic myosin in adult fibers reflects an extreme step or overshoot reaction in their stimulation-induced fast-to-slow transition.

The reaction of myotubes with anti-embryonic and anti-fast/neonatal myosins was a common finding. The inconsistent reaction of myotubes with anti-slow myosin could either point to different developmental stages or indicate that, due to different cell lineage commitments, not all of them converted to slow-twitch fibers. The frequent combination of adult myosins together with neonatal and/or embryonic myosin heavy chains in small IC fibers (Figs. 22–24) could indicate that they are newly developing fibers past the myotube stage. However, reactions for adult myosins together with embryonic and/or neonatal myosins may also occur in large IIB and especially in IIA fibers. Some of the slight or moderate reactions with the anti-embryonic myosin in type-IIA and -IIB fibers (Fig. 28) may be akin to the cross reaction with fast myosin seen in normal muscle. However, significant amounts of embryonic and/or neonatal myosins should be present in the 4% of the IIB fibers and the 20% of the IIA fibers that not only reacted with anti-fast/neonatal myosin, but also with anti-neonatal/embryonic and anti-embryonic myosins (Fig. 28). These fibers either represent newly formed type-IIA and -IIB fibers derived from myotubes, or existing type-IIA and -IIB fibers, which transiently express embryonic and neonatal myosins. The assumption that they stem directly from non-adapting and cell lineage-specific myotubes could explain why, in the longest stimualted muscles, there was a slight to moderate increase in the number of IIB fibers, after they had markedly decreased during the earlier phases of chronic stimulation. However, the possibility cannot be excluded that transforming adult fibers temporarily express embryonic and neonatal myosin heavy chains, similarly to what might occur in surviving IIA fibers during the early stages of denervation (Schiaffino et al. 1988).

An interesting finding was the strong reaction of degenerating fibers with the anti-neonatal/embryonic myosin. It is unlikely that deteriorating fibers are capable of activating protein synthesis, especially of a type not native to the fiber. It is more likely that previously unaccessible epitopes are exposed, probably by proteolysis or unfolding, and that these kryptic determinants react with the anti-neonatal/embryonic myosin. It cannot be excluded that the strong anti-neonatal/embryonic reaction is due to a reaction with an epitope of nonmuscle myosin in phagocytes invading the degenerating muscle fiber. In any event, the anti-neonatal/embryonic myosin would appear to be a useful marker in detecting degenerating myofibers.

The appearance of uncommon immunohistochemical subtypes in contralateral muscles agrees with earlier observations (Srihari et al. 1981; Reichmann et al. 1983; Staron et al. 1987). The reasons for these changes remain obscure. It may be speculated that they are caused by reflex activity originating in the stimulated side. However, it may also be that these changes originate from unusual long-term positions of the animal leading to altered loads on the contralateral foot dorsiflexors.

In conclusion, the present results indicate that the stimulation-induced fast-to-slow transformation is a complex process with the coexistence of several myosin heavy chain isoforms in the same fiber. The coexistence of fast and slow myosin heavy chain isoforms, but also of isoforms normally present only in developing or regenerating skeletal muscle fibers, may indicate that transforming fibers either sequentially or simultaneously express various myosins. Sequential and simultaneous expression of embryonic, neonatal and adult myosin isoforms may be related in some fibers to degeneration-regeneration processes (Maier et al. 1986; Maier and Pette 1987). However, it may also result from a temporary reactivation of genes that are normally silent in adult skeletal muscle fibers. Finally, and in conjunction with the latter possibility, it may occur because myonuclei along the length of a fiber are not synchronized in their phenotypic expression during the transformation process.

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