A developmentally regulated disappearance of slow myosin in fast-type muscles of the mouse

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Histochemistry and immunocytochemistry using an antibody to adult rat slow-type myosin demonstrated that about 10% of the fibers in the mouse extensor digitorum longus and semimembranosus muscles contain slow myosin during the first month after birth. In adult animals, these muscles have only 0–0.8% slow myosin-containing fibers. These results demonstrate a developmentally linked disappearance of an adult-type myosin, and show that the adult phenotype of muscle fibers is not necessarily determined before birth as previously suggested.

Histochemistry *Immunocytochemistry* *EDL* *Semimembranosus* *129 Mouse*

1. INTRODUCTION

The predominant myosin isozymes found in developing skeletal muscles differ from those present in adult muscles. These developmentally regulated embryonic (or fetal) and neonatal isozymes can be distinguished from the adult forms principally by the heavy chain subunit as demonstrated by protein chemical [1–7], immunochemical [3,4,7–9] and molecular cloning [10–12] approaches. A sequential appearance and disappearance of the embryonic and neonatal isozymes has been described for both fast- and slow-type muscles [3–5,8,9,13–15].

In addition to these predominant myosin forms, a minority of fibers in developing fast muscles have been reported to contain slow myosin at fetal and newborn stages [14,16–18]. These fibers may correspond to primary generation fibers (i.e., those formed first during fetal development; see [19]). It has been suggested that these same fibers will contain slow myosin in adult muscles [14,16,17].

We show that developing fast muscles in the mouse have a small proportion of slow myosin-containing fibers, most of which are not present in the corresponding adult muscles. This is the first demonstration of a developmentally linked disappearance of an adult-type myosin. Since primary generation fibers have been demonstrated to occur in mouse muscles [19], these results show that neither they nor those fibers which contain slow myosin in the neonatal period necessarily become slow-type fibers in adult mouse muscles.

2. MATERIALS AND METHODS

Mice of the 129 ReJ strain were used. Phenotypically normal (+/dy) and homozygous dystrophic (dy/dy) animals were identified at 4, 7 and 14 days of age by examination of spinal roots as in [20]. Genotypically dystrophic animals were included in this study since the EDL and soleus muscles show no morphological abnormalities at 7 days when examined by light microscopy [21,22]. Histochemical ATPase staining was performed as described [23] with preincubation at pH 4.3 carried out at room temperature for 40 min. Characterization of the neonatal myosin antibody and methods for antigen and antibody purification, solid phase

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enzyme immunoassay, immunoblotting and indirect immunofluorescence have already been described [15,24,25].

3. RESULTS

3.1. *Characterization of the antibody to slow myosin*

A rabbit antibody was prepared by injecting SDS-denatured heavy meromyosin [15] made from rat soleus muscle myosin. The affinity purified immunoglobulins react in a solid phase enzyme immunoassay about 5–10-times more strongly with slow myosin than with neonatal or adult fast myosin preparations and about 20–100-times more strongly than with embryonic myosin. The values for neonatal and fast myosin are minimum estimates since these preparations contain some slow myosin and therefore part of the reactivity observed will be due to the presence of slow myosin. Results from immunoblotting using rat myosins also show that the immunoglobulins react specifically with the heavy chain component of slow myosin. When used in immunocytochemistry, the purified antibody selectively stains slow- but not fast-type fibers in adult rat muscle, and does not react with fibers containing embryonic and neonatal myosin in developing rat muscles (not shown).

Table 1

Histochemical slow-type fibers in the EDL muscle at various ages

Age	No. of mice	No. of fibers containing slow myosin[a]	%[b]
4–14 days	5	131 ± 12.7	10
2 months	7	0	0
	3	2.3 ± 0.6	0.2
4 months	3	0	0
	7	7.0 ± 2.7	0.8

[a] Determined as the fibers reacting strongly positive in the histochemical ATPase reaction after acid preincubation at pH 4.3, and expressed as mean ± SD

[b] Based on the following values for total fiber number: 1320 fibers for ages 4–14 days [21], 1060 for 2 months and 920 for 4 months of age [34]

3.2. *Presence of slow myosin-containing fibers in young mouse muscles*

The muscles used in this study were the extensor digitorum longus (EDL), the semimembranosus (SM) and the soleus. In 20 adult animals examined (2–4 months of age), no slow fibers could be demonstrated by standard histochemical techniques in the EDL muscle of 10 mice while the remaining mice had less than 10 slow fibers per muscle (table 1). Similar results have been reported by others for the same mouse strain [26] and for the EDL muscle of C57Bl mice [27]. In a total of 30 mice of 2–6 months of age, no SM muscle was found to contain slow fibers. The soleus muscle is normally considered to be rich in slow fibers, and in mice about 50–60% [27] react histochemically as typical type I slow fibers; and approximately this proportion is found in the 129 ReJ strain used here.

The antibody to slow myosin described above was used in immunocytochemical studies on muscles taken from mice of different ages. Fig.1 shows the immunocytochemical staining on the adult EDL, SM and soleus muscles in an animal in which only the soleus muscle contains fibers which stain with the slow antibody. In young mice (1 month or less of age) however, these 3 muscles always have fibers which contain slow myosin. Examples of immunocytochemical results with muscles taken from a 7-day-old animal of genotype dy/dy are shown in fig.2. We have found that almost all fibers in muscles from 4- and 7-day-old mice, and most fibers in 14-day-old ones, react strongly with an antibody to rat neonatal myosin (not shown). Therefore, these observations demonstrate that the slow antibody is reacting with a myosin present only in a minority of the fibers and that it is not cross-reacting with mouse neonatal myosin which is also present at these ages.

At 4, 7 and 14 days, the intensity of slow antibody staining in the EDL and SM muscles is indistinguishable from the intensity observed in soleus muscles of the same animals. In addition, most of the fibers that react with the slow antibody also demonstrate a strong ATPase reaction after pH 4.3 preincubation, characteristic of slow myosin-containing fibers. This histochemical reaction on developing muscles indicates that the slow myosin is not simply a minor component of these

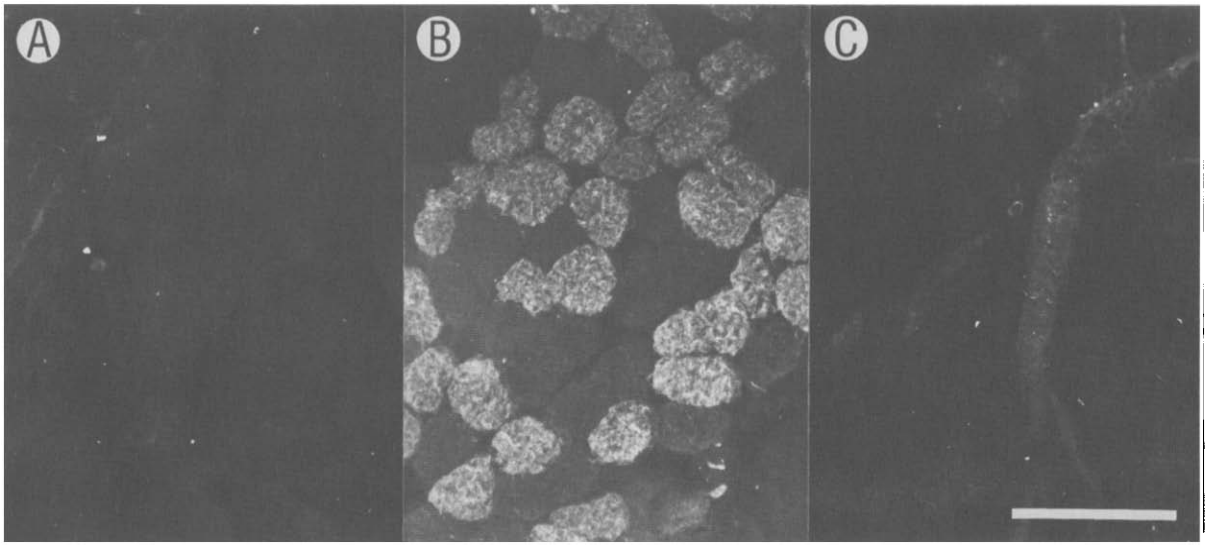

Fig.1. Indirect immunofluorescence using the antibody to slow myosin on EDL (A), soleus (B) and semimembranosus (C) muscles from a 4-month-old normal mouse. Scale bar, 100 μm.

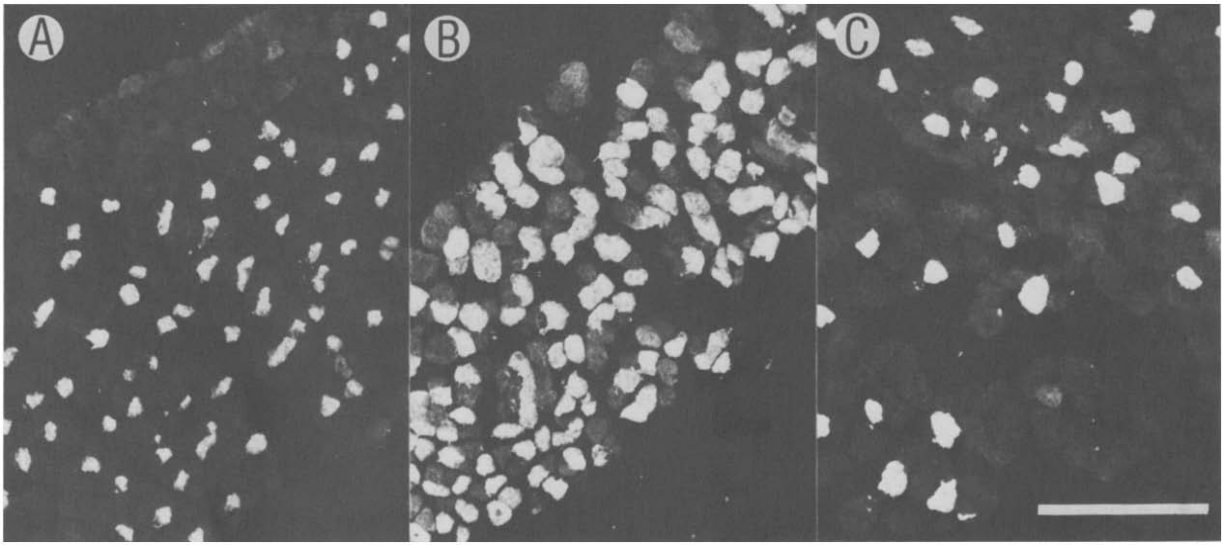

Fig.2. Indirect immunofluorescence using the antibody to slow myosin on EDL (A), soleus (B) and semimembranosus (C) muscles from a 7-day-old dy/dy mouse. Scale bar, 100 μm.

EDL and SM fibers. However, slow myosin is probably not the only myosin present since those fibers that react with slow antibody also react with the neonatal antibody (not shown).

We determined the numbers of fibers in the EDL muscles which stain darkly after preincubation at pH 4.3 in the histochemical ATPase reaction. These values decrease from 10% of all fibers at ages 4–14 days to 0–0.8% at 2–4 months of age (table 1). We have not accurately determined the total numbers of fibers or slow-myosin containing fibers in the SM muscles of young mice, although at 4–14 days of age greater than 100 fibers per muscle were found to react strongly with the slow myosin antibody and were positive for myosin ATPase after preincubation at pH 4.3.

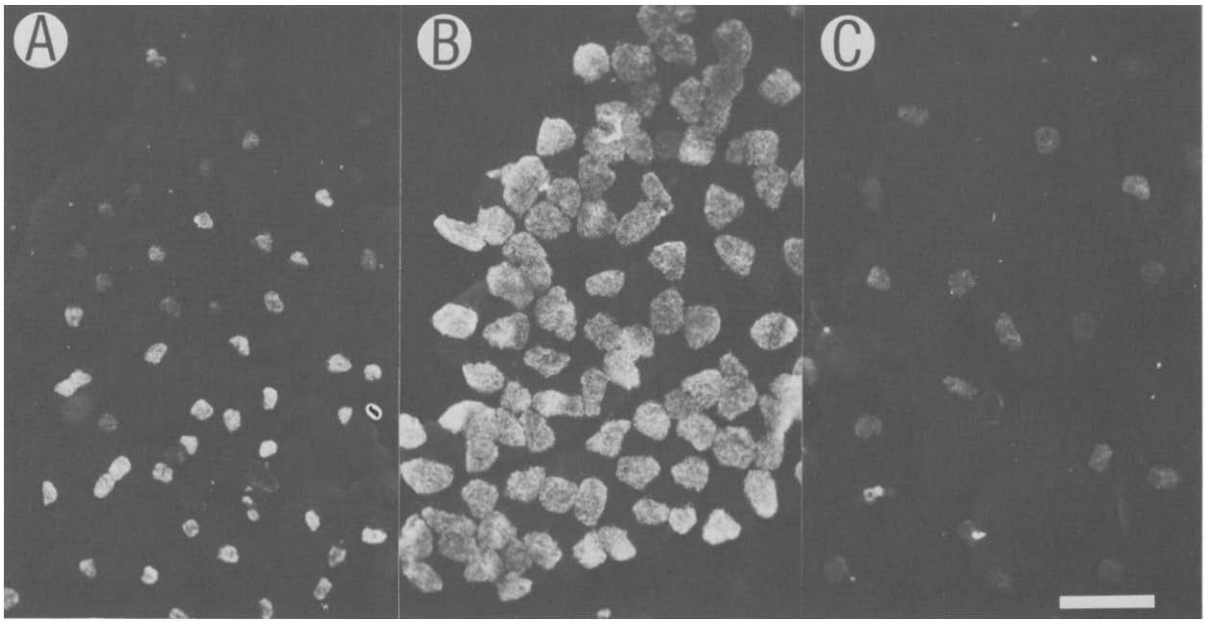

Fig.3. Indirect immunofluorescence using the antibody to slow myosin on EDL (A), soleus (B) and semimembranosus (C) muscles from a 28-day-old normal mouse. Scale bar, 100 μm.

At 28 days after birth, slow myosin-containing fibers are still readily detectable by immunocytochemistry in the soleus, EDL and SM muscles (fig.3). The staining is weaker in the fibers of the SM compared to those of the EDL or soleus, and some fibers in the EDL stain more weakly than others. This suggests that slow myosin is lost at different rates in different fibers and muscles.

4. DISCUSSION

We have found that the EDL and SM muscles of the mouse always have a contingent (greater than 100 fibers per muscle) of slow myosin-containing fibers during the first month after birth. The same muscles contain no (SM) or less than 10 (EDL; table 1 and [26,27]) slow fibers at adult ages. The immunocytochemical results demonstrate therefore an age-related disappearance of an apparently adult myosin type, a situation which has not been documented previously in developing muscles.

These results are particularly relevant to the question of how slow muscle fibers arise when they are present in adult muscles. It has been suggested by others [14,16,17] that those fibers which appear earliest in developing muscles are a specific subpopulation of fibers that contain slow myosin in fetal and newborn stages. Since the number of these slow myosin-containing fibers in the fetal rat EDL is about the same as in the adult EDL, it was concluded that these primary generation fibers are precursors of slow-type adult fibers [16,17]. Based on these results, authors in [14] have emphasized that the future adult phenotype of individual fibers in the hindlimb muscles is precisely determined at the fetal stage.

However, it is evident from our observations that those fibers which contain slow myosin in the newborn mouse do not necessarily become slow fibers in the adult. Likewise, primary generation fibers of the mouse EDL do not become slow-type fibers in adult muscle. Although authors in [19] have found that the EDL muscle in the 129 ReJ mouse strain used here contains 250–280 primary generation fibers, many adult mice have no slow fibers and the remainder have less than 10 per muscle (table 1 and [26]). We do not know if the slow myosin-containing fibers that we have identified correspond strictly to the primary generation fibers.

Our results suggest that two processes could con-

ceivably be occurring in muscles which do contain slow fibers in the adult: slow myosin could be lost in some fibers, as shown here, while being induced in others (see, e.g., [28]). These two processes could go unnoticed in muscles which contain slow fibers in the adult since the myosin content of a single fiber cannot be followed throughout development. In this respect, the choice of the mouse EDL and SM muscles was fortuitous, since the disappearance of slow myosin can be observed without the complicating presence of a large contingent of slow fibers in these adult muscles.

In adult muscles, the induction of slow myosin appears to be a nerve-dependent process [29]. The role of the nerve may be to impose a continuous pattern of activity on the muscle; when this is done experimentally, both heavy and light chains of slow myosin will accumulate in a previously fast muscle [30,31]. In newborn rat muscles however, the nerves are not capable of the same type of tonic stimulation [32] although nerve–muscle contact is certainly established at these times. Therefore, it is possible that the presence of slow myosin in fetal or newborn animals is independent of nerve influence.

If the development of slow motor-nerve properties is a postnatal phenomenon, then the population of fibers containing slow myosin in newborn muscles might overlap with nerves of a developing slow motor unit in those muscles where such units occur (e.g., rat or mouse soleus, rat EDL). This could lead to some slow myosin-containing fibers being innervated by a slow motor neuron with the result that slow myosin is maintained into adult stages. A further possibility is that slow myosin-containing fibers and slow nerves might arise separately and then be actively matched during development [33]. These mechanisms could reconcile the different observations concerning the fate of slow myosin-containing fibers in the EDL muscles of young rats and mice: in the rat, slow motor units clearly develop and are present in the adult muscle, whereas in the mouse they often are not.

Even if innervation by slow nerves accounts for the maintenance of slow myosin-containing fibers found in certain newborn muscles, the results reported here demonstrate that the adult phenotype is not necessarily determined before birth. Further experiments on mouse muscles, where the disappearance of slow myosin can be unambiguously observed, may provide information on the possible mechanisms controlling this disappearance.

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