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Increased density of satellite cells in the absence of fibre degeneration in muscle of myotonic mice

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Summary. A mutant mouse with a hereditary myotonia, 'arrested development of righting response', ADR, was investigated with respect to mononucleated cell populations in skeletal muscle. Upon enzymatic dissociation of different muscles from mice aged between 15 and 120 days, a 3- to 5-fold higher yield of mononucleated cells per muscle fresh weight was obtained from mice with the ADR syndrome than from control mice. Clonal cell culture showed that the absolute number of cells with myogenic potential was increased and that mutant clones had shorter generation times than wild-type controls. Morphological differentiation of ADR myotubes was indistinguishable from that of the controls. Light microscopy confirmed the presence of increased numbers of mononucleated cells per muscle volume. At the ultrastructural level, there were 3.3 times as many satellite cells (the myogenic stem cells of mature muscle) per myofibre nucleus in ADR than in controls. Because no fibre degeneration was observed in the ADR mutant, we conclude that the enlarged mutant satellite cell pool is not a result of compensatory proliferation but is a consequence of fibre-type transformation and/or delayed maturation of the myotonic muscle.

Key words: Neuromuscular disease – 'Arrested development of righting response' ADR – Satellite cell – Muscle culture – Myotonia

Satellite cells (Mauro 1961) represent myogenic stem cells in mature muscle; these cells can be activated, using various stimuli, to proliferate and fuse into myotubes (review: Campion 1985; Schmalbruch 1985). It is generally accepted that satellite cells are quiescent in healthy mature muscle and become activated to multiply following muscle damage, during compensatory muscular hypertrophy, or following fibre degeneration caused by intoxication, hormone inbalance, or hereditary disorders. It is therefore not surprising to find more satellite cells, many of which are in the process of division, as a regenerative response in muscular dystrophies such as Duchenne dystrophy of man (Wakayama and Schotland 1979; Cullen and Watkins 1981; Cullen 1986). However, satellite cell numbers are not affected in another neuromuscular disease, the Fukuyama type of congenital muscular dystrophy (Terasawa 1986).

In the mouse, many neuromuscular mutations have been described (Peters 1986), some of which may serve as animal models for human hereditary muscle diseases. In the present context, satellite cell pools and their possible relation to regenerative processes and to muscle fibre types are of particular interest. A decrease of satellite cell density has been observed in neonatal mice affected by the mutation 'motor endplate disease', med[1] (Jockusch et al. 1980).

Here we have investigated the mutation 'arrested development of righting response', adr[1], of the mouse (Watts et al. 1978; Watkins and Watts 1984); in the homozygous condition this mutation leads to a myotonia (Mehrke 1985; Mehrke et al. 1986a, b). ADR[1] muscle has a uniformly red phenotype (Watts et al. 1978; Füchtbauer et al. 1985), which like other biochemical abnormalities (Stuhlfauth et al. 1984) is probably a secondary consequence of the myotonic condition (Reininghaus et al. 1986).

In the course of establishing primary muscle cultures from ADR muscle, it was noted that higher yield of mononucleated cells were obtained from mutant than from control muscle. Because tissue dissociation is subject to a number of poorly controlable factors, we investigated, using various methods, whether more mononucleated cell types are present in ADR muscle than in control muscle, and whether satellite cells are involved in this change.

With respect to muscle activity and the relative proportions of oxidative and glycolytic fibres, the ADR syndrome represents a natural perturbation of the balanced state of muscle. The ADR mutant thus allows the interrelationships between muscle fibre properties and those of the satellite cell population to be studied. Whereas descriptive data concerning satellite cell densities are available for human diseased muscle, an experimental analysis, e.g. by muscle transplantation, is only possible in an animal model.

The present study was undertaken to quantify and characterize the changes in satellite cell densities in a hereditary myotonia of the mouse and thus pave the way to an experimental analysis of the genetic and physiological control of satellite cell densities in skeletal muscle.

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[1] Lower-case symbol refers to the allele of the gene, upper-case symbol to the phenotype

Materials and methods

Animals. A2G mice carrying the recessive autosomal mutation adr were originally obtained from Drs R.L. and D.L. Watts, London. Homozygous adr/adr animals (designated 'ADR') could be recognised from post-natal day 8–9 onward (Watkins and Watts 1984) and were usually compared with sex-matched phenotypic wild-type (+/adr or +/+) littermates (designated as 'control' or 'wild-type', WT, animals). Recently, it was found that adr and another murine mutation "myotonia", mto (Heller et al. 1982), are allelic (Jockusch and Bertram 1986). For the purpose of comparison, the mutation 'X-linked muscular dystrophy' (mdx, Bulfield et al. 1984; Dangain and Vrbová 1984) was investigated.

Preparation and culture of cells. Myogenic cells were obtained from minced vastus lateralis or tibialis anterior muscles by dissociation for 80 min at 37° C in 1.5 mg/ml collagenase, 2.0 mg/ml dispase, and 0.5 mg/ml DNase in 10% (v/v) newborn calf serum in Ham's F12-medium (cf. Mehrke 1985). The yield of mononucleated cells (myoblasts, fibroblasts, pericytes, adipocytes, endothelial cells) per mg muscle was about 10^3 to 10^4 cells, depending on age and genotype of the donor.

Cells were seeded on tissue culture plastic coated with rat tail collagen, either at low density ($1–5 \times 10^3$ cells per 60 mm ∅ dish) for clonal analysis or at higher density ($1–5 \times 10^4$ cells per 35 mm ∅ dish) for differentiation studies. The growth medium was 79% (v/v) Ham's F12 medium with 20% (v/v) foetal calf serum (Gibco/KC Biological) and 1% (v/v) chicken embryo extract (preparation modified after Konigsberg 1971). The fusion medium was Dulbecco's modified Eagle's medium (DME) with 5% (v/v) foetal calf serum and 5% (v/v) horse serum (Gibco, KC Biological). Sera were heat-inactivated (20 min at 56 °C). All media contained 100 I.U./ml of penicillin and 100 µg/ml streptomycin. The culture medium was renewed every second day. Cells were kept at 37° C under 5% CO_2 or 10% CO_2 for F12 or DME based medium, respectively (cf. Blau and Webster 1981; Yasin et al. 1981).

Light microscopy. Shock-frozen muscles were cut at their thickest region. Cryostat sections (8 µm) were fixed with acetic acid/alcohol (1:3) and stained with the fluorescent dye, acridine orange (Miike 1983) or by the improved trichrome method (Engel and Cunningham 1963). For evaluation of fibre circumference, enlarged photographs of stained cryostat sections were processed with a Videoplan computer (Kontron). To screen for possible degeneration, acid-phosphatase staining was performed according to Lojda et al. (1976). Cell cultures were fixed in 3.7% paraformaldehyde in calcium- and magnesium-free phosphate-buffered saline (20–60 min at 23° C) and stained with haematoxylin and eosin.

Electron microscopy. Anaesthetized animals were perfused intracardially with 3% glutaraldehyde, 1% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2; 20° C). Dissected muscles were fixed for 2 h in the same fixative (4° C), post-fixed in 1% OsO_4 and processed according to standard procedures. Muscle sections were taken in alternating semi- and ultrathin serial series from the following region: tibialis anterior about 20% distally from its proximal insertion point, vastus lateralis about 20% proximally from its distal insertion point, soleus at its thickest regions.

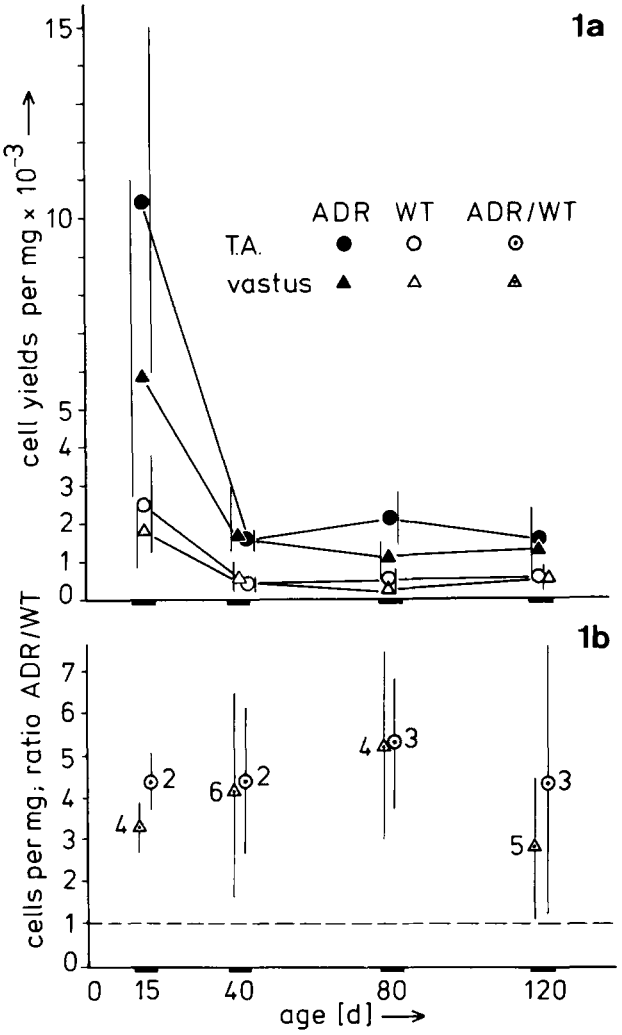


Fig. 1 a, b. Cell yields from vastus and anterior tibial (*T.A.*) muscle as a function of age and genotype. **a** Freshly dissected muscles were weighed, minced and enzymatically dissociated. Mononucleated cells were counted and related to muscle fresh-weight. Each point represents averaged values ±S.D. from 2–6 independent experiments. **b** Ratio (ADR to wild-type, *WT*) of cell counts calculated for each pair of muscles. The density of mononucleated cells is 3–5 times higher in the ADR mutant. *Points* show means of ratios ±S.D., with numbers of independent experiments

Sections were evaluated by an unbiased observer (not knowing the source of material) on the screen of a Zeiss 109 electron microscope. Number of myofibres and myonuclei were counted at a primary magnification of 3000× and each fibre was checked for satellite cells. Potential satellite cells were identified at magnifications of 7000× and 20000× by the following criteria: (1) position within basal lamina of muscle fibre, (2) constant intercellular gap between muscle fibre and satellite cell, (3) lack of any extracellular matrix-like material in gap, (4) high content of heterochromatin in nucleus. Cells of doubtful identity were photographed at 7000× and evaluated at magnifications of 21000×. More than 50% of the investigated sections were recounted by another unbiased observer to validate the accuracy of the first observer.

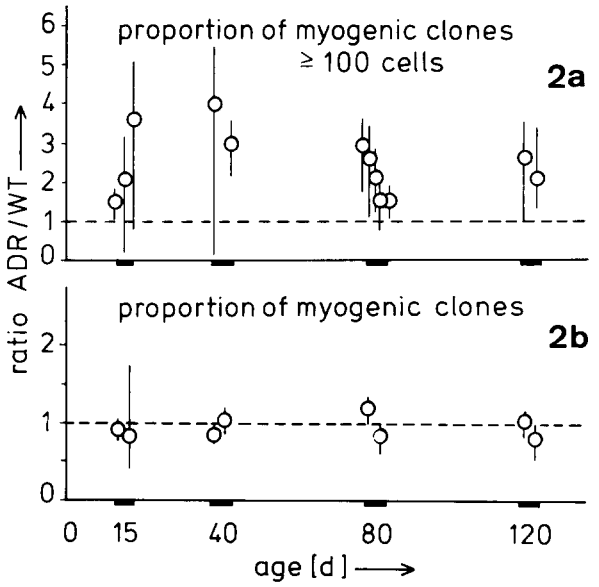


Fig. 2a, b. Clonal cell cultures from ADR and wild-type (*WT*) muscle. Values are presented as ratio ADR/wild-type. Each *point* includes counts of 20–600 clones from one individual experiment with minimal and maximal values indicated by *bars*. **a** Proportion of myogenic clones from vastus muscle with ≧100 cells. Proportion of large myogenic clones is increased in ADR. **b** Proportion of myogenic clones among all clones; vastus and anterior tibial muscle. The absolute values of the proportions "myogenic clones/all clones" decreased from about 75% at day 15 to 50% in the adult muscle

For statistical evaluation, sections comprising ≧100 fibres were scored and numbers of satellite cells were related to numbers of fibre nuclei.

Results

Cell yields. When muscle from ADR animals of different ages was digested in parallel with wild-type muscle, higher yields of mononucleated cells were obtained from the mutant, both per muscle and per muscle fresh weight (Fig. 1a, b). Because ADR muscles on the average weighed only 60 to 70% of wild-type muscles, the differences between the cell yields per fresh weight were more pronounced than per organ values. The absolute yields per fresh weight declined with age, both in mutant and in control animals (Fig. 1a). The ratio of mutant to wild-type cell yields for both vastus or tibialis anterior muscles varied between 3 and 5 (Fig. 1b).

Differentiation and growth properties. Cultures from ADR- and wild-type cells had the ability to form myotubes when taken from 15- to 120 d-old mice (Figs. 2, 3). The proportions of myogenic clones obtained from vastus and anterior tibial muscles were examined at different ages. No significant difference was detectable between ADR and wild-type muscle (Fig. 2b).

Myogenic clones from vastus muscle were examined with respect to their ability to form large clones (≧100 cells) within 8 days. A significantly higher proportion of large clones was observed in mutant muscles (Fig. 2a) than in wild-type controls whereas fibroblast clones from the same samples showed no differences.

When the generation times of myoblasts and fibroblasts from 15–120 d-old animals were determined in clonal culture, shorter generation times of myoblasts were observed in 15 d ADR animals compared with controls. These differences were also statistically significant in 40 d- and 80 d samples ($P \leqq 0.05$). In 120 d specimens, no significant difference could be established.

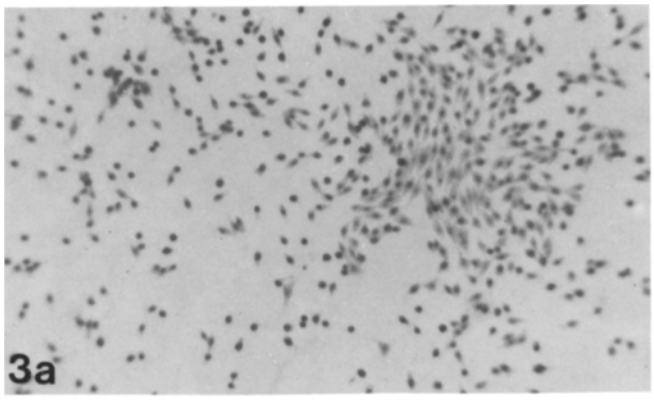
Density of nuclei in muscle tissue. The mononucleated cells obtained by tissue dissociation included satellite cells, fibroblasts, adipocytes, pericytes and endothelial cells. Fluorescent staining of nuclei showed that there was a higher density of nuclei (1.7 fold) in ADR- than in control muscle (Fig. 4a, b). Electron-microscopical investigation showed that the number of nuclei within ADR-muscle fibres was elevated by about 10% (cf. Table 2), and thus contributed only in part to the difference in the density of all nuclei.

A quantitative analysis of the number of fibre-associated and non-associated nuclei is given in Table 1.

Density of satellite cells. Because there exists no reliable light-microscopical method to count satellite cells, ADR- and control muscles were investigated at the ultrastructural level. Satellite cells were identified by their specific position within the basal lamina of the muscle fibre (Fig. 5a, b) from which they were separated by a narrow cleft (ca. 30–60 nm) that was free from any discernible material. Satellite cells in ADR- and control muscle displayed typical features, such as a high ratio of nucleus-to-plasma volume, high content of heterochromatin and a distinct nucleolus. The satellite cell membrane showed caveolae, which occurred frequently on the basal-lamina face and were less numerous on the side adjacent to the muscle membrane (Fig. 5b). Within the cytoplasm, ribosomes and polyribosomes were the most frequent orgenelles. Size, frequency and morphology of cell organelles all indicated that satellite cells of mutant and control animals were physiologically and mitotically quiescent. Although no mitotic figure was observed in this study, a single satellite cell (out of 133 examined) with a centrosome was seen in ADR muscle and in another case two heterochromatic areas without the limiting nuclear membrane. No qualitative differences in the ultrastructural features of satellite cells were seen between mutant and wild-type.

Quantitative evaluation of wild-type tibialis and vastus muscle both in young and mature animals showed a mean ratio of satellite cells-to-fibre nuclei of 1.2% (range, 0.3% to 1.7), whereas in the ADR mutant, the number of satellite cells was increased by a factor of 3.3 (mean, 4.0%; range, 2.4% to 6.2%; Table 2; Fig. 6). There was no overlap between the distribution of satellite cells in ADR and wild-type in any muscle and at any age examined. The soleus, a partially slow, purely oxidative muscle, which in the mouse consists of roughly equal numbers of type-I- and type-IIa fibres (Laarse et al. 1984; Reininghaus et al. 1986), exhibited satellite cell densities that were high, but independent of the genotype (Table 2; Fig. 6).

The earliest age at which the ADR mouse can safely be recognized is 9 d postnatal. A preliminary analysis showed that the number of satellite cells is 3–5 times higher in 9-d-old as compared to 18-d muscles with no significant difference between mutant and wild-type.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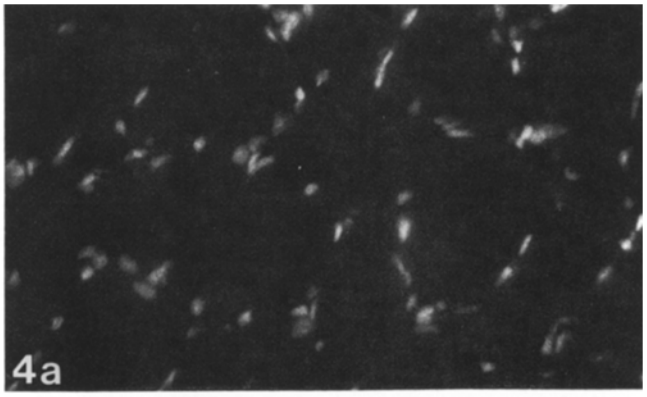
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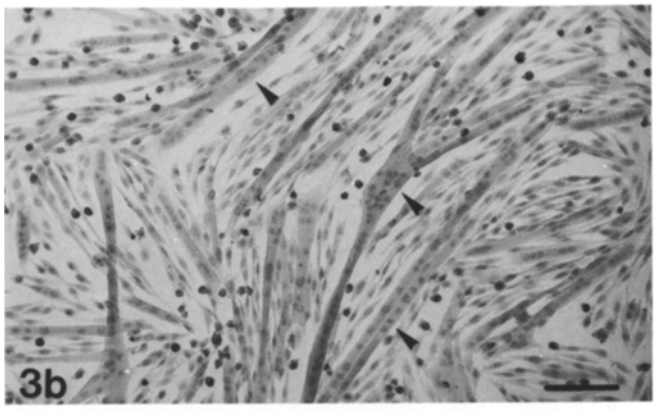
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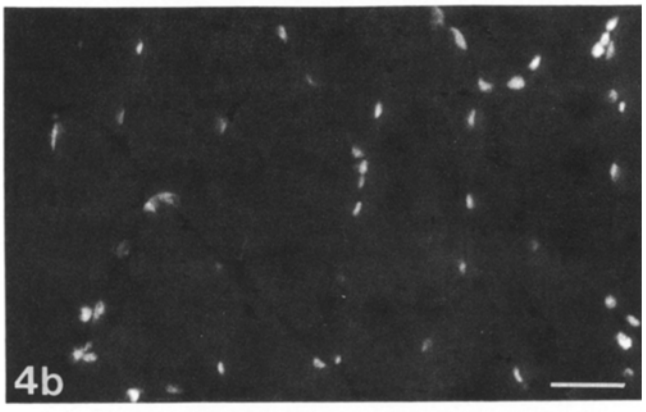
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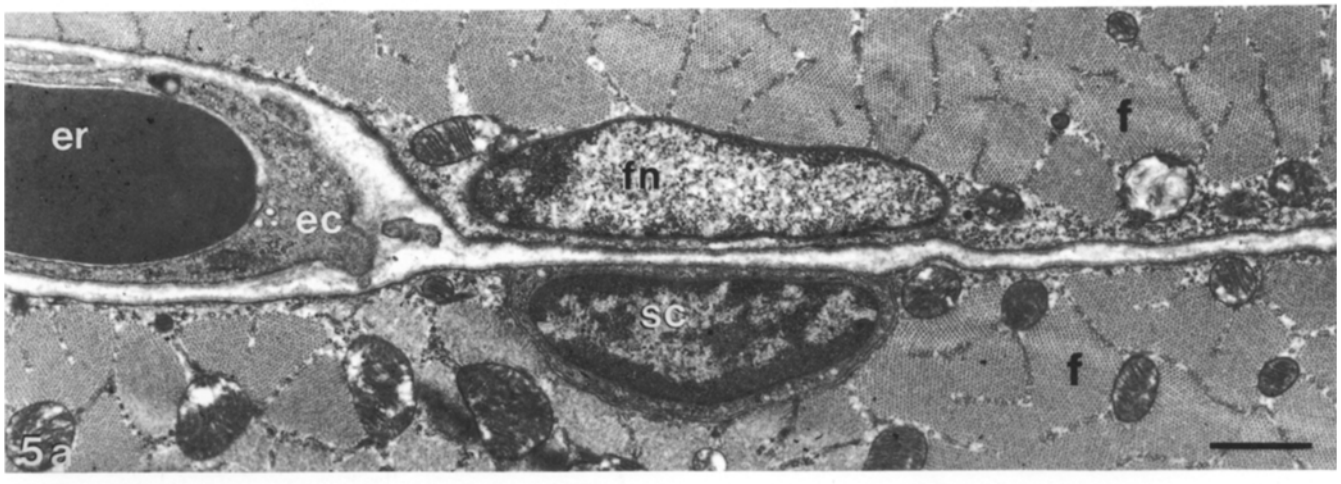
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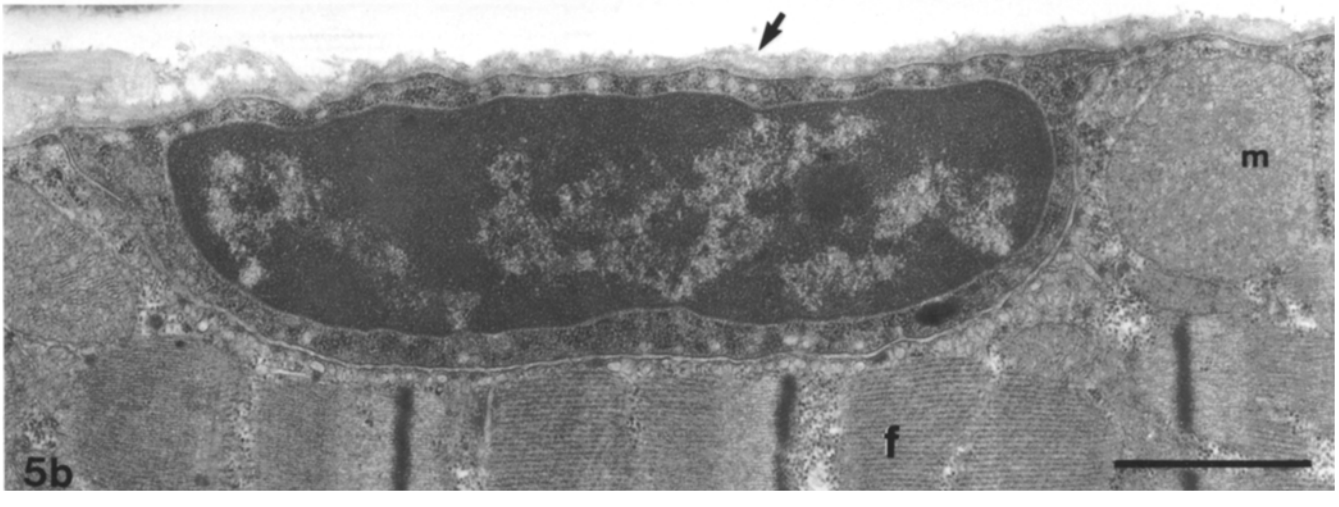
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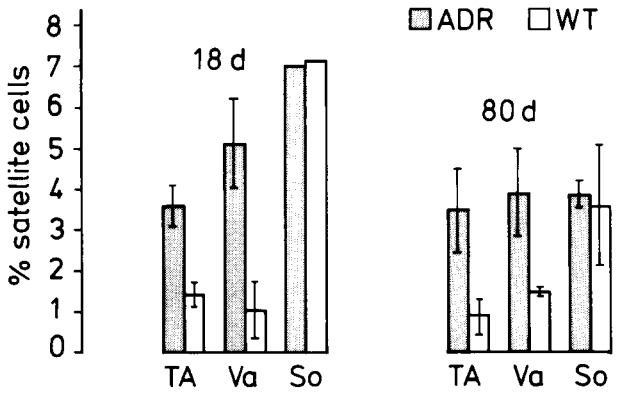

Fig. 6. Satellite cell densities deduced from quantitative electron microscopy of ADR and wild-type (WT) muscles. Percentage of satellite cells (=satellite cell nuclei $\times 100$/fibre nuclei) is increased by a factor of 3.3× in mutant anterior tibial (*TA*) and vastus (*Va*) muscles, whereas no clear difference is present in the purely oxidative soleus (*So*) muscle. Data taken from Table 2. Bar: minimum/maximum value

Table 1. Number of fibres, fibre nuclei and fibre-associated nuclei determined from trichrome-stained 8-µm cryostat sections from tibialis anterior of 80-d (animal I) and 82-d (animal II)-old mice. Total numbers given in brackets. *WT* wild-type

	Animal No.	ADR	WT	Ratio ADR/WT
Total nuclei per 0.0015 mm^3 sectional volume	I.	170	98	1.7
Mean fibre-diameter	I.	44±7 µm	51± 7 µm	0.9
	II.	45±6 µm	49±10 µm	
Number of fibre-associated nuclei per mm fibre circumference	I.	12 (121)	7 (67)	1.56
	II.	14 (689)	10 (715)	

Possible signs of degeneration. The method used to count nuclei, i.e., acridine orange fluorescence, should also have revealed any degeneration and regeneration of muscle fibres (Miike 1983).

In accordance with previous investigations (Watts et al. 1978), no sign of fibre degeneration was observed. This was verified by staining for acid phosphatase. An intense staining for acid phosphatase indicated fibre atrophy in regions of muscles taken from MDX mice at the age of 4–6 weeks (cf. Dangain and Vrbová 1984). In ADR- as in control muscle, there was no positive staining for acid phosphatase above background. However, in very rare cases ($\leq$0.5%), single fibres of ADR muscle showed signs of fibre splitting, a phenomenon commonly held as a sign of regenerative processes (cf. Bradley 1979).

We have examined animals from day 9 onward and found no sign of fibre degeneration at the ultrastructural level, with the exception of one single degenerating fibre in a 80 d ADR tibialis anterior muscle (out of 4546 examined mutant fibres).

In addition, a small proportion of fibres with centrally situated nuclei indicative of immature or regenerated fibres were observed both in ADR- (ca. 2.7%) and in control muscle (ca. 1.8%). Fibres with central nuclei were preferentially located near the perimysium.

Discussion

Using a combination of cell dissociation, clonal cell culture, and light- and electron microscopy, we have observed elevated numbers of mononucleated cells and specifically of satellite cells in skeletal muscle of a mouse mutant with hereditary myotonia.

The higher density of non-fibre nuclei in the ADR mutant could be caused by a smaller average diameter of muscle fibres (like in slow red muscle) and/or a higher density of blood vessels. Indeed, the mean fibre diameter in ADR tibialis anterior muscle is lower (45 µm) than that of control animals (59 µm; cf. Table 1). However, this difference is too small to explain the observed differences in cell yields, assuming a proportionality of numbers of mononucleated cells to fibre surface.

Satellite cells may occur in increased numbers as a result of either (1) the incorporation of fewer satellite cells into the muscle fibre or, (2) a previous activation by degenerative/regenerative processes, as in Duchenne dystrophy of man or, (3) an intrinsically larger number of quiescent satellite cells because of a different overall fibre-type composition. The latter can be demonstrated when comparing the soleus and extensor digitorum longus of the rat (Gibson and Schultz 1982) and is confirmed by this study in the mouse (Fig. 6; Table 2).

At best sporadic signs of degeneration have been observed in ADR muscle, and these are in no way comparable to those seen in X-linked muscular dystrophy of the mouse, the mutation studied for comparison. Furthermore, there is no increase in the proportion of satellite cells showing ultrastructural signs of proliferation in ADR muscle.

For chronically stimulated rabbit fast muscle, a set of

Fig. 3a, b. Differentiation of myogenic clones from 80 d ADR vastus muscle in culture. **a** Myogenic clone after 5 days in growth medium: only mononucleated myoblasts are present. **b** Myogenic clone at day 12 of culture, 16 h after change to fusion medium: myoblasts have started to fuse to myotubes (*arrowheads*). Haematoxylin/eosin-staining; ×100; scale bar: 100 µm

Fig. 4a, b. Number and distribution of nuclei in mutant and control muscle. Within frame shown, the number of nuclei is 1.7 times higher in (**a**) ADR than in (**b**) wild-type specimen. 80-d tibialis anterior; acridine-orange staining of 8-µm cryostat section; epifluorescence optics; ×500; scale bar: 20 µm

Fig. 5a, b. Satellite cell and muscle fibre in 80 d tibialis anterior of ADR. **a** Nucleus of quiescent satellite cell (*sc*) displays high heterochromatin content in comparison to fibre nucleus (*fn*). *er* erythrocyte; *ec* endothelial cell of capillary; *f* muscle fibre; ×13600; scale bar: 1 µm. **b** Satellite cell enclosed within basal lamina (*arrow*) of muscle fibre (*f*) with poorly developed organelles indicating inactive state. *m* mitochondrium; ×25800; scale bar: 1 µm

Table 2. Electron-microscopical evaluation of satellite-cell density in tibialis anterior (*Tib. ant.*) and vastus muscle of 18 d- and 80 d-old mice. Pooled data show that the number of satellite cells is increased by a factor of 3.3 × in fast muscle of the ADR mutant, but unchanged in the soleus muscle (cf. Fig. 6). Each result is based on 2–4 individual counts with absolute numbers given in brackets. *SCN* satellite cell nuclei; *FN* fibre nuclei; *F* fibres

	Animal No.	ADR		Wild-type	
		SCN/FN	SCN/FN/F	SCN/FN	SCN/FN/F
Tib.ant.	III.	4.1%	(9/218/292)	1.7%	(3/181/263)
	IV.	3.1%	(12/394/621)	1.1%	(5/435/687)
		$\bar{x}=3.6\%$		$\bar{x}=1.4\%$	
18d Vastus	III.	4.0%	(11/273/348)	0.3%	(1/359/459)
	IV.	6.2%	(25/401/602)	1.7%	(13/764/1223)
		$\bar{x}=5.1\%$		$\bar{x}=1.0\%$	
Tib.ant.	V.	4.5%	(13/288/466)	1.3%	(3/235/321)
	VI.	2.4%	(14/586/686)	0.4%	(1/255/479)
		$\bar{x}=3.5\%$		$\bar{x}=0.9\%$	
80d Vastus	V.	5.0%	(11/219/337)	1.5%	(3/196/273)
	VI.	2.8%	(8/288/380)	1.4%	(4/291/443)
		$\bar{x}=3.9\%$		$\bar{x}=1.5\%$	
Fast muscle		$\bar{x}=4.0\%$		$\bar{x}=1.2\%$	
18d Soleus	IV.	7.0%	(16/230/312)	7.2%	(32/447/513)
80d Soleus	V.	4.2%	(8/189/256)	5.1%	(8/157/179)
	VI.	3.5%	(6/172/246)	2.1%	(4/189/209)
		$\bar{x}=3.9\%$		$\bar{x}=3.6\%$	
Soleus		$\bar{x}=5.5\%$		$\bar{x}=5.4\%$	

changes in muscle phenotype has recently been described (Maier et al. 1986). Similar changes including a uniform oxidative phenotype and elevated numbers of mononucleated cells are found in the ADR fast muscle. Because atrophying myofibres, infiltration by phagocytotic cells, replicating satellite cells and myotubes have been observed in the case of chronically stimulated muscle, the elevated numbers of mononucleated cells have been attributed to ongoing degeneration/regeneration caused by the stimulation (Maier et al. 1986). These features have not been observed in our investigation of ADR muscle, which included animals from day 9 onward. Thus, an activation of the satellite cell population by degenerative/regenerative processes does not seem a likely explanation for the elevated levels of satellite cells in ADR muscle.

From enzyme-histochemical observations, it is known that ADR fast muscle displays a uniform 'red' character rather than the usual white/red 'checkerboard' pattern of wild-type fast muscle (Watts et al. 1978; Füchtbauer et al. 1985; Reininghaus et al. 1986). In the case of anterior tibial and vastus muscle this is a result of a change from a glycolytic to an oxidative character observed in about 50% of the ADR fibres. In view of the observation that 'red' muscles have an intrinsically higher number of satellite cells (cf. Fig. 6), one could argue that the fibre transformation in ADR shifts the satellite cell densities of partially glycolytic muscles towards those of the completely oxidative soleus. This possibility is consistent with our data (Table 2; Fig. 6).

Satellite-cell densities decrease during postnatal maturation of muscle (man: Schmalbruch and Hellhammer 1976; rat: Gibson and Schultz 1983; mouse: Schultz 1974; Snow 1977; this study, cf. Fig. 6). Thus it might be possible that the myotonic muscle is delayed in maturation and that high satellite-cell densities represent one aspect of its relatively immature state. This hypothesis is supported by the clonal cell culture experiments showing that myogenic clones from ADR muscle proliferate like clones from younger wild-type animals. However, an important control, i.e. clonal cultures from normal soleus, has not been done because of the scarcity of the material. The curves of cell yields versus age (Fig. 1) do not suggest a developmental delay in ADR muscle. However, the capacity of the myogenic cells to produce large clones is retained longer in the mutant than in the control (cf. Fig. 2a). The results of cell yields, clonal cell culture, and of quantitative electron microscopy generally are in agreement regarding the satellite-cell population. However, at the age of 18 days, larger numbers of myogenic cells (compared with adult muscle) are found after cell dissociation than by analysis at the ultrastructural level. This discrepancy, which does not affect the described differences between myotonic and normal muscle, cannot be explained at present.

In conclusion, the increased satellite-cell density in mouse myotonia is not a result of a compensatory response to fibre degeneration, but arises rather from a shift in fibre-type and/or an immature state of the diseased muscle.

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