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Formation of new muscle fibres and tumours after injection of cultured myogenic cells

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Summary

We examined the effects of implantation of cultured myogenic cells from a permanent cell line into soleus muscles of histocompatible adult mice. Myogenic cells (10^6 or 10^4) were implanted into intact muscles, muscles frozen with liquid nitrogen, paralysed with botulinum toxin or reinnervated after long-term (seven months) denervation. Formation of numerous muscle fibres in myogenic cell-injected muscles raised the total number of fibres up to ten times above control by four weeks. Larger effects were found in freeze-damaged than in paralysed muscles. The new fibres had small calibers, considerable length ($>$ 1.3 mm, maximum distance over which serial sections were made), were multinucleated and were oriented parallel to the large-diameter fibres of the host muscles. In some experiments β-galactosidase, introduced into myogenic cells via retroviral transfection, was detected in small and large muscle fibres 4–20 weeks after implantation, indicating survival of the grafted cells and formation of mosaic (host-donor) and new fibres of donor origin. Muscle weight increased significantly and, rather surprisingly, a parallel increase was found in isometric tetanic tension of isolated nerve-muscle preparations; thus tension per mg muscle tissue was not different from normal. By eight weeks reduction of acetylcholine sensitivity and down-regulation of neural cell adhesion molecule to normal were observed, indicating that synaptic transmission at the new fibres was mature. After different periods of time (5–20 weeks, depending on the subclone used) tumours developed in most but not all injected limbs (37 out of 39). The tumours were destructive to the muscles and were classified as *rhabdomyosarcomas*. Prior to tumour formation, neural cell adhesion molecule positive cells reappeared in the muscles; since the myogenic cells initially produced differentiated muscle fibres, it appears that malignant growth is induced by factors *in vivo*. Thus, at present the outcome of such implantation is unpredictable.

Introduction

In experiments on reinnervation of mouse muscle following long-term denervation we observed that recovery of soleus muscle force is delayed and incomplete (Irintchev *et al.*, 1990). We speculated that in part this might be due to exhaustion of the satellite cell pool during the denervation period as shown by Anzil and Wernig (1989) for long-term denervated frog muscles. Thus we tested whether injection of myogenic cells (MCs) grown *in vitro* could contribute to the *functional* recovery of diseased muscles in adult animals.

It has been shown that injection of normal myoblasts into dystrophin-deficient muscles converts some of the host fibres to dystrophin-positive as a result of cell fusion and expression of the donor gene (Partridge *et al.*, 1989, Karpati *et al.*, 1989, Law *et al.*, 1990b, for a review see Partridge, 1991). Positive functional results have been reported after myoblast injections into muscles of dystrophic mice (*dy/dy* mutant, Law *et al.*, 1988a,b; 1990a) and cell implantations already have been attempted as a therapeutic approach in humans ('myoblast transfer therapy', Law *et al.*, 1990b).

We report on the fate of MCs from a permanent cell line injected into cryo-damaged, botulinum-paralysed or long-term denervated and reinnervated muscles, and on positive functional effects of MC injection. We also find, however, that tumours regularly develop in limbs injected with MCs. A brief account of the experimental results has been published (Irintchev *et al.*, 1991).

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Materials and methods

Myogenic cell cultures

Primary cultures were established according to the method of Blau and Webster (1981) from thigh muscles of 2- to 3-week-old Balb/c mice. Myoblasts were cultured in Waymouth's medium supplemented with 10% foetal calf serum in 5% CO_2 atmosphere. After five passages cloning was performed and one of these clones (designated as F8B) appeared to be transformed into a permanent line (stable for over 60 passages). F8B cells were transfected with plasmid vectors pRSVneo for Neomycin resistance (Gorman, 1985) and pCH110 (Pharmacia, LKB) containing the β-galactosidase gene (LacZ) of *E. coli* (Gorman *et al.*, 1982; Kruczek & Doerfler, 1983; Knebel *et al.*, 1985). Different transfected subclones (27, 34, 61) and non-transfected F8B cells were used in the experiments. Prior to implantation, the transfected cells were tested histochemically for β-galactosidase (β-Gal) activity (Sanes *et al.*, 1986) and found positive, while no β-Gal activity was detectable later on frozen sections from MC-injected muscles. In some animals myoblasts from the C2C12 line, transfected retrovirally with the β-Gal gene (C2nlsBAG cells, produced and provided by Simon M. Hughes, Marilyn Travis and Helen M. Blau, Stanford) were used. Most of these cells contain both cytoplasmic and nuclear β-Gal.

Animal treatment and cell injection

Female CD2F1 hybrid mice (Balb/c ♀ × DBA ♂, Zentralinstitut für Versuchstiere, Hannover) were used. These mice are histocompatible with the F8B MCs, which orginate from Balb/c mice, for the MHC H-2 locus (H-2^d). Some CBA/J mice which are histoincompatible (MHC H-2^k) were also used. C2nlsBAG cells (originally derived from C3H mice) were implanted into muscles of C3H or CBA/J mice (both MHC H-2^k, obtained from Charles River Wiga, Sulzfeld, Germany). The animals were kept in standard cages in an environmentally controlled room and received water and food *ad libitum*. Animal treatments were performed under deep halothane/nitrous oxide (used for repeated nerve freezing) or pentobarbitone anesthesia (Nembutal, 60 mg per kg body weight; cell implantation, muscle-nerve preparation). All experiments were performed in accordance with the German laws for care and protection of experimental animals.

In one group of mice ($n = 10$) the right sciatic nerve was repeatedly frozen at 2-week intervals to denervate muscles over a period of seven months (see Irintchev *et al.*, 1990). Following a reinnervation period (time after the last freezing) of approximately nine months, MCs (subclones 27 and 34) were implanted into the right soleus muscle ($1.6–6.4 \times 10^6$ cells in phosphate-buffered saline, PBS, per muscle) at three points along the length of the muscle using glass micropipettes.

In a second group ($n = 15$), muscle paralysis and axonal sprouting were induced by injection of botulinum toxin Type A. The toxin (10 ng ml^{-1} PBS) was injected into the calf muscles with a microsyringe at a dosage of 25 pg per g body weight. MCs (subclone 61, approximately 10^4 or 10^6 cells in 4 μl PBS) were implanted with 26S gauge needles (outer diameter 0.47 mm) on 5 or 10 μl Hamilton microsyringes into the paralysed muscles one day later. Muscles were penetrated along the longitudinal axis in a caudo-rostral direction and the cells were injected while withdrawing the needle.

In a third group ($n = 15$) the right soleus muscle was frozen along its whole length with a metal rod (tip area of approximately 1 mm^2) precooled in liquid nitrogen. Serial cross-sections from three muscles removed 1–3 days after freezing showed damage to all muscle fibres. When freezing, care was taken not to cause mechanic damage to the soleus nerve or the blood supply. MCs were injected as described above for paralysed muscles immediately after thawing.

Control groups for each type of experiment consisted of age-matched animals which received the same treatment as the experimental groups but, instead of MC suspension, PBS only was injected in the same volume.

In vitro tension measurements

Isometric tension measurements were performed *in vitro* as described previously (Badke *et al.*, 1989; Irintchev *et al.*, 1990). The intact left and the experimental right solei were isolated with the nerve and directly- and indirectly-elicited twitch and tetanic tensions (20, 50 and 100 Hz) were measured at optimum pretension in aerated Tyrode's solution at 25° C. Acetylcholine-evoked muscle contracture (5 and 50 mg ACh perchlorate per litre Tyrode's solution) were also measured.

After the contraction measurements the muscles were blotted dry and weighed. The left and right soleus muscles from the same animal were then fixed side by side on a piece of liver (see Irintchev *et al.*, 1990) and frozen as a single block in isopentane precooled with liquid nitrogen.

Histological procedures

Frozen cross-section (10 μm) from soleus muscles were cut in series at two defined levels about 2 mm apart: at the beginning of the intracapsular region of the most proximal muscle spindle (where about half of the fibres are present in a single cross-section) and in the endplate region (where all muscle fibres are present, Vaughan & Goldspink, 1979). Sections were stained with 1% Toluidine Blue/1% Borax and for myofibrillar ATPase after acid (pH 4.3) and alkali (pH 10.3) preincubation (Butler & Cosmos, 1981; Guth & Samaha, 1970), ACh esterase (AChE) (Karnovsky & Roots, 1964), ACh receptors (AChR) (rhodaminated α-bungarotoxin), β-Gal (Sanes *et al.*, 1986), laminin and the neural cell-adhesion molecule (N-CAM) (indirect immunofluorescence), and combined esterase–neurofilament staining (see below). Immunofluorescence stainings were performed with characterized rat monoclonal antibodies (anti-mouse laminin–LAM-1, ICN Biomedicals and anti-mouse N-CAM–clone H-28 developed by Dr C. Goridis, Marseille, a gift from Dr W. Wille, Köln). Sections (5 μm for laminin, 10 μm for N-CAM) were incubated with the primary anti-body for 1 h (laminin, room temperature) or overnight (N-CAM 4° C) at dilution of 1:100. Streptavidin-biotin detection system (Jackson Immunoresearch) was used to visualize immunoreactive sites: anti-rat IgG–biotin (1:200, 1 h at room temperature) followed by streptavidin-FITC (1:200, 30 min). Laminin immunostaining was performed on sections stained for myofibrillar ATPase activity (pH 4.3 or 4.6). A

combined esterase–neurofilament staining was used to verify that the endplates in the experimental muscles were innervated. Esterase was visualized in cryostat sections fixed in Baker's formol-calcium by a direct azocoupling method using Naphthol-AS-D-acetate as substrate and hexasonium-*p*-rosaniline as coupler (see Lojda *et al.*, 1976, incubation time 10 min at room temperature). Esterase-stained sections were subsequently incubated over night at 4° C with a diluted (1:1000) monoclonal mouse antibody iC8 to neurofilaments (Vitadello *et al.*, 1986, kindly provided by Fidia Research Laboratories, Abano Terme, Italy), followed by peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch, 1:200, 1 h at room temperature) and peroxidase staining using 4-chloro-1-naphthol as substrate. With this procedure synaptic esterase is visualized in a red colour, axons are stained dark blue. There is a good distinction between the two colours even when axons appear entirely surrounded by the esterase staining at endplates.

Overlapping videoprints from ATPase-stained sections (pH 4.3) were glued together to reconstruct the whole cross-sections (final magnification × 380 or × 610) and used as 'hard' copies for quantitative evaluations. Total numbers of muscle fibres and fibre type distributions were determined in cross-sections from the endplate regions as described previously (Wernig *et al.*, 1990, 1991b). Mean orthogonal diameters (mean of the longest axis and a short one passing through the middle of the longest at right angle) were measured in some muscles with a digitizing tablet (Wernig *et al.*, 1990). All fibres in a cross-section were evaluated.

Statistical analysis

Differences between means of two groups was tested with independent or paired Student's *t*-test. For multiple comparisons between groups, Tukey's test was performed if analysis of variance (ONEWAY) had proved significant effects of the treatments (groups tested: experimental PBS-injected, experimental MC-injected and intact contralateral muscles). Significance level was set at 5%

Results

FORMATION OF NEW MUSCLE FIBRES IN MC-INJECTED MUSCLES

Numerous small muscle fibre profiles were observed in cross-sections of soleus muscles 2–20 weeks after injection of F8B MCs (Figs 1 and 8). The small muscle fibres most likely originated from the injected MCs since small fibres were absent or infrequent in reinnervated, paralysed or frozen PBS-injected muscles (Figs 2 and 8, Table 1), and in some muscles (see below) small fibres contained β-Gal, the marker introduced into myogenic cells (Figs 3 and 4). By 4–20 weeks the total number of fibre profiles in the endplate region of MC-injected muscles was considerably higher than in intact contralateral or treated control muscles (Table 1, see also Fig. 19 for change in fibre numbers with time). A small but significant increase in the number of muscle fibre profiles was also detectable in frozen and PBS-injected solei (Table 1, Fig. 8a) probably as a result of imperfect regeneration (split and/or branched fibres; see Ontell & Feng, 1981, Ontell *et al.*, 1982,

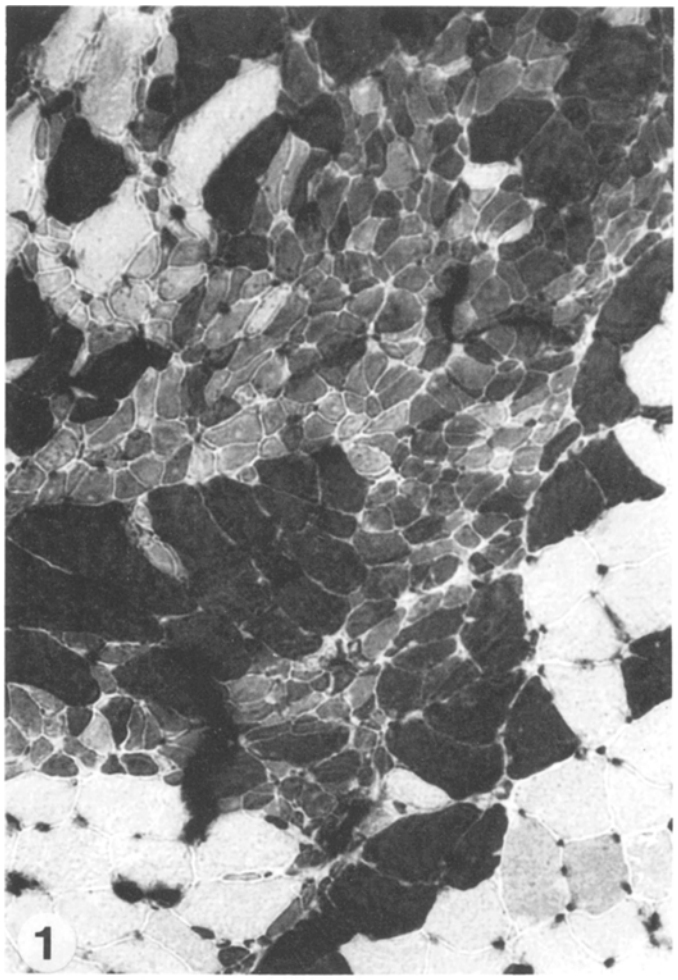
Table 1. Number of muscle fibres in cross-sections through the endplate region of MC-injected and control soleus muscles 4–20 weeks after treatment.

	Experimental muscles	*Intact contralateral muscles*
	Paralysed – PBS	
mean ± SD	776 ± 61	831 ± 109
range	671 – 854	618 – 966
n	7	7
	Paralysed – MCs	
mean ± SD	3718 ± 1184*	793 ± 74
range	2307 – 5594	725 – 911
n	6	6
	Frozen – PBS	
mean ± SD	922 ± 72*	735 ± 30
range	820 – 1050	697 – 780
n	7	6
	Frozen – MCs	
mean ± SD	6344 ± 2994*	792 ± 71
range	3068 – 9321	692 – 846
n	6	4

* significantly different ($P < 0.05$, paired *t*-test) from intact contralateral muscles.

Figs 1–2. Frozen cross-sections (10 μm) from reinnervated soleus muscles five weeks after MC- (Fig. 1) or PBS-injection (Fig. 2). Numerous small fibres (mean diameter 9.5 μm ± 3.6 SD, number of evaluated fibres = 605) surround large diameter fibres (32 ± 6.7 μm, $n = 113$) in the MC-injected muscle (Fig. 1). Such small fibres are not present in the PBS-injected control muscle (Fig. 2). Stained for myofibrillar ATPase activity (preincubation at pH 10.3). Scale bar = 40 μm for both figures.

Figs 3–4. β-Gal staining in soleus muscles of a CBA/J (Fig. 3, incubation time 5 h) and a C3H mouse (Fig. 4, incubation time 18 h) studied at 8 and 19 weeks, respectively, after freezing and injection of C2nlsBAG myoblasts (cells transfected retrovirally for both cytoplasmatic and nuclear β-Gal). Both cytoplasmic and nuclear (arrow) staining are seen in small and large fibre profiles at eight weeks (Fig. 3). At 19 weeks (Fig. 4) nuclear staining is detectable in many fibres while the cytoplasmic activity has almost completely disappeared. Scale bar = 20 μm for both figures.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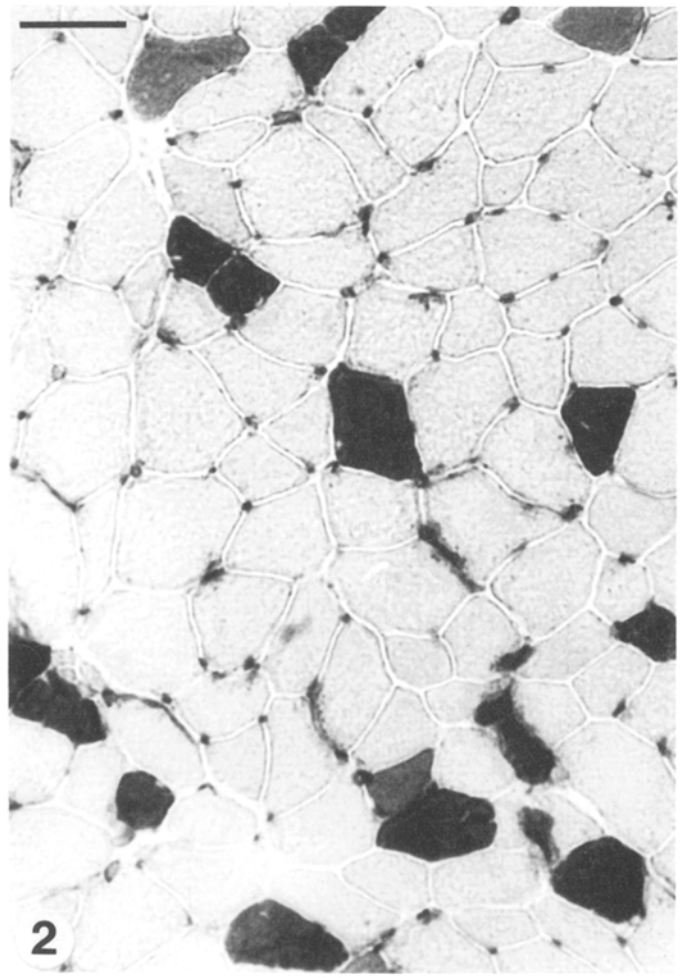
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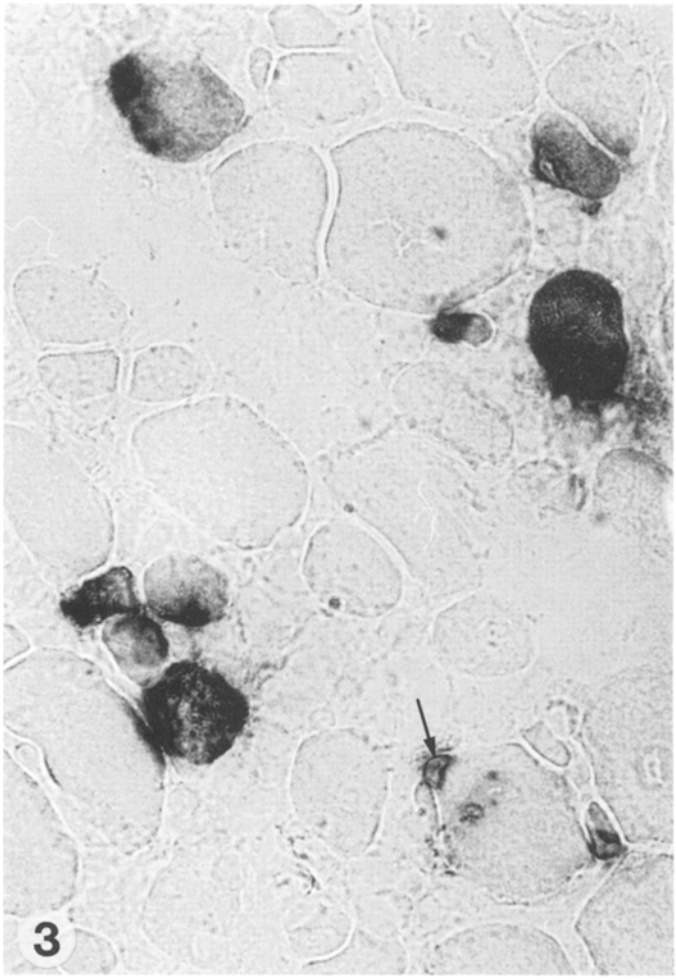
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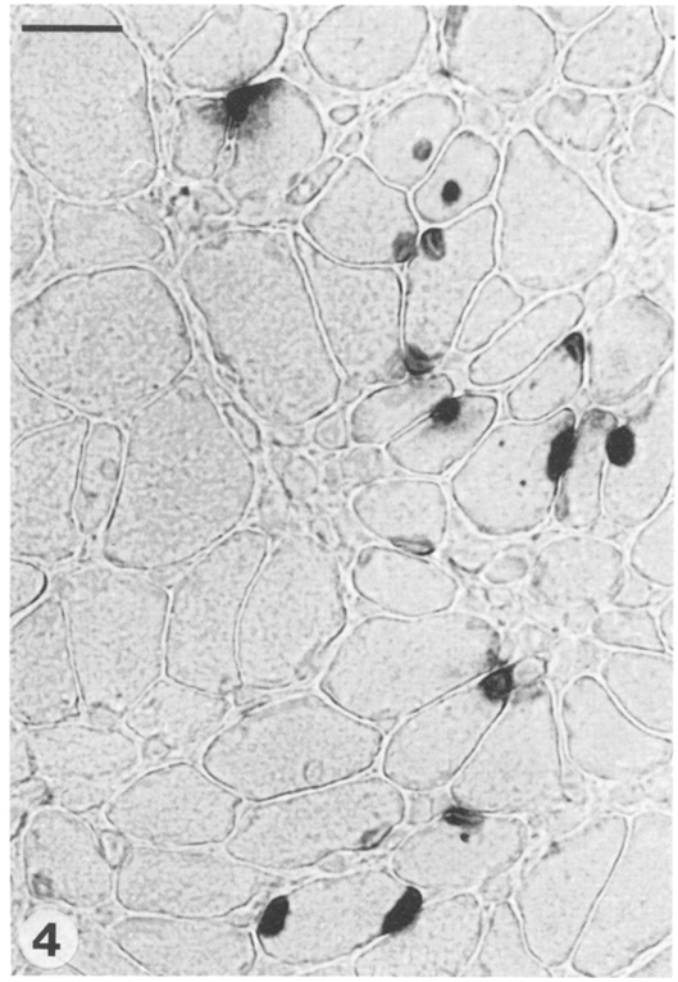
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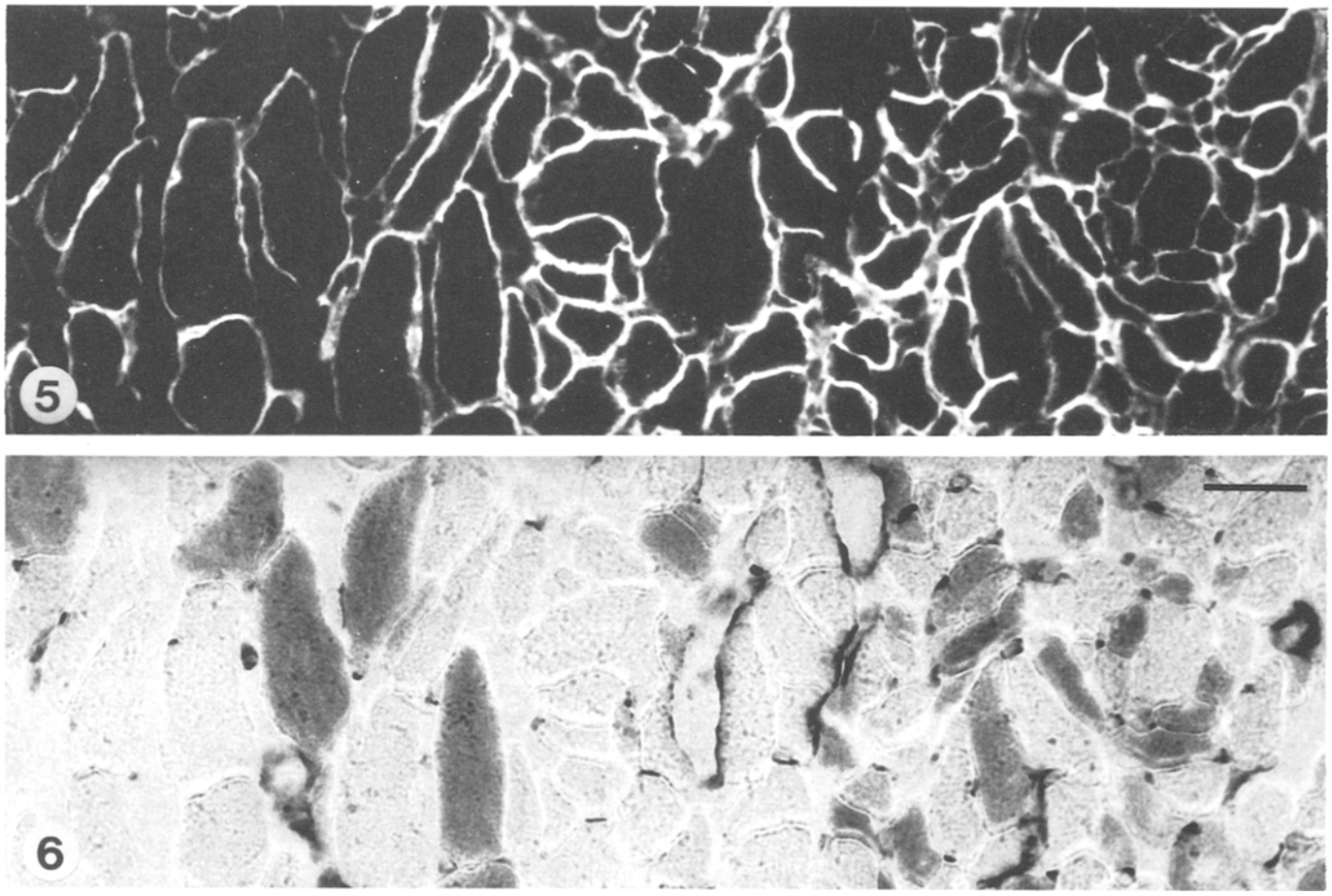


Figs 5–6. Double staining for myofibrillar ATPase (pH 4.3, Fig. 6) and laminin (Fig. 5, FITC fluorescence) of a cross-section shows the presence of basal lamina sheaths around small and large fibres eight weeks after injection of MCs in a paralysed muscle. Scale bar = 25 μm.

Wernig *et al.*, 1990, for a review see Schmalbruch, 1985).

While cells from the F8B subclones stained for β-Gal in the culture dish, no activity could be detected histochemically in sections from MC-injected muscles 2–5 weeks post-injection. This was obviously due to disappearance of the plasmid-induced gene or down-regulation of its expression after differentiation *in vivo*, since after injection of retrovirus-transfected MCs (C2nlsBAG cells) into C3H and CBA/J mice many small and large muscle fibres stained positive for β-Gal (Figs 3 and 4).

Several lines of evidence showed that the new cells in MC-injected muscles are fully differentiated muscle fibres. First, all small profiles were positively stained for myofibrillar ATPase after alkali and/or acid pre-incubation (see below). Secondly, a basal lamina sheath was found around every fibre profile (Figs 5 and 6). Further, the small fibres had considerable lengths (> 700 μm in Fig. 7 and > 1300 μm in 39 of 48 fibres smaller than 15 μm in a similar muscle studied 16 weeks post-injection, data not shown; see also below for further estimations). Finally fibres were innervated and considerably contributed to muscle force (see below).

MUSCLE INNERVATION

Staining for AChE (Fig. 9), AChR (Figs 10–12) and neurofilaments (Figs 13–15) showed that the new muscle fibres were innervated by 4–8 weeks. In frozen, but not in paralysed, MC-injected muscles at 4 weeks the whole cross-sectional surface of individual small fibres was stained with rhodaminated α-bungarotoxin due to the presence of extrasynaptic receptors (Figs 10 and 11). After longer periods extrasynaptic staining disappeared completely (Fig. 12).

To assess the maturity of this innervation we used immunostaining for the N-CAM. This molecule is expressed by aneurally developing myotubes and is down-regulated with maturation of innervation during early postnatal development but re-expressed in denervated muscle fibres (Covault & Sanes, 1985, 1986). No cells stained positively for N-CAM in intact contralateral muscles and frozen or paralysed muscles four weeks after PBS injection. Neural-cell adhesion molecule positive small fibres were observed in frozen

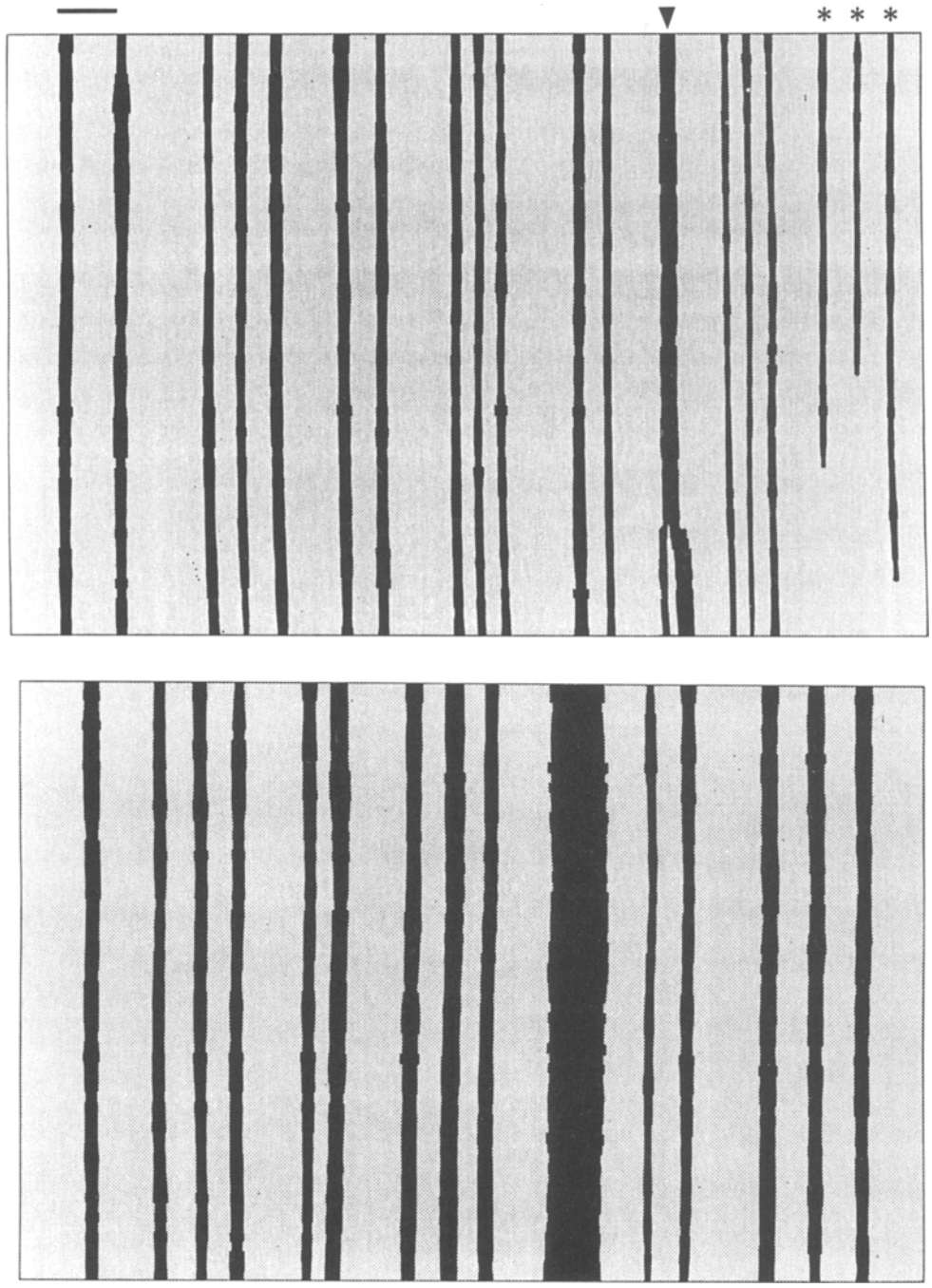


Fig. 7. Schematic reconstruction of muscle fibres in a frozen and MC-injected muscle four weeks after treatment. A group of muscle fibres (one large-diameter, lower panel, and 33 small-diameter fibres) were traced in 5 μm serial cross-sections stained for mATPase (pH 4.6) over a distance of 700 μm. Except for three fibres which ended distally (asterisks) all small fibres could be traced along the entire distance studied. Branching was observed only in one fibre (arrowhead). Reconstruction is based on the mean orthogonal diameters of the fibres determined in every second section. Scale bar = 40 μm.

MC-injected solei 4 weeks after the implantation (Fig. 16). The immunoreactivity was strongly reduced in two muscles and completely absent in three muscles after survival periods of 8–16 weeks (Fig. 17). However, at the time when tumours started to develop (see below), N-CAM positive cells were again detectable in MC-injected solei ($n = 3$, Fig. 18). Paralysed MC-injected muscles contained fewer N-CAM positive fibres at four weeks post-injection which indicated a more rapid and complete innervation of new muscle fibres after paralysis with botulinum toxin (see also above for similar findings with extrasynaptic receptors).

In sections from the proximal third of paralysed or

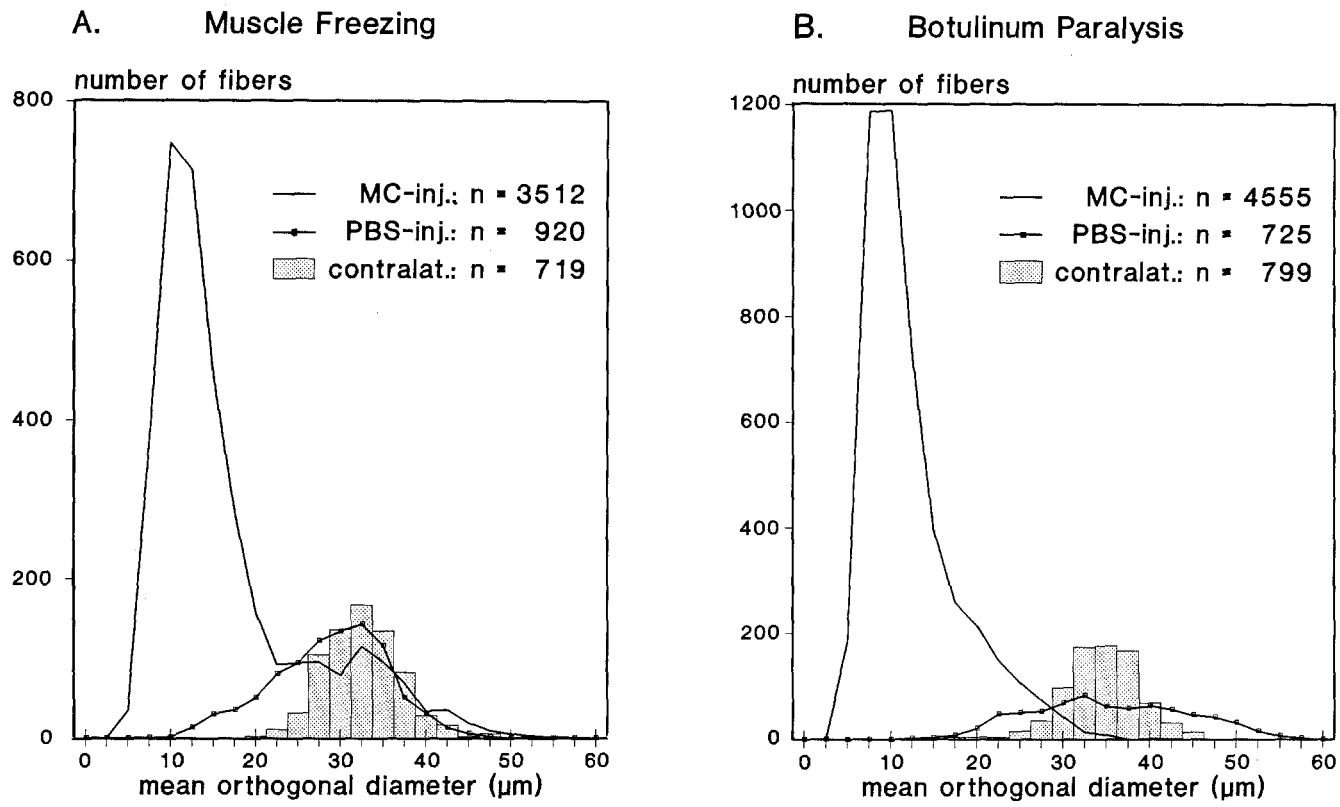


Fig. 8. Frequency distributions of fibre diameters in frozen (Fig. 8A) or paralysed (Fig. 8B) soleus muscles eight weeks after treatment. MC-injected muscles are compared with treated but PBS-injected and intact contralateral (to the PBS-injected) muscles. Each histogram represents data from one muscle; all fibre profiles (n) in complete cross-sections through the endplate region were measured.

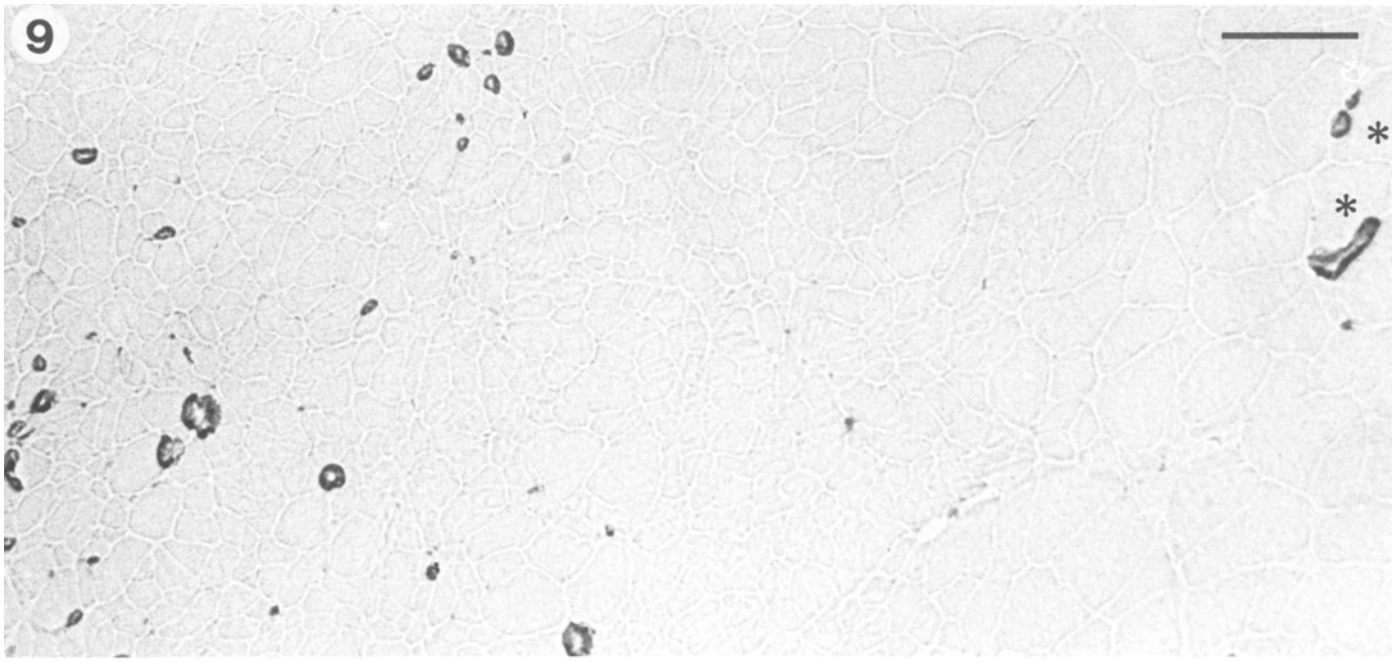
frozen and MC-injected muscles (see Materials and methods for level definition) most of the small fibres were N-CAM negative by eight weeks. Since no endplates were found in this region the small fibres are continuous over a distance of at least and possibly more than 2 mm (distance between levels of sectioning). If this were not the case and the fibres present in the proximal third of the muscle were disconnected from the endplate-bearing segment, the former should continue to express N-CAM (comp. Covault & Sanes, 1985, for expression of N-CAM in muscles denervated for more than eight weeks).

FIBRE TYPES IN MC-INJECTED MUSCLES

In paralyzed or frozen muscles injected with clone 61 the majority of the fibres were Type II at four weeks post-treatment (Fig. 19, range 2889–7636 fibres, compare with 141–294 in treated – paralysed or frozen and PBS-injected – control muscles, $n = 6$); there were substantial amounts of undifferentiated fibres (448–2083 *versus* 88–313 in treated control solei), while the absolute numbers of Type I fibres (271–531) were similar to treated control muscles (166–552). Thus, it appears that most of the new fibres initially express Type II characteristics. At two and four months post-injection total number of fibres and number of Type II fibres were significantly smaller than at four weeks (Fig. 19); at the same time the absolute number of Type I fibres was increased. Besides initial loss of muscle fibre profiles these results indicate ongoing remodelling of motor units with increasing predominance of Type I motoneurons (see Discussion).

Fig. 9. Acetylcholine esterase staining of synapses in a cross-section from a paralysed and MC-injected muscle four weeks after treatment. Numerous small fibres bear endplates (left side of the picture). In the upper right corner two large fibres with endplates (asterisks) are seen. Scale bar = 50 µm.

Figs 10–12. Acetylcholine receptor staining (rhodaminated α-bungarotoxin) of cross-section taken from two muscles at four weeks (Figs 10 and 11) and 16 weeks (Fig. 12) after freezing and MC-implantation. Fig. 10: in an area of the muscle containing small (right half) and large fibres (left half) numerous synapses are labelled; note the small size of the synapses on the small fibres and the extrasynaptic staining of small fibres on the right. Fig. 11: extrasynaptic and synaptic staining in small fibres from the muscle shown in Fig. 10 at higher magnification. Fig. 12: in an area containing small fibres synaptic but not extrasynaptic staining is visible. Sections shown in Figs 10–12 were stained simultaneously in the same staining solution. Scale bars = 40 µm.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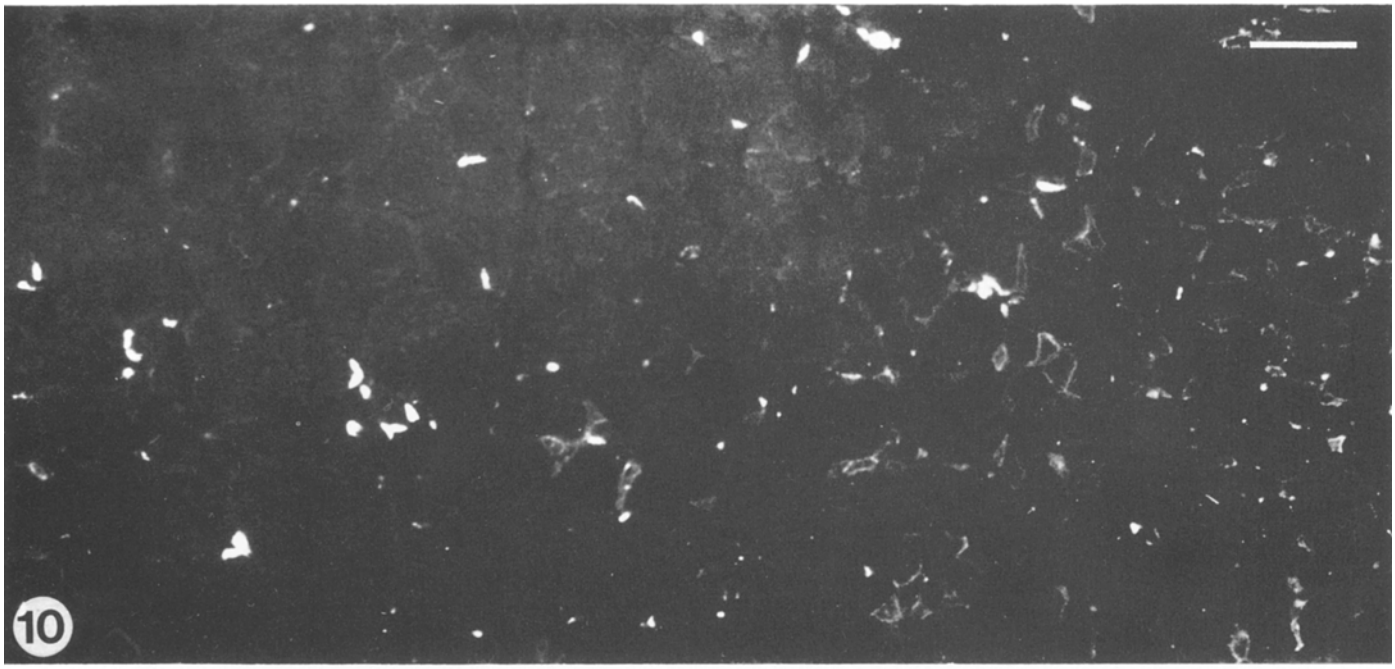
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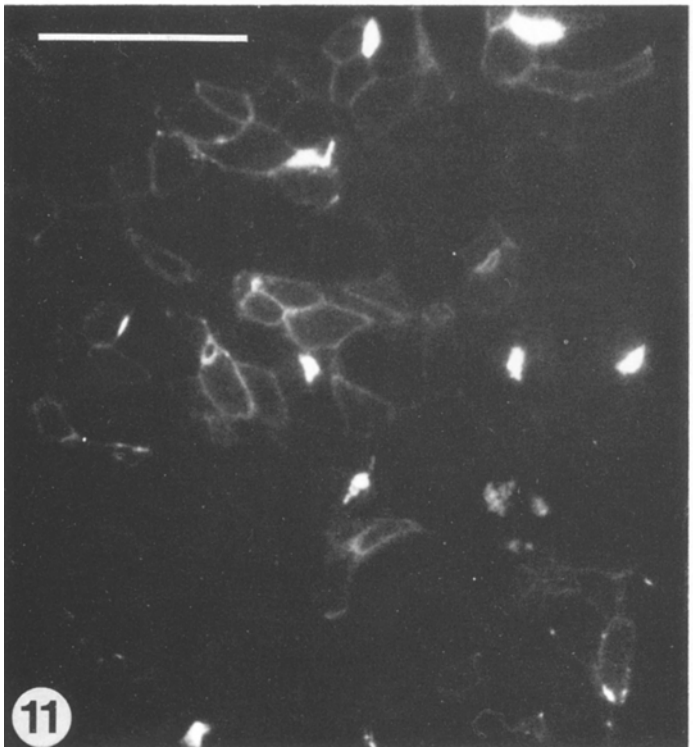
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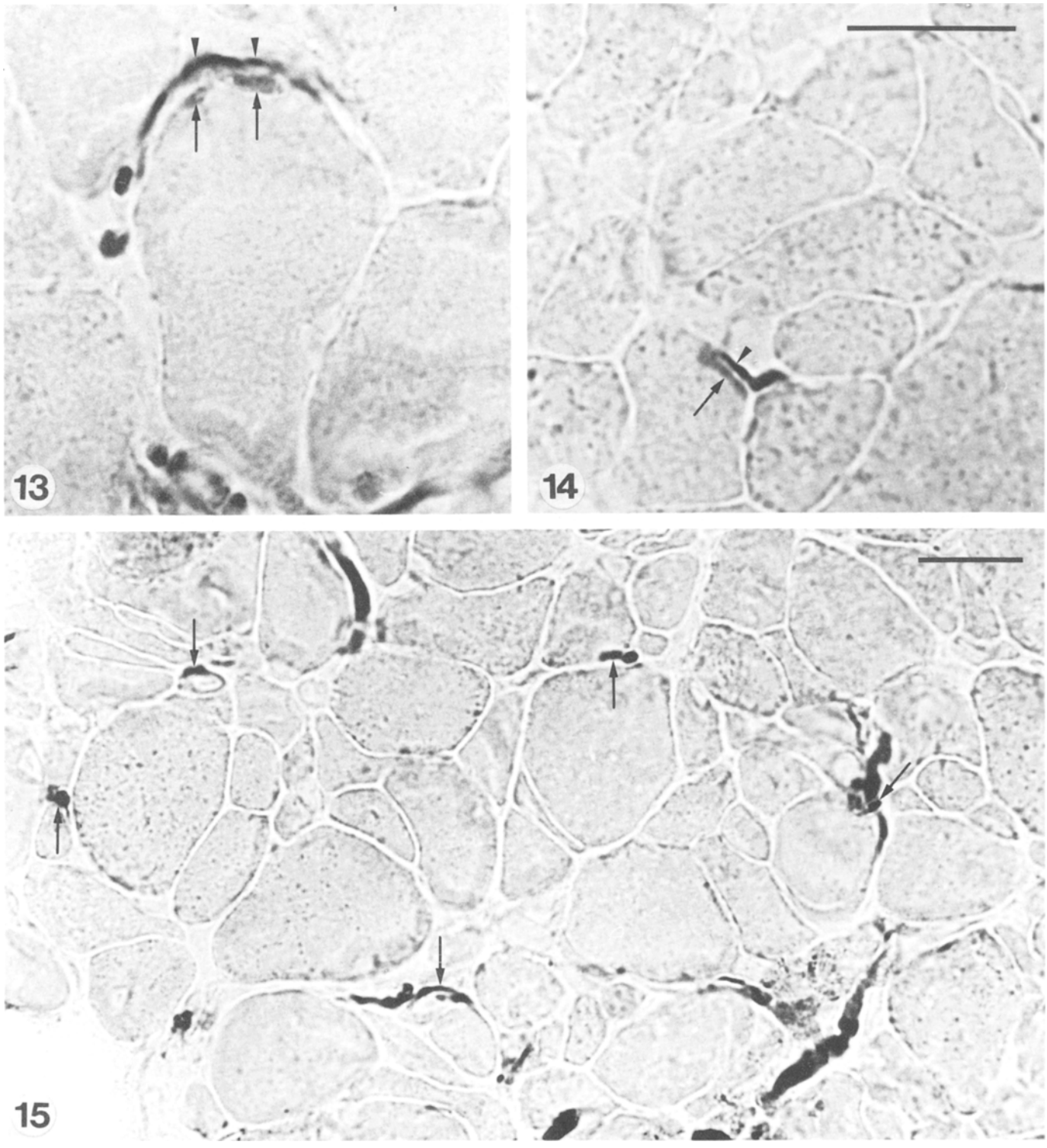
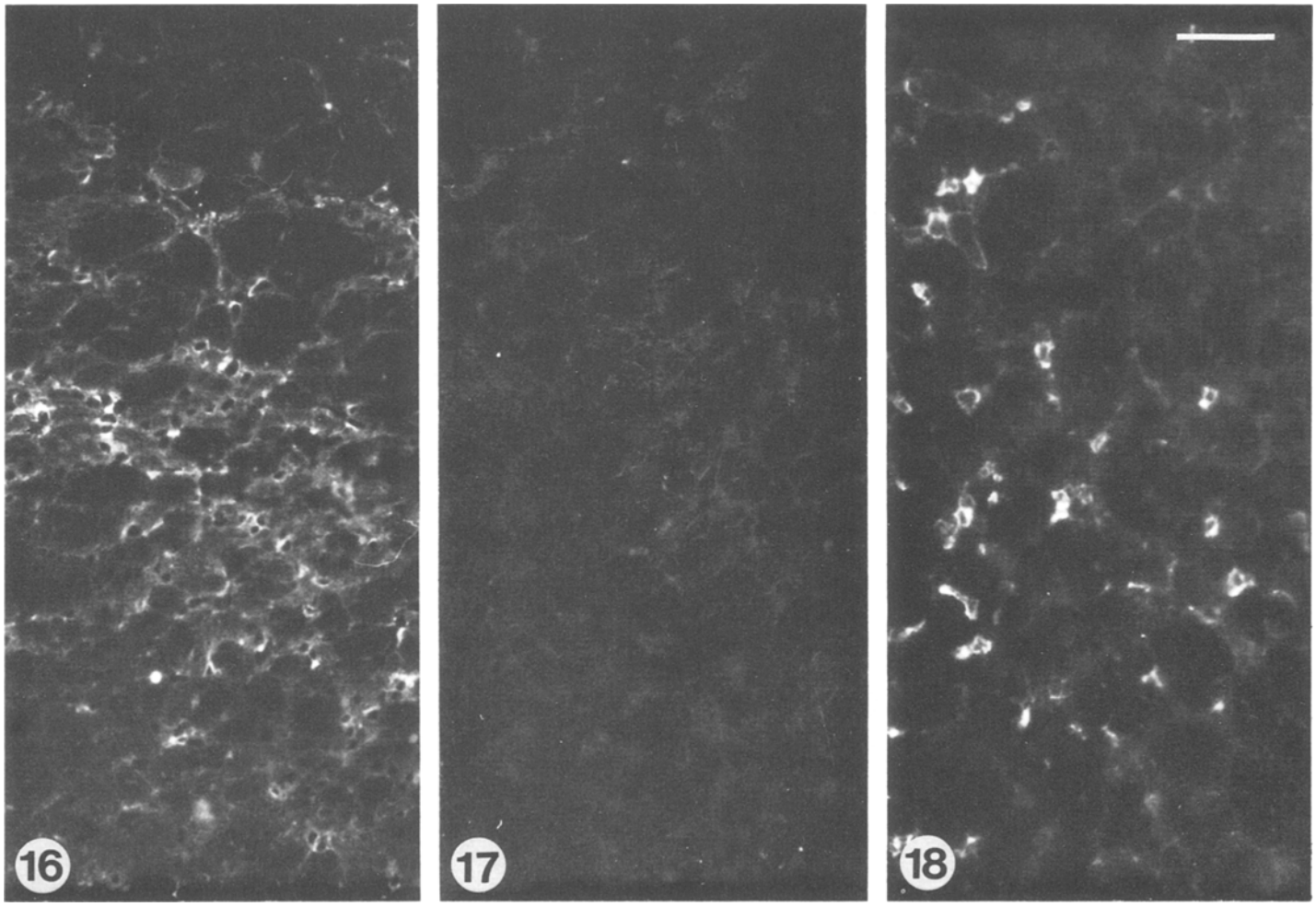


Figs 13–15. Combined esterase–neurofilament staining of cross-sections from the endplate regions of a paralysed and MC-injected soleus (Figs 14–15, studied eight weeks post-injection) and of the intact contralateral soleus of the same animal (Fig. 13). One selected synapse from each muscle with clear spatial deliniation of the esterase (arrows) and the axon terminal staining (arrowheads) are shown in Figs 13 and 14. An area of the MC-injected muscle which contains small and moderately large fibres is shown at lower magnification in Fig. 15. Endplates in which both esterase and neurofilament staining were seen are marked with arrows. Note the small diameter of the innervated fibres. Scale bars = 20 μm.



Figs 16–18. Neural cell adhesion molecule immunofluorescence staining of cross-sections from three frozen and MC-injected soleus muscles 4 (Fig. 16), 16 (Fig. 17) and 17 (Fig. 18) weeks after the treatment. At four weeks N-CAM immunoreactivity was detectable in small, but not large fibres (Fig. 16). With further maturation of innervation N-CAM immunostaining completely disappeared (Fig. 17). However, when tumours started to develop (areas in soleus containing polymorphic cells or more advanced stages when visible tumour masses were present in the limb) N-CAM immunoreactivity was again detectable in the muscles (Fig. 18). Scale bar = 40 μm.

FUNCTIONAL EFFECTS

Frozen muscles

PBS-injected frozen muscles recovered normal tetanic and specific tension and weight within four weeks post-freezing (Table 2) while twitch tensions remained consistently smaller than in the contralateral muscles 4–18 weeks post-treatment ($P < 0.05$, paired t-test). Acetylcholine-evoked contractures, which indicate the presence of extrasynaptic ACh receptors (see Irintchev *et al.*, 1990) were higher than in intact contralateral muscles at four weeks but not later (Table 2), while nerve-evoked tensions were not significantly smaller than directly evoked ones; this indicates complete innervation which is probably still immature at four weeks (see Badke *et al.*, 1989; Irintchev *et al.*, 1990).

The injection of MCs resulted in a large increase in maximum tetanic and twitch tension and muscle weight (Table 2), and surprisingly, specific tension (maximum tension per unit muscle weight) was not different from normal (Table 2). At four weeks a clear deficit in indirectly *versus* directly evoked tension (means 135 *versus* 247 mN, $P < 0.05$, t-test) was present, and ACh-evoked contractures were larger than normal (Table 2). Also typical for immature innervation (see Badke *et al.*, 1989), indirect but not muscle-evoked tetanus showed depression in the course of a 2 s stimulation (not shown).

At 8–18 weeks after MC-injection, not only the directly-evoked tension but also the indirect tetanic force were significantly larger than in frozen control and contralateral normal muscles (Table 2). The difference between nerve- and directly-evoked tensions present at four weeks has disappeared, as has elevated ACh-sensitivity, all indicating maturation of innervation.

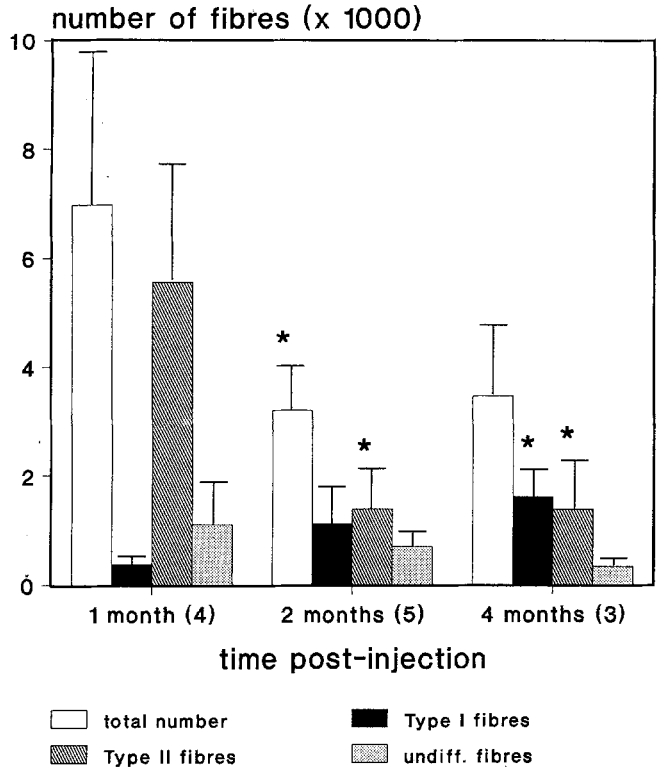


Fig. 19. Absolute total numbers of muscle fibres and numbers of Type I, Type II and undifferentiated fibres (means + SD) in frozen or paralyzed and MC-injected muscles one, two and four months after treatment (number of muscles studied are given in parenthesis). Asterisks indicate significant differences from the values at one month (Tukey's test).

Paralysed muscles

Paralysed PBS-injected muscles were not different from intact contralateral muscles at four weeks except for a higher ACh-sensitivity (data not shown). The effect of injection of MCs into paralysed muscles was markedly less effective than in frozen muscles. In six muscles studied 4–20 weeks post-implantation maximum tetanic tension was on the average 24% higher than in intact contralateral muscles (207 ± 19 *versus* 167 ± 22 mN, $P < 0.05$, paired t-test; compare with an overall increase of 63% in frozen muscles). A comparable increase was found in muscle weight (15.7 ± 2.0 *versus* 12.8 ± 1.8 mg, $P < 0.05$, paired t-test) and specific tension was normal. The deficits at four weeks in the nerve-evoked tension were smaller than in frozen muscles (data not shown).

Reinnervated muscles

Soleus muscles reinnervated after long-term denervation show incomplete recovery of muscle force and weight (Irintchev *et al.*, 1990). Similar results were obtained in the present study: four muscles, reinnervated after denervation periods of seven months, were studied 2–5 weeks after PBS injection; they were lighter (86% of contralateral muscles, 9.2 ± 1.6 mg *versus* 10.2 ± 2.0 mg, $P < 0.05$, paired t-test) and somewhat weaker (79% of control, 108 ± 10 mN *versus* 137 ± 32 mN, n.s.) than the intact contralateral solei. The effects of MC-injection could be monitored only at

Table 2. Contractile parameters and muscle weight of soleus muscles 4–18 weeks after freezing and injection with phosphate-buffered saline (PBS) or myogenic cells (MC), and of intact contralateral muscles

	Four weeks post-injection			*8–18 weeks post-injection*		
	Frozen PBS-injected	*Frozen MC-injected*	*Intact contralateral*	*Frozen PBS-injected*	*Frozen MC-injected*	*Intact contralateral*
Tetanic tension, P_o (direct stim., mN)	127 ± 12	$247 \pm 58^{*+}$	135 ± 22	164 ± 12	$257 \pm 58^{*+}$	171 ± 21
Tetanic tension (nerve stim., mN)	106 ± 35	135 ± 29	130 ± 23	$157 \pm 8^{¶}$	$223 \pm 11^{*+}$	$168 \pm 13^{§}$
Twitch tension (direct stim., mN)	$34 \pm 2^{+}$	$80 \pm 29^{*+}$	43 ± 7	$37 \pm 3^{+}$	$55 \pm 17^{*}$	46 ± 4
Twitch tension (nerve stim., mN)	$29 \pm 8^{+}$	41 ± 6	42 ± 7	$35 \pm 2^{+¶}$	$48 \pm 11^{*}$	$46 \pm 3^{§}$
ACh contracture (50 mg l^{-1} percentage P_o)	$10.7 \pm 1.6^{+}$	$13.8 \pm 1.1^{+}$	5.2 ± 1.9	4.9 ± 0.6	5.0 ± 2.4	3.9 ± 0.9
Muscle weight (mg)	11.9 ± 0.3	$22.5 \pm 4.3^{*+}$	11.0 ± 1.6	12.0 ± 1.5	$21.8 \pm 6.4^{*+}$	12.1 ± 1.2
Specific tension ($N\,g^{-1}$)	10.6 ± 1.2	10.9 ± 1.3	11.8 ± 1.6	13.8 ± 0.9	12.2 ± 1.9	$14.3 \pm 1.3^{§}$
n	3	3	6	4	4	7

Results are means ± SD Tukey's test:
* significantly different ($P < 0.05$) from PBS-injected muscles,
+ significantly different from contralateral muscles ($P < 0.05$).
§ $n = 6$
¶ $n = 3$.

Table 3. Tumour formation by clones of myogenic cells in different experimental conditions

Clone no.	Transfection	Dosage (cells)	Manupulation and muscle	Injected limbs	First appearance of tumours in inj. limbs at weeks post-implantation			Without tumours after > 20 weeks
					5–10	10–15	15–20	
			Histocompatible hosts (CD2F1 mice)					
F8B	none	10^6	Intact (5)* or paralysed (4) soleus	9	6	1	0	2+
F8B/34	β-Gal	$10^4/10^6$	Reinnervated (3), frozen (4) or paralysed (4) soleus; intact tib.ant. (5)*	16	16	0	0	0
F8B/27	β-Gal	about 10^6	Reinervated soleus	2	2	0	0	0
F8B/61	β-Gal	$10^4/10^6$	Frozen (7) or paralysed (5) soleus	12	0	1	11	0
			Histoincompatible hosts (CBA/J mice)					
F8B/61	β-Gal	10^6	Frozen soleus	3	0	0	0	3§

* The right soleus and the left tibialis anterior were injected in the same animals; in all other groups the injection was unilateral.
+ Animals survived for 34 weeks after implantation.
§ Animals survived for 21 weeks after implantation.

very early post-injection periods (2–5 weeks) because the MC clones used (27 and 34, see Table 3) caused early development of tumours. All four MC-injected muscles were significantly heavier than the contralateral muscles (13.8 ± 2.1 *versus* 10.6 ± 0.6 mg, $P < 0.05$, paired *t*-test) and developed tensions similar to the intact muscles (126 ± 22 *versus* 125 ± 22 mN).

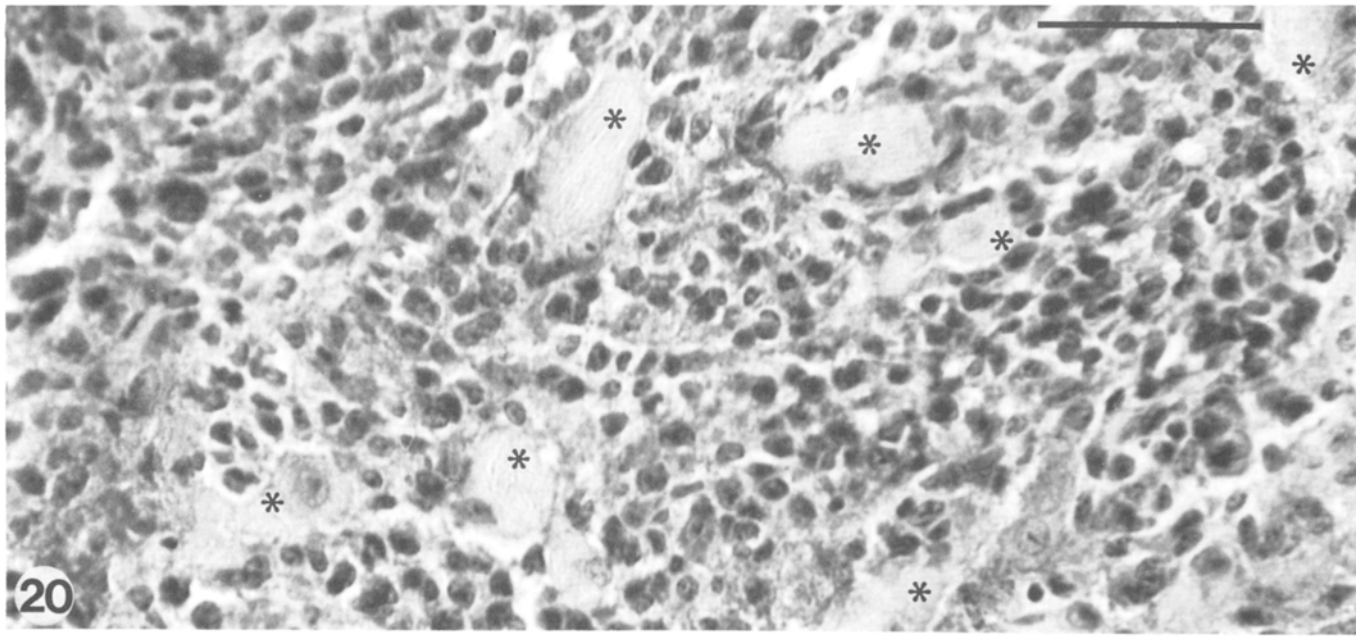
FORMATION OF TUMOURS AFTER INJECTION OF MCs

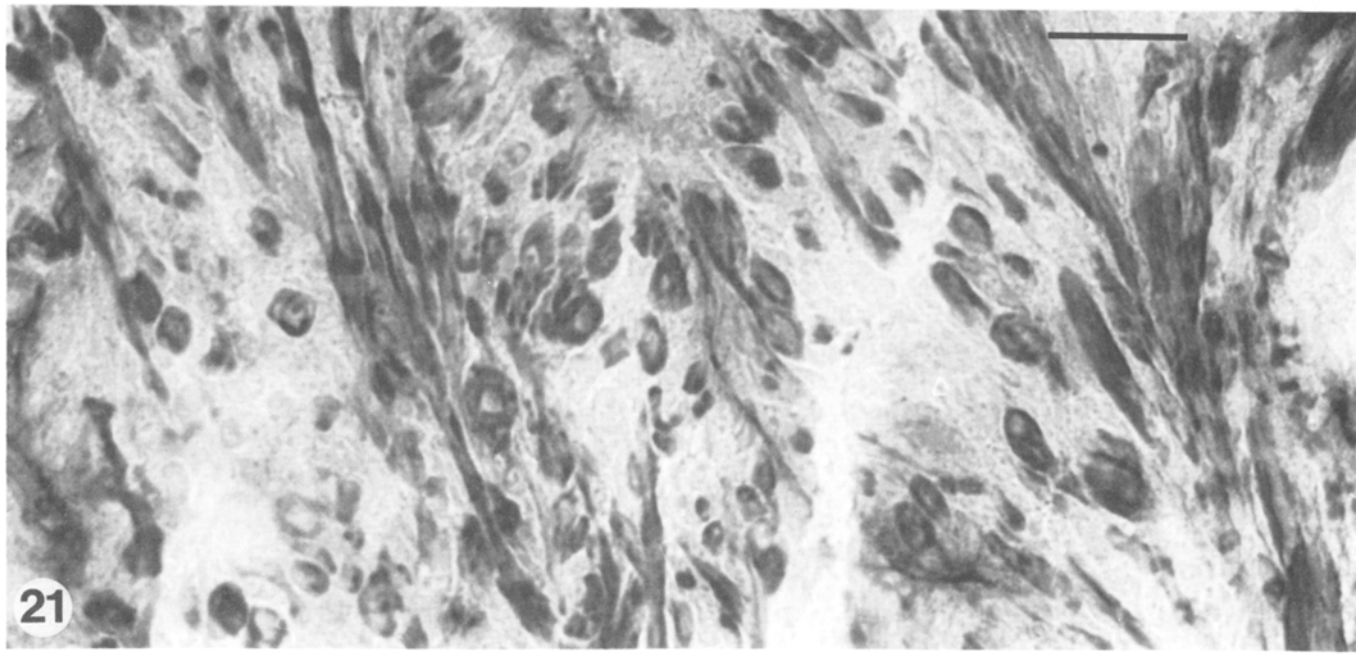
Alongside the positive functional effects reported above, we consistently observed tumour formation 5–20 weeks after injection of MCs. By the second month post-injection, swellings first developed in injected limbs (Table 3) and large tumour masses (1.6–3.7 g, $n = 5$) formed within a few weeks. The tumours were primarily located between the limb muscles and the skin but were destructive to the underlying muscles and were classified as *rhabdomyosarcomas* (Dr. U. Pfeifer, personal communication). In frozen sections from tumour masses muscle fibres, some containing β-Gal (Fig. 22), were observed running in all directions and surrounded by numerous cells which, unlike the fibres did not stain for myofibrillar ATPase activity (Figs 20–22).

In contrast to the other cell clones, clone 61 produced tumours only at late periods (4–5 months post-injection) (Table 3). While these inter-clone differences could not be explained, it appears (see Table 3) that neither the plasmid transfection, dosage, size of the injected muscle nor type of manipulation provides the primary reason for the malignant growth. In MC-injected paralysed solei of the two tumour-free limbs small muscle fibres were present at 34 weeks, although in low numbers, indicating successful implantation. Injection of MCs into muscles of histo*incompatible* mice (Table 3) did not produce tumours within 20 weeks and no increase in the total number of fibres was found. In current experiments with β-Gal-transfected cells from a different line (C2nlsBAG cells) tumours develop in previously frozen muscles of C3H mice (3 of 4 limbs within 20 weeks, Fig. 22).

Discussion

Implantation of MCs into frozen or paralysed adult muscles resulted in a significant increase in muscle force and mass 4–20 weeks post-implantation. This was obviously due to the formation of numerous new muscle fibres (up to ten times the normal number) and not to hypertrophy of intrinsic fibres (see Fig. 8). The new fibres most likely formed from the implanted myogenic cells since small (and large) fibres in muscles implanted with C2nlsBAG cells contained β-Gal, the marker for the myogenic cells (c.f. Hughes & Blau, 1990). The lack of histochemically detectable β-Gal activity in muscles injected with the F8B subclones is probably due to a stop in expression of the β-Gal gene or reduction below detectable levels. Interestingly, also after implantation of C2nlsBAG cells, cytoplasmic but not nuclear β-Gal decline. Expression of donor and host, but no heterodimeric glucose-6-phosphate isomerase isoenzymes in MC-injected dystrophic muscles has recently been observed (Morgan *et al.*, 1990), which too indicates formation of new fibres.


20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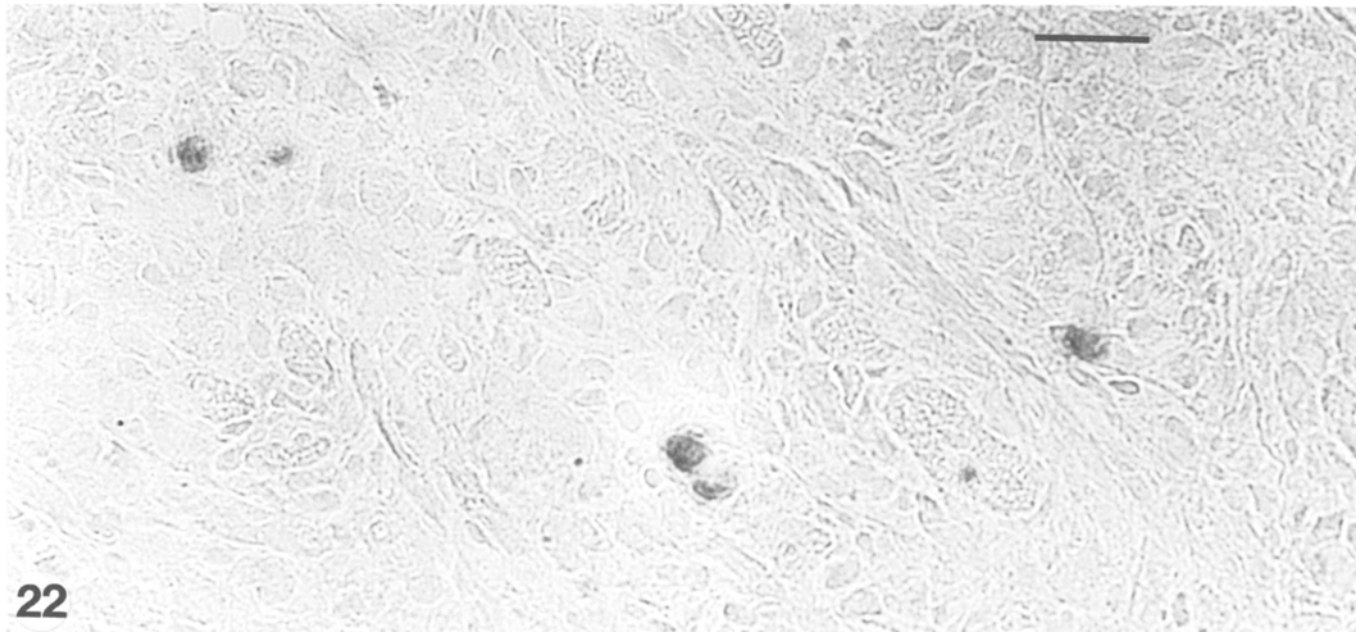
22

A decline in the number of fibres in MC-injected muscles between four and eight weeks post-injection was found. Since no signs of fibre degeneration and phagocytosis were observed at four weeks, we can assume that this decline is due to ongoing fusion of fibres or myotubes.

Rather surprisingly, the new muscle tissue in MC-implanted muscles appeared to give good functional results as inferred from the normal specific force (tension per unit weight). Evidence, both direct (tracing of small muscle fibres) and indirect (N-CAM immunostaining), was found that the new fibres are long and oriented parallel to the intrinsic fibres, which helps to explain the good functional performance of the enlarged muscles. Interestingly, in paralysed MC-injected muscles fewer large fibres develop than in paralysed controls (Fig. 8B). The stimulus for fibre growth is most likely connected to the demands on force production for soleus muscle during normal motor activity; it is possible, therefore, that the numerous new small fibres produce enough contractile force so that the atrophic host fibres after formation of new endplates lack this stimulus. In paralysed control muscles, on the other hand, this stimulus causes hypertrophy of some fibres (as compared to control muscles, Fig. 8B) apparently as compensation for the fraction of small atrophic fibres also present in this muscle. In frozen muscles (Fig. 8A) damaged host fibres rapidly regenerate to their full size, presumably prior to mature innervation of the new fibres, and together with the developing new fibres cause an overproduction of muscle mass and potential force. Obviously, in frozen muscles additional mitogenic factors are active which are less pronounced or missing in reinnervated or paralysed MC-injected muscles (Bischoff, 1986, 1990; Partridge, 1991). It is noteworthy that since all small fibres become innervated, motor units have in the average enlarged by factors as high as 10; this is well in line with motor unit enlargements observed after partial denervation (Thompson & Jansen, 1977).

New fibres were maturely innervated after eight weeks and indirectly-evoked tensions were larger than in control muscles. The maturation of synaptic transmission was also evident from the changes in muscle fibre types. New fibres originating from clone 61 initially stained as Type II fibres, possibly indicating that these cells are committed to express this type. The later decrease in Type II and increase in Type I fibres (Fig. 19) can be entirely explained by a larger reinnervation capacity of Type I motoneurons and continuing remodelling of motor innervation also found after reinnervation (Irinchev *et al.*, 1990) and muscle damage (Wernig *et al.*, 1991a,b). Interestingly, with clone 34, small fibres initially expressed both Type I and Type II characteristics (i.e. Type IIC, 2–5 weeks post-injection, results not shown), indicating clear inter-clone differences.

Tumour formation after myoblast implantation has not been reported before. Establishment of a permanent cell line implies transformations in the genome which 'immortalize' the cells, a process which has been interpreted as the initial stage in the complex genesis of tumours (Mougneau *et al.*, 1984; Bishop, 1987). Therefore, our results do not necessarily apply also to primary cultures. However, the striking point in this context is the fact that some weeks before the development of tumours, differentiation of the injected cells seemed complete, including the development of functional nerve–muscle connections and the down-regulation of N-CAM. Neural cell adhesion molecule reappeared in muscles at about the time when tumours occurred indicating fresh cell proliferation. All these observations indicate that MCs are not *a priori* tumourigenic and malignant growth is induced by factors *in vivo*. This is supported by the finding that all injected limbs contained small fibres but not all (37 of 39) produced tumours. Alternatively but less likely, a few transformed myoblasts – which have escaped detection – continued to proliferate and eventually formed the tumours.

In conclusion, the implantation of MCs into adult muscles produces large numbers of new muscle fibres which acquire functional innervation and lead to an increase in muscle force and mass. Mitogenic factors in the injured muscle may be the explanation for the larger effects in the cryo-damaged than in the paralysed muscles. All clones of myogenic cells used, however, eventually produced tumours.

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Figs 20–21. The histological appearance of a tumour which had developed subcutaneously over the tibialis anterior muscle. Fig. 20: The tumour consists of muscle fibres (asterisks) which show cross-striations and are surrounded by numerous cells with darkly stained nuclei of variable size. Cryostat section, Toluidine Blue staining. Fig. 21: Staining for myofibrillar ATPase activity (preincubation at pH 10.3). Small fibres, but not the surrounding cells, stain for the ATPase activity. Note the lack of uniform orientation of the fibres (compare with Fig. 1). Scale bars = 40 μm.

Fig. 22. β-Gal staining (dark deposits) of a tumour grown in the limb of a C3H mouse injected with C2nlsBAG myoblasts. β-Gal positive cells in the tumour prove its origin from the injected cells. Scale bar = 20 μm.

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