Functional effects of myoblast implantation into histoincompatible mice with or without immunosuppression

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1. The goals of this study were to evaluate the immunogenicity of myogenic cells (MCs) (1) immediately after implantation into regenerating muscles, and (2) following their maturation under initial immunosuppression. Implanted mouse soleus muscles were evaluated by isometric tension recordings *in vitro* followed by histological investigations on frozen sections.

2. Implantation of non-histocompatible myoblasts into cryodamaged soleus muscles of CBA/J mice induced immune rejection which caused large and permanent deficits in muscle force: 4–42 weeks postimplantation maximal tetanic tension was 50–60% that of intact or regenerated cryodamaged control muscles without tendency for recovery or histological signs of muscle regeneration. Specific tension (force per unit muscle weight) was also significantly reduced.

3. On frozen sections, only 62 ± 12% of the total area was desmin-positive, that is, occupied by muscle fibres, *versus* 90 ± 4% in regenerated and 92 ± 3% in intact muscles. Also, the total number of muscle fibre profiles was significantly reduced.

4. Under immune suppression with cyclosporin A (CsA), large muscles developed within 4 weeks. Following CsA withdrawal, muscle weight and force, in addition to desmin-positive areas on cross-sections, gradually declined over several months despite continual regeneration, indicating retarded immune rejection.

5. Initial application of CsA for 8 weeks after implantation, instead of 4 weeks, did not result in better survival of the implants, nor did a higher initial dose of CsA (100 instead of 50 mg kg^{-1} day^{-1}). Prolonged continuous application of a reduced dose (25 mg kg^{-1} day^{-1}) did not prevent muscle wasting but caused an additional delay.

6. It is concluded that histoincompatible myoblasts are highly immunogenic and that immune rejection causes large and permanent muscle deficits indicating elimination of host muscle tissue. Initial transient immunosuppression protects the incompatible cells, but after withdrawal, prolonged immune rejection and retarded muscle wasting occur.

Implantation of normal muscle precursor cells (myoblast transfer) is considered a potential therapy for inherited muscle diseases, aiming to counteract loss of both muscle tissue and strength (Partridge, 1991; Morgan & Partridge, 1992; Hoffman, 1993). In previous experiments we found that myoblast implantation could indeed be highly effective in improving muscle functional characteristics: in one experiment it was shown that implanted myogenic cells (MCs) from a permanent cell line formed thousands of new muscle fibres that became innervated and contributed to largely increased muscle tension (Wernig, Irintchev, Härtling, Stephan, Zimmermann & Starzinski-Powitz, 1991). The major prerequisite for such positive functional effects is that the donor tissue is tolerated by the host. Immunogenicity of muscle tissue has been considered to be relatively low, since mature skeletal muscle fibres normally do not express proteins of the major histocompatibility complex (MHC) (Ponder, Wilkinson, Wood & Westwood, 1983; Appleyard, Dunn, Dubowitz & Rose, 1985; Karpati, Pouilot & Carpenter, 1988). However, implantations into mouse muscles (Watt, Morgan & Partridge, 1991; Wernig & Irintchev, 1992) and recent results from clinical trials (Huard, Roy, Bouchard, Malouin, Richards & Tremblay, 1992; Roy, Tremblay, Huard, Richards, Malouin & Bouchard, 1993), indicate that immune reactions may occur even in MHC-compatible hosts, thus posing questions as to the functional efficacy of myoblast transfer (see also Partridge, 1991; Morgan & Partridge, 1992).

The functional consequences of an immune reaction in implanted muscles are not known. Observations by Partridge and co-workers (Morgan, Coulton & Partridge, 1987; Watt *et al.* 1991) indicate reduced amounts of surviving host tissue after rejection of non-histocompatible and histocompatible MCs. In the present study we implanted F8B61 myoblasts, known to produce muscle tissue in histocompatible animals (CD2F1 mice, MHC H-2^d; Wernig *et al.* 1991), into regenerating muscles of non-histocompatible CBA/J mice (MHC H-2^k) and we found a surprisingly high degree of force reduction which appeared permanent. In order to prevent this functional impairment, some animals were treated with the immunosuppressive cyclosporin A (CsA), shown previously to protect muscle grafts and implanted MCs (Watt, Partridge & Sloper, 1981; Watt, Morgan & Partridge, 1984; Law & Goodwin, 1988). Immunosuppression was limited to a period of 4 weeks postimplantation to allow differentiation of the implanted cells into muscle fibres (Wernig *et al.* 1991) and downregulation of MHC antigen expression (Wernig & Irintchev, 1992). Throughout this study, the expression of the neural cell adhesion molecule (NCAM) and enhanced basophilic staining with Toluidine Blue (basophilia due to enlarged numbers of ribosomes) were taken to indicate regenerating muscle fibres (see Wernig, Irintchev & Weisshaupt, 1990; Irintchev, Salvini, Faissner & Wernig, 1993). Using contractile muscle force as the main criterion for transplantation success, we found that CsA prevented immune rejection and led to significantly retarded muscle wasting after withdrawal.

Preliminary results of this work have been published as an abstract (Irintchev, Wernig & Lange, 1992).

METHODS

Animals and animal care

Inbred female CBA/J mice, purchased from Charles River Wiga (Sultzfeld, Germany) or bred in the laboratory and kept under standard laboratory conditions, were used for experiments at the age of 4–7 months. Treatment of the animal was in accordance with German laws for the protection of experimental animals.

Experimental procedures

Several groups of experimental animals received daily subcutaneous injections of 50 mg kg^{-1} CsA (Sandimmun, Sandoz, Basel, Switzerland; diluted 1:5 with 0·9% NaCl). Control mice were injected with similar volumes (10 μl (g body wt)$^{-1}$) of saline. This CsA dose is immunosuppressive for the mouse (Klaus & Kunkl, 1983). After 7 days of treatment, both control and immunosuppressed animals were anaesthetized with fentanyl (Fentanyl-Janssen, Janssen, Neuss, Germany), fluanisone (Sedalande, Delalande, Cologne, Germany) and diazepam (Valium 10 Roche, Roche, Grenzach-Wyhlen, Germany) (0·4, 2·0 and 5·0 mg kg^{-1} I.P., respectively) (Green, 1975) and the right soleus muscle was exposed along its entire length. Myogenic cells of the permanent cell line F8B61 (Balb/c origin, H-2^d), which are incompatible with the CBA/J mice (H-2^k), were implanted immediately after freezing and thawing of the muscles *in situ* (Wernig *et al.* 1991). Freezing was performed using a cryode, a metal rod with a flat end (3 × 0·7 mm), pre-cooled in liquid nitrogen; the cryode was applied onto the exposed soleus muscle, midway between the tendons, once for 10 s. After thawing the muscle, the wound was closed with 7/0 Ethilon polyamide threads (Ethicon, Norderstedt, Germany). Each muscle received 4 μl suspension containing 10^6 cells in phosphate-buffered saline (PBS). In two groups of animals, treatment with CsA or saline was continued for 4 weeks after implantation ($n = 16$ and 14) and in two other groups, for 8 weeks ($n = 9$ and 7). In a control group ($n = 8$) the soleus muscle was frozen and thawed *in situ* without cell implantation or drug treatment. Experiments were performed 28–295 days after cryodamage and cell implantation, or cryodamage alone. Intact age-matched animals served as an additional control group ($n = 6$).

In another group of animals ($n = 8$) with unilateral cell implantations, 100 instead of 50 mg kg^{-1} CsA was injected daily 1 week prior to, and 4 weeks after, implantation. These animals were investigated 165–211 days after CsA withdrawal. In another group of animals ($n = 10$), the initial CsA dose of 50 mg kg^{-1} was reduced to 25 mg kg^{-1} after the fourth post-implantation week and CsA medication continued until experiments were performed 62–231 days after cell implantation.

In vitro isometric tension measurements

Contraction measurements were performed as previously described (Irintchev, Draguhn & Wernig, 1990; Wernig *et al.* 1990; Wernig *et al.* 1991). Soleus muscles with nerves were dissected from anaesthetized animals and brought into aerated Tyrode solution. Muscles, mounted in lucite chambers and connected to a force transducer, were stimulated directly with a pair of silver electrodes in the bath (direct muscle stimulation). Nerves were stimulated with suction electrodes (indirect muscle stimulation). Measurements were performed at optimal muscle length at which maximal twitch tension was produced. Single pulses (duration of 0·5 ms for muscle and 0·1 ms for nerve stimulation) and tetani (20, 50 and 100 Hz for 2 s) were used for direct and indirect muscle stimulation. Voltage amplitudes were set at twice the lowest value sufficient to cause a plateau in twitch force (final values 20–25 V for direct and 5–8 V for indirect muscle stimulation).

Temperature was kept at 25 °C (maximum deviation ± 0·5 °C) throughout the measurements to prevent O_2 and glucose deficits of inner fibres under *in vitro* conditions (Segal & Faulkner, 1985). Muscles and nerves were stimulated alternately with single pulses and tetani. The sequence of measurements on any muscle was kept constant in all experiments with adequate time between single measurements (minimum of 3 min). Signals were stored in a digital oscilloscope (HM 208; Hameg, Frankfurt, Germany) and plotted on paper. The degree of innervation was estimated from tetanic tension via nerve *versus* direct stimulation at 100 Hz.

After the contraction measurements, muscles were gently blotted and weighed with parts of the distal and proximal tendons present.

Histology and immunocytochemistry

Muscles were placed on pieces of turkey liver, pinned at approximately resting length and rapidly frozen in isopentane, pre-cooled in liquid nitrogen. The liver pieces, having been fixed and stored in 10% formalin for up to 6 months, were washed thoroughly in running tap water for several hours, and in 0·9% NaCl solution for at least 24 h prior to use (Irintchev *et al.* 1990). Turkey liver was used because of its low connective tissue

content. Serial cross-sections (6 μm) were collected on chrome–gelatin-coated slides and stained with Toluidine Blue or antibodies.

Antibody stainings of NCAM and desmin, alone or in combination, were used to visualize specific cell types (see below). Immunofluorescent stainings were performed as previously described (Irintchev, Zeschnigk, Starzinski-Powitz & Wernig, 1994). Acetone-fixed tissue sections were overlaid with 20% normal goat serum in PBS (30 min at room temperature (18–22 °C)) and incubated with the primary antibodies either overnight at 4 °C (NCAM) or for 1 h at room temperature (desmin). Primary antibodies were diluted in PBS–carrageenan solution (PBS containing 0·7% w/v lambda carrageenan, a non-gelling gelatin reducing background levels, obtained from Sigma, Deisenhofen, Germany (see Sofroniew & Schrell, 1982) and 0·02% w/v sodium azide). The antibody to desmin (clone D33, purified mouse IgG; Dako, Hamburg, Germany,) was used at a concentration of 1 μg ml^{-1}, and the antibody to NCAM (rat IgG, hybridoma supernatant, clone H-28; Hirn, Pierres, Deagostini-Bazin, Hirsch & Goridis, 1981; Gennarini, Rougon, Deagostini-Bazin, Hirn & Goridis, 1984) was diluted 1:100. After having been washed with PBS, sections were incubated with species-appropriate biotin-conjugated second antibody (1:200 in PBS–carrageenan, 1 h at room temperature) and rinsed with PBS. Finally, slides were overlaid with 5-((4,6-dichlorotriazin-2-yl)amino)-fluorescein (DTAF) streptavidin (1:200 in PBS) for 30 min, washed and embedded in Fluoromount (Southern Biotechnology Associates, Birmingham, IL, USA). Second antibodies and streptavidin conjugates were purchased from Jackson Immunoresearch Laboratories (Dianova, Hamburg, Germany).

For double NCAM–desmin labelling, staining for NCAM was performed as described above, after which sections were incubated with primary antibody to desmin for 1 h, washed and overlaid with goat anti-mouse IgG, conjugated with rhodamine.

Control incubations included omitting the primary or secondary antibody, and substituting the primary antibody with non-immune rat or mouse IgG (5 μg ml^{-1}).

Quantitative procedures

Desmin, an intermediate filament protein expressed in striated muscle cells (see Irintchev *et al.* 1994), was used to differentiate muscle and non-muscle cells and to measure the cross-sectional areas occupied by muscle and non-muscle tissue. Area measurements (total area and desmin-positive area) were performed on complete muscle cross-sections (one per muscle). Videoimages, taken at the lowest magnification (5 × 1·25, Axiophot; Zeiss, Oberkochen, Germany) with a Silicon Intensified Target (SIT) video camera (model C2400; Hamamatsu, Hamamatsu City, Japan), were enhanced with an image processor (Argus-10; Hamamatsu) so that both background and specific immunofluorescence were visible. Measurements of digitized images were performed with Argus-10 software. For total area, the outer contours of the muscle cross-section were encircled and the area measured. In a second measurement, the desmin-negative and desmin-positive areas were encircled and measured separately. At the magnification used, visible desmin-negative areas amount to some 10% of the total area (see Results) and represent perimysial spaces and large vessels (desmin-positive smooth muscle cells in blood vessels, recognized at higher magnification, were treated as desmin-negative). Repeated measurements ($n = 10$) of one muscle cross-section (total area only) indicated a satisfactory reproducibility (coefficient of variation, 11·4%).

Regenerating muscle fibres, recognized in Toluidine Blue-stained sections as small diffusely basophilic muscle fibre profiles (Wernig *et al.* 1990, see blue-violet stained profiles in Fig. 7), were quantified directly under the microscope (×40 objective lens). NCAM, which is expressed in non-innervated cross-striated muscle cells irrespective of the degree of maturation (myoblast, myotube or muscle fibre; Covault & Sanes, 1985; Moore & Walsh, 1986; Schubert, Zimmermann, Cramer & Starzinski-Powitz,

Table 1. Contractile parameters and weights of freeze-damaged mouse soleus muscles with or without implantation of incompatible MCs (F8B61 in CBA/J mice) and of intact muscles

	Group no.	Maximum tetanic tension (mN)	Tetanic tension nerve stim. (mN)	Maximum twitch tension (mN)	Twitch tension nerve stim. (mN)	Wet muscle weight (mg)	Specific tension (N g^{-1})	No. of muscles/ animals
Regeneration								
Regenerated muscles	1	130 ± 22	126 ± 24	26 ± 6*	26 ± 6	10·4 ± 1·6	12·5 ± 1·5	8/8
Intact contra-lateral muscles	2	147 ± 13	145 ± 14	39 ± 5	39 ± 5	10·1 ± 1·2	14·6 ± 1·3	8/8
Rejection								
Regenerated MC-implanted muscles	3	74 ± 20†	66 ± 22‡	14 ± 6†	13 ± 4‡	9·3 ± 2·2	8·2 ± 2·6†	21/21
Intact contra-lateral muscles	4	148 ± 13	146 ± 28	35 ± 5	35 ± 5	10·0 ± 1·9	15·2 ± 2·0	19/19
Intact animals	5	141 ± 16	—	36 ± 4	—	10·3 ± 2·2	14·1 ± 2·5	12/6

Regenerated muscles were studied 11–15 weeks after cryodamage, regenerated MC-implanted 4–42 weeks after cryodamage and cell implantation. Values are means ± standard deviations. Significantly different ($P < 0·05$, one-way ANOVA–Tukey's test for groups 1, 3 and 5) from: * intact muscles (Group 5), † regenerated (Group 1) and intact (Group 5) muscles. ‡ $n = 19$

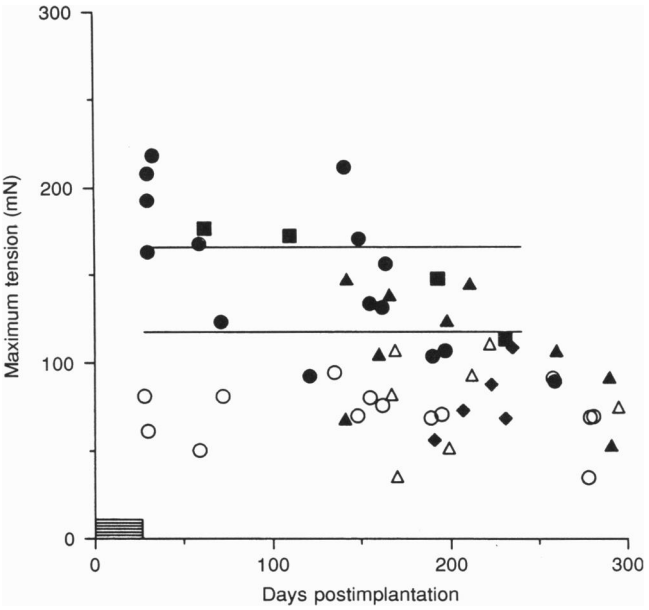

Figure 1. Muscle tension
Maximum tetanic tension of myoblast-implanted soleus muscles of animals treated for 4 weeks (indicated by shaded bar) with 50 mg kg^{-1} CsA (●), 8 weeks (▲), 100 mg kg^{-1} CsA (◆), continuously with CsA (4 weeks, 50, then 25 mg kg^{-1}, ■) or 0·9% NaCl for 4 (○) or 8 weeks (△). Lines show the range for muscles of completely untreated age-matched control animals (12 muscles, 6 animals).

1989; Irintchev *et al.* 1994), was used as an additional marker for regenerating fibres. Counting was performed directly under the microscope (40 × 1·25, Zeiss Axiophot microscope) on sections double-stained for NCAM and desmin: for each muscle a single complete cross-section was evaluated.

The total number of muscle fibre profiles was evaluated from complete muscle cross-sections reconstructed from videoprints (Toluidine Blue staining, final magnification ×382). Owing to split and branched fibres, the number of profiles is inevitably higher than the numbers of muscle fibres; no attempt was made to correct for this (Irintchev & Wernig, 1987; Wernig *et al.* 1990).

Statistical analysis

Multiple comparisons of group mean values were performed with one-way analysis of variance and a subsequent Tukey's test (Wallenstein, Zucker & Fleiss, 1980). To reduce the number of comparisons, groups of no interest were excluded from the analysis (e.g. intact contralateral muscles in Table 1). For all comparisons the accepted level of significance was $\leqslant 0{\cdot}05$. Throughout the text, mean group values are given with standard deviations.

RESULTS

Implantation without immunosuppression

Implantation of incompatible MCs into cryodamaged muscles caused a pronounced and permanent muscle weakness lasting throughout the period of observation (1–10 months postimplantation; Table 1; Fig. 1, open symbols). In addition, there was a significant reduction in force production per unit muscle weight (Tables 1 and 2).

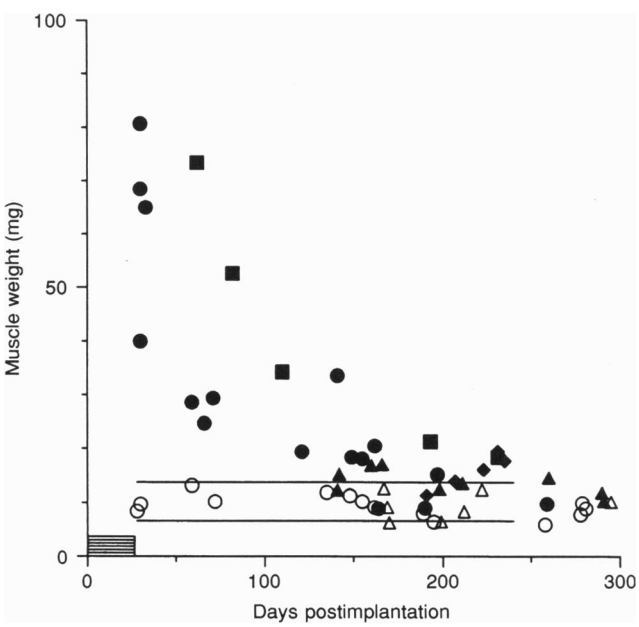

Figure 2. Muscle weight
Wet muscle weight of myoblast-implanted soleus muscles. See Fig. 1 for details.

Table 2. Desmin-positive area and number of muscle fibres

	Desmin-positive cross-sectional area (mm^2)	Total cross-sectional area (mm^2)	Desmin-positive to total area (%)	Total number of muscle fibre profiles §
Regenerated muscles	0·74 ± 0·05 (6)	0·82 ± 0·08 (6)	90 ± 4 (6)	980 ± 181 * (7)
Regenerated MC-implanted muscles	0·34 ± 0·09 † (13)	0·57 ± 0·18 † (13)	62 ± 12 † (13)	527 ± 158 † (12)
Intact muscles ‡	0·84 ± 0·12 (6)	0·92 ± 0·11 (6)	92 ± 3 (6)	756 ± 45 (9)

Histological analysis of regenerated (11–15 weeks after cryodamage), regenerated MC-implanted (4–40 weeks after cryodamage and implantation of F8B61 histoincompatible cells without immunosuppression) and intact muscles. Total and desmin-positive cross-sectional areas were measured from digitized videoimage-enhanced recordings of complete muscle sections stained with antibodies to desmin (see Methods). Total numbers of muscle fibre profiles were determined on videoprints from complete cross-sections stained with Toluidine Blue. Significantly different ($P < 0·05$, one-way ANOVA–Tukey's test) from: * intact muscles, † regenerated and intact muscles. ‡ Areas were measured in sections from intact muscles contralateral to regenerated, numbers of muscle fibre profiles were determined in sections from muscles of untreated age-matched CBA/J mice. § Components of split fibres were counted individually. Values are means ± standard deviations; numbers in parentheses refer to the number of muscles.

In accordance with previous observations (Wernig *et al.* 1991), functional recovery after freeze injury alone was remarkably good; only maximum twitch tension was smaller than in intact control muscles (Table 1).

Contralateral intact soleus muscles were similar to muscles of age-matched intact animals (ANOVA and Table 1) indicating no functional consequences of the contralateral manipulation.

Implantation under CsA protection

Immunosuppression during the first 4 weeks post-implantation allowed formation of large muscles with larger than normal contractile forces (first 4 muscles in Figs 1 and 2). Immediately after CsA withdrawal, muscle wet weight was larger than normal by a factor of 4–8, while maximum tension appeared elevated only by a factor of 1·2–1·6, which is in line with the observed less favourable muscle architecture (compare Fig. 6).

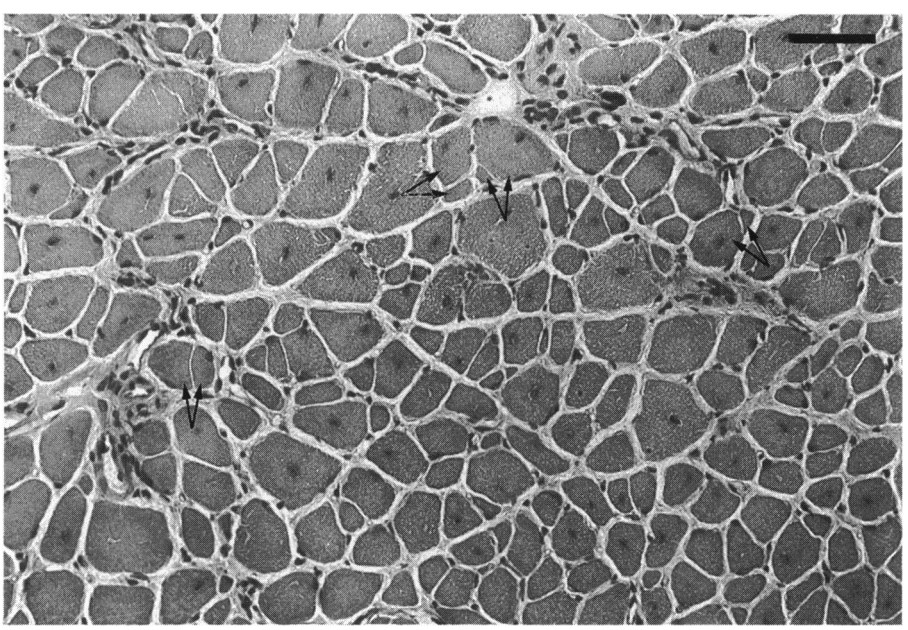

Figure 3. Split fibres and centralized myonuclei after regeneration

Toluidine Blue staining of a cross-section from a regenerated control muscle 12 weeks after cryodamage. Numerous split muscle fibres, detectable as small fragments complementing, as a whole, the size and outlines of a large fibre (arrows), and centralized muscle nuclei are present. Note the lack of fibrosis. Scale bar, 50 μm.

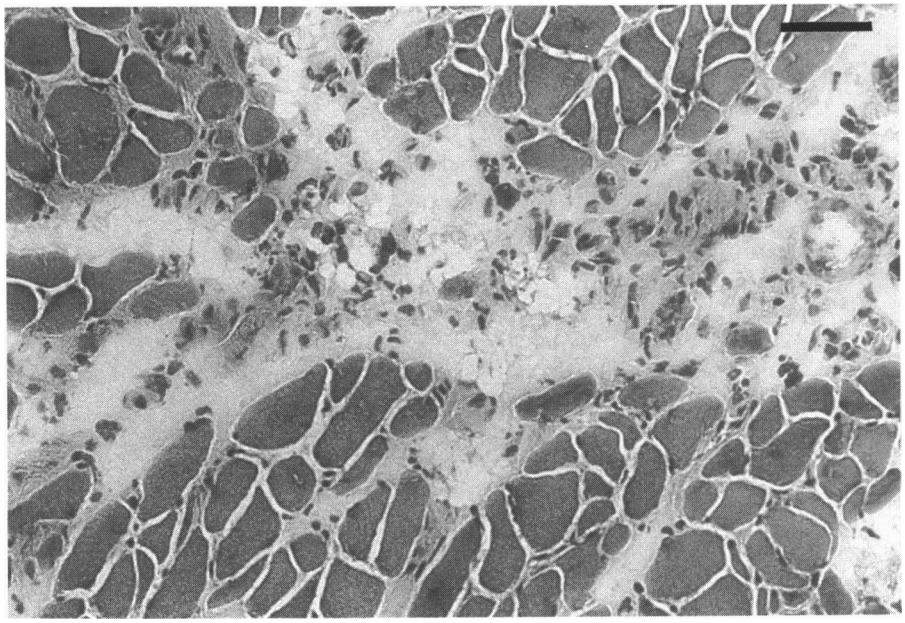

Figure 4. Fibrosis after immune rejection

Cross-section from a regenerated muscle 27 weeks after implantation of incompatible MCs without immunosuppression. Note the fibrotic area in the centre devoid of muscle fibres and the absence of cellular infiltrates. Scale bar, 50 μm.

In the period following CsA withdrawal (8–42 weeks postimplantation) a gradual reduction in tension and mass occurred, with tension eventually declining below the values of intact controls (Figs 1 and 2, filled symbols). Specific tension was below normal (7 ± 3 *versus* 14 ± 3 N g^{-1}, $n = 29$ in both samples). The degree of innervation (estimated from tetanic tension evoked via nerve *versus* direct stimulation) was near normal (median = 92 *versus* 98% in contralateral controls, $n = 19$ in both samples).

Effects of prolonged initial treatment, higher dose or maintained application

Prolonged initial application of CsA for 8 instead of 4 weeks did not result in a better survival of the implants following CsA withdrawal (Figs 1 and 2, ▲).

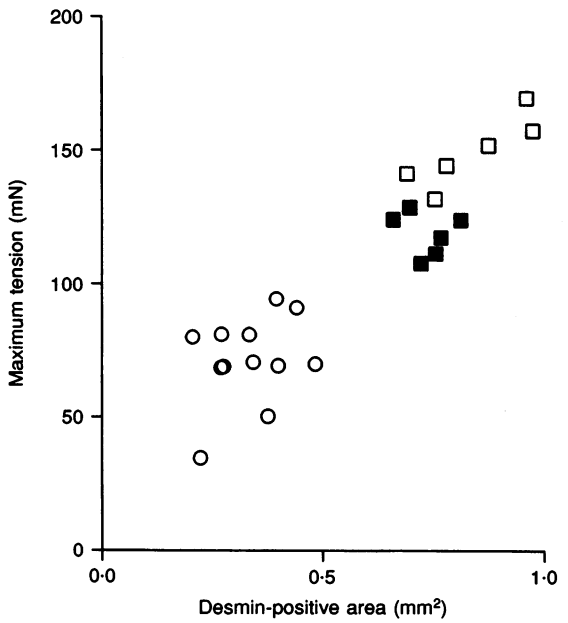

Figure 5. Maximum tetanic tension as a function of desmin-positive area

Shown are three groups of muscles: cryodamaged MC-implanted muscles (○), cryodamaged control muscles (■) and intact muscles contralateral to cryodamaged control muscles (□). Least-squares linear analysis indicated a high coefficient of correlation ($r = 0{\cdot}91$, $n = 24$) with a slope significantly different than zero ($P < 0{\cdot}0001$, Student's two-tailed t test).

Eight animals were treated with a higher dose of CsA (100 mg kg^{-1} day^{-1}) during the postimplantation period (4 weeks). Probably as a result of infection, two of these animals died during the period of treatment, one died 3 months later. The other five animals, studied for approximately 5–7 months after CsA withdrawal, did not show a more effective long-lasting protection of the grafted cells than that achieved by the regular CsA dosage (Figs 1 and 2, ◆).

Seven animals with MC implants were treated continuously with CsA, 50 mg kg^{-1} day^{-1} for the first 4 weeks postimplantation and 25 mg kg^{-1} day^{-1} thereafter. In two of these animals, rhabdomyosarcomas developed in the injected limb 20 weeks after cell implantation (see Wernig *et al.* 1991) and a third mouse died for no known reason. In the remaining four animals studied for 2–7 months postimplantation, there was a tendency for a somewhat delayed loss in muscle weight while other parameters were not different (Figs 1 and 2, ■).

Morphological features of implanted and control regenerated muscles

Two long-lasting morphological signs of regeneration, centralized myonuclei and split fibres (Irintchev & Wernig, 1987; Wernig *et al.* 1990), were consistently observed in regenerated muscles after cryodamage (Fig. 3). Neither total cross-sectional nor desmin-positive areas (an estimate of the regenerated muscle tissue) were significantly reduced when compared with intact muscles (Table 2). Regenerating basophilic fibres as well as NCAM-positive fibre profiles were rare, indicating that regeneration of the muscle was completed.

MC-implanted muscles of unsuppressed animals

MC-implanted muscles of unsuppressed animals 1–10 months after implantation contained large areas of connective and fatty tissue devoid of desmin-positive muscle cells and with reduced total numbers of muscle fibre profiles (Fig. 4, Table 2). No hypertrophy of individual muscle fibres was present (Figs 3 and 4). Surprisingly, no regenerating basophilic and only a few NCAM-positive muscle fibres were found after the second month postimplantation (9 muscles) suggesting that despite heavy loss of muscle tissue, regeneration was no longer taking place.

Maximum tetanic tension in all three experimental groups correlated well with the desmin-positive cross-sectional area (correlation coefficient (r) = 0·91, Fig. 5) and accordingly tension developed per unit of desmin-positive area was similar (163 ± 19 mN mm^{-2} in regenerated control muscles, 207 ± 93 mN mm^{-2} in MC-implanted regenerated muscles and 180 ± 15 mN mm^{-2} in intact muscles, n = 6, 12 and 6, respectively, $F > 0{\cdot}41$, one-way ANOVA).

CsA-protected implanted muscles

CsA-protected implanted muscles studied 1–4 days after drug withdrawal had cross-sectional areas severalfold

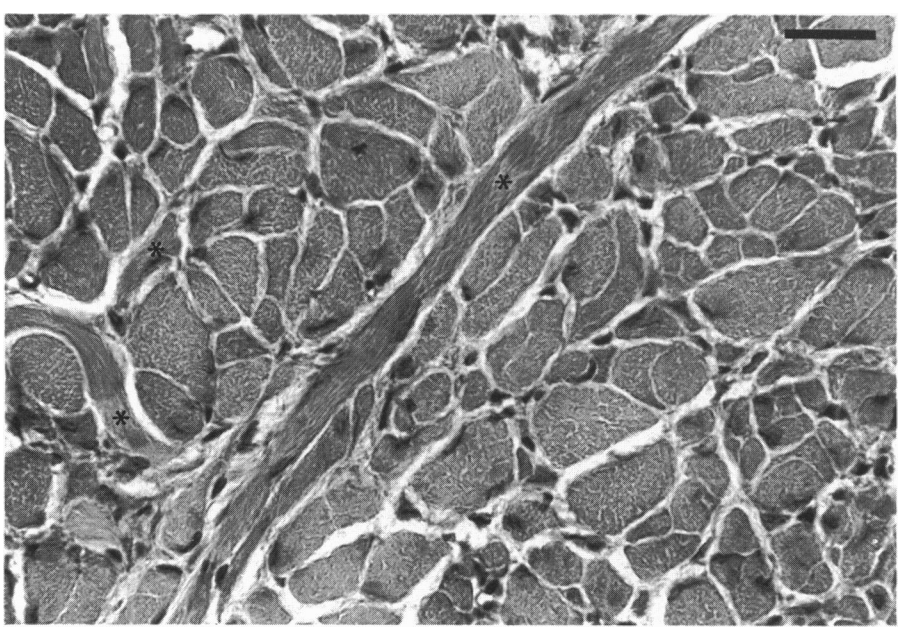

Figure 6. Implant protection by immunosuppression

Toluidine Blue staining of a cross-section from an MC-implanted muscle 3 days after the last CsA injection (30 days postimplantation). Numerous small- and large-diameter muscle fibre profiles are oriented parallel to the length axis of the muscle but some run perpendicular or oblique (*). Cellular infiltration is not conspicuous. Scale bar, 25 μm.

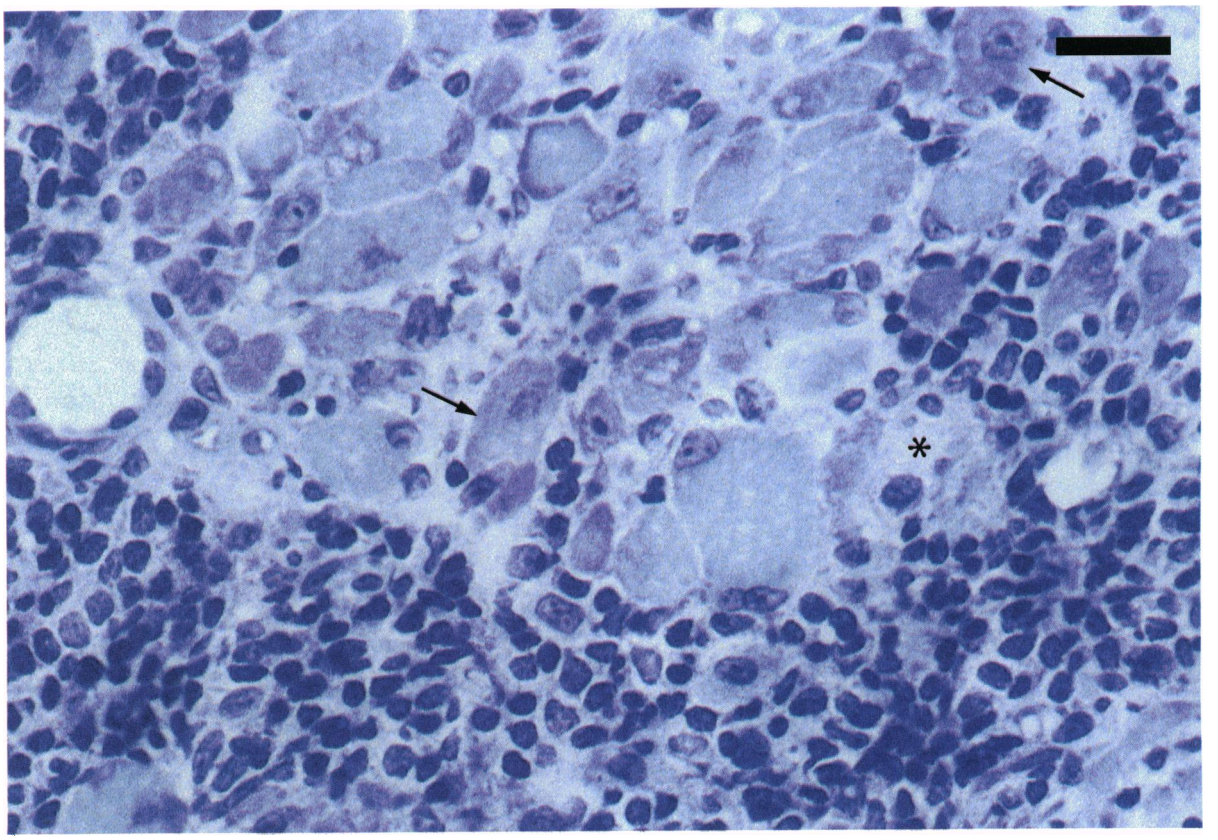

Figure 7. Immune reaction with muscle damage and reactive regeneration after CsA withdrawal
Toluidine Blue stained cross-section of a MC-implanted muscle 1 month after CsA withdrawal (2 months postimplantation). Massive infiltration of darkly stained mononuclear cells, regenerating muscle fibres (small profiles with central nuclei and blue-violet basophilic cytoplasm, some indicated by arrows), and a necrotic fibre (*, recognized in serial sections) are seen. Scale bar, 20 μm.

larger than controls; however, in contrast to previous implantations in which smaller muscles had developed within the same time period (Wernig *et al.* 1991), muscle fibre profiles running perpendicular or oblique to the length axis of the muscle were present (Fig. 6). This abnormal orientation predicts that such fibres do not contribute to, or perhaps even hinder, force generation of muscles (see above). The total number of muscle fibre profiles counted in single cross-section was much higher than normal (6500 in the muscle with both the smallest cross-sectional area and weight).

At longer periods after CsA withdrawal, extensive cellular infiltration and muscle fibre necrosis occurred. Muscles contained large areas, varying in size, occupied by connective tissue (more than 1/3 of the cross-sectional area in some cases) and fewer fibres than before (3300 and 2500 fibres in two muscles at 2 and 5 months, respectively; Fig. 8). In contrast to non-immunosuppressed implanted muscles (see above), muscle wasting and regeneration still continued 2–9 months postimplantation; this was evident from the presence of necrotic fibres (Fig. 7, asterisk), and, simultaneously, of small basophilic regenerating fibres (Fig. 7, arrows) (range for numbers of basophilic profiles in complete cross-sections, 2–38, median = 8, $n = 11$ muscles). Accordingly, numbers of NCAM-positive muscle fibre profiles were also elevated (range 2–78, median = 43, $n = 9$ muscles, as compared to 0–7, median = 0, in nine non-immunosuppressed muscles 2–10 months after MC implantation, $P < 0{\cdot}001$, Wilcoxon–Mann–Whitney test) (Fig. 9). These observations may be interpreted as reactive regeneration caused by the continual muscle damage and/or the continual proliferation of implanted cells.

DISCUSSION

Recent evidence shows, in both man and mouse, that implantation of muscle precursor cells can cause an immune reaction, independent of the degree of matching MHC (Morgan *et al.* 1987; Huard *et al.* 1991; Watt *et al.* 1991; Wernig & Irintchev, 1992; Huard *et al.* 1992; Roy *et al.* 1993; see also Morgan & Partridge, 1992). These observations indicate that the induction of tolerance by

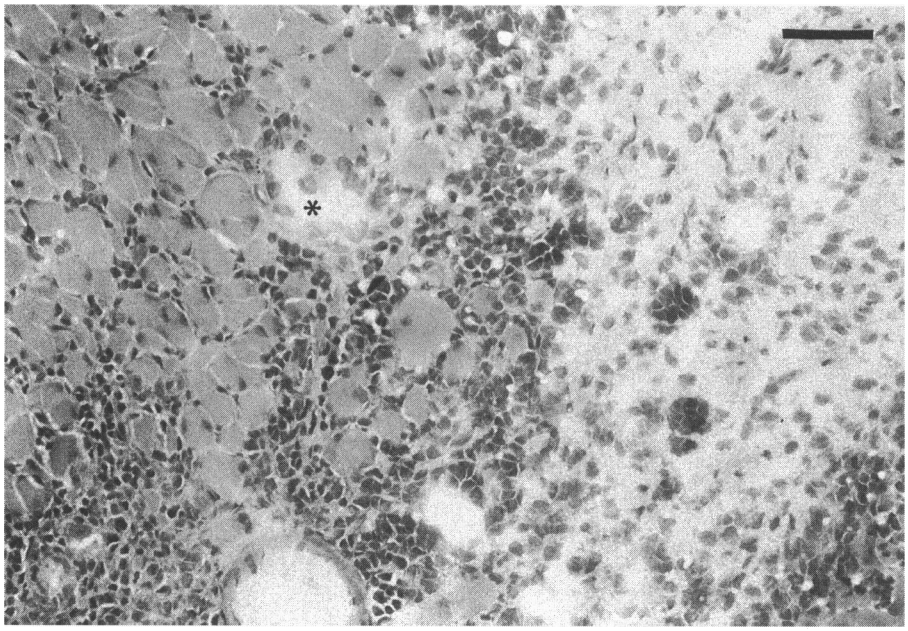

Figure 8. Late fibrosis and persistent infiltration after CsA withdrawal

Toluidine Blue stained cross-section of a MC-implanted muscle 4 months after CsA withdrawal (5 months postimplantation). Large area containing only fibrous tissue is seen on the right-hand side. Mononuclear cellular infiltrates surrounding muscle fibres are conspicuous. *, necrotic fibre. Scale bar, 50 μm.

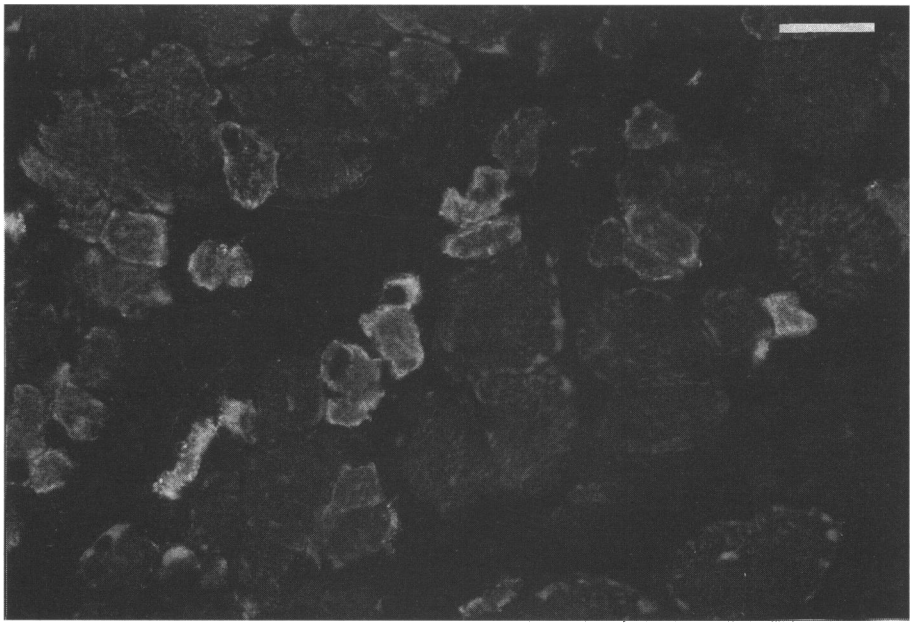

Figure 9. Persistent regeneration after CsA withdrawal

Cross-section from the muscle shown in Fig. 8 stained with antibody to NCAM (DTAF immunofluorescence). Small, presumably regenerating, muscle fibre profiles, some of which have centralized nuclei as seen on adjacent Toluidine Blue stained sections (not shown), are NCAM positive. Scale bar, 20 μm.

either antigen-specific (still not available) or non-specific (e.g. immunosuppressive therapy) means might usually be required to secure the survival of implanted cells. The effectiveness of immunosuppression with CsA has been shown experimentally (Watt *et al.* 1984; Law & Goodwin, 1988); however, clinical trials with Duchenne muscular dystrophy patients gave controversial results: one group consistently observed dystrophin-positive progeny of the implanted muscle cells (Law *et al.* 1990, 1992) while others failed to find donor dystrophin in HLA-matched and immunosuppressed patients (Gussoni *et al.* 1992; Karpati *et al.* 1993; see also Hoffman, 1993). These results pose the question as to how immunogenic myoblast implants are, and by which means rejection can be prevented.

It may be expected that myoblasts implanted into H-2 mismatched hosts would be rapidly rejected due to expression of class I MHC antigens (Hohlfeld & Engel, 1990). In the present study we do not have a direct marker for the implanted cells, but low muscle mass, low fibre numbers and small cross-sectional area, strongly suggest rejection within the first 4 weeks. This was directly demonstrated after implantation of β-galactosidase-transfected C2 myoblasts (H-2^k) into muscles of C57Bl/6J mice (H-2^b) which show lymphocyte infiltration and MHC class I expression in the first weeks but subsequent total loss of β-galactosidase-positive cells (Wernig & Irintchev, 1992; A. Wernig & A. Irintchev, unpublished results). We do not know whether in the present study some donor cells have survived, as was the case after an unexpected immune response to C2C12 cells in MHC-matched mice (Wernig *et al.* 1991; Wernig & Irintchev, 1992) or after implantation of non-compatible primary MCs from CBA/J mice into non-tolerant A mice (Watt *et al.* 1991).

Quite unexpectedly, the present results show that implantation of clonal myoblasts into cryodamaged muscles of non-tolerant mice has an adverse effect on muscle force (reduction by 40–50% of control values). Since the desmin-positive cross-sectional area was significantly reduced compared with regenerated control muscles, it appears that not only donor cells, but also regenerating host muscle tissue has been removed. One explanation is that host and donor cells have formed numerous hybrid fibres (see e.g. Partridge, Morgan, Coulton, Hoffman & Kunkel, 1989; Partridge, 1991; Watt *et al.* 1991) which have been destroyed because of expression of donor antigens on the entire fibre surface. In addition, cytokines, like tumour necrosis factor (TNF) produced during immune rejection, may cause deterioration of muscle tissue including that of the host (Goodman, 1991; see also Wernig & Irintchev, 1992). The fact that muscles do not recover within many months, and that regeneration is absent, might indicate that the satellite cell pool is severely exhausted after several cycles of degeneration and regeneration during the immune response.

CsA was effective in protecting the grafted cells during the 4 week period of treatment; however, much larger muscle masses developed than after implantation of the same myoblast line into histocompatible CD2F1 mice in a previous study (Wernig *et al.* 1991). The reason for this difference is not known, but it could be related to a higher proliferative potential of the present clone or, for some reason, better survival and proliferation of myoblasts after implantation. The large muscle masses which formed under CsA produced relatively little specific force (force per unit muscle weight); it is possible that too many MCs implanted at a single site could produce spatially less well ordered (as observed), and thus functionally poor muscles. In addition, we cannot exclude the possibility that direct muscle stimulation, and oxygen and glucose supply of the inner parts of the muscles, were insufficient *in vitro* due to the large muscle masses (see Segal & Faulkner, 1985), thus leading to incorrect tension parameters. Noteworthy though, is the fact that enlarged muscles did not show signs of tumour formation (e.g. large numbers of undifferentiated cells); in fact, in contrast to compatible mice in which rhabdomyosarcomas regularly form within some 20 weeks (Wernig *et al.* 1991), this was the case only with continuous CsA application.

The time period of CsA protection was chosen to allow maturation and downregulation of MHC expression in MCs to occur (Wernig *et al.* 1991; Wernig & Irintchev, 1992). Nevertheless, subsequent withdrawal of CsA was followed by massive, though largely retarded, immune rejection. The mechanisms of this response are presently under study. Preliminary results indicate that also 4 and presumably 8 weeks after MC implantation, some muscle fibre profiles express donor MHC class I molecules which might induce the immune reaction (A. Irintchev, M. Zweyer & A. Wernig, unpublished observations). The fact that muscle wasting is retarded, and rejection reaction prolonged, might be due, at least in part, to reparative proliferation of host and donor satellite cells, as suggested by the continual presence of small basophilic and NCAM-positive fibres.

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