Successful Histocompatible Myoblast Transplantation in Dystrophin-deficient mdx Mouse Despite the Production of Antibodies against Dystrophin

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Abstract. Myoblast transplantation has been considered a potential treatment for some muscular disorders. It has proven very successful, however, only in immunodeficient or immunosuppressed mice. In this study, myoblasts from C57BL10J +/+ mice were transplanted, with no immunosuppressive treatment, in the tibialis anterior of fully histocompatible but dystrophin-deficient C57BL10J mdx/mdx mice. One to 9 months after transplantation, the success of the graft was evaluated by immunohistochemistry. All the transplanted mice (n = 24) developed dystrophin-positive fibers following transplantation. Depending on myoblast cultures, transplantations, and time of analysis, the mice presented 15 to 80% of dystrophin-positive fibers in transplanted muscles. These fibers were correctly oriented and they were either from donor or hybrid origin. The dystrophin-positive fibers remained stable up to 9 months.

Possible humoral and cellular immune responses were investigated after grafting. Antibodies directed against dystrophin and/or muscle membrane were developed by 58% of the mice as demonstrated by immunohistochemistry and Western blotting. Despite the presence of these antibodies, dystrophin-positive fibers were still present in grafted muscles 9 months after transplantation. Moreover, the muscles did not show massive infiltration by CD4 cells, CD8 cells, or macrophages, as already described in myoblast allotransplantations. This lack of rejection was attributed to the sequestrated nature of dystrophin after fiber formation. These results indicate that myoblast transplantation leads to fiber formation when immunocompetent but fully histocompatible donors and recipients are used and that dystrophin incompatibility alone is not sufficient to induce an immunological rejection reaction.

SKELETAL muscle fibers are multinucleated giant syncitia formed during embryogenesis and growth by the fusion of hundreds or even thousands of precursor cells named myoblasts (Allbrook, 1981; Campion, 1984). Some of these myoblasts do not immediately participate in the fusion process but remain attached to muscle fibers under the basal lamina sheet as quiescent stem cells; these are termed satellite cells, and their role is to contribute to fiber regeneration after metabolic or mechanical lesions (Campion, 1984). This biological particularity has been used during the last 15 years in attempts to alleviate some muscular genetic diseases, such as Duchenne muscular dystrophy (DMD)[1] (Law et al., 1990; Partridge, 1991; Karpati et al., 1993; Tremblay et al., 1993). Moreover, the implantation of genetically modified myoblasts has been explored for the treatment of genetic, inherited metabolic deficiencies (Blau et al., 1993).

The mdx mouse, which constitutively lacks full-length dystrophin (Dys) expression because of a single-point mutation (Sicinski et al., 1989), is a good biochemical animal model for DMD and has been widely used to test the efficacy of various transplantation protocols, and successful myoblast transplantation procedures in mdx mice were first reported by the group of Partridge et al.(1989). After injection, the normal myoblasts containing the normal Dys gene fuse together and/or with host myoblasts to form muscle fibers expressing Dys (Karpati et al., 1989; Partridge et al., 1989; Morgan et al., 1990, 1993; Huard et al., 1994*a*,*b*; Kinoshita et al., 1994*a*,*b*).

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1. *Abbreviations used in this paper*: Ab, antibody(ies); DMD, Duchenne muscular dystrophy; Dys, dystrophin; EDL, extensor digitorum longus; MHC, major histocompatibility complex; NK, natural killer; TA, tibialis anterior.

To date, the results obtained in human clinical trials have been disappointing. Dys-positive fibers have been obtained in some patients (Huard et al., 1991, 1992; Law et al., 1990; Tremblay et al., 1993), and the normal Dys-coding mRNA has been found in two patients after transplantation (Gussoni et al., 1992). Transient strength increases have been reported (Tremblay et al., 1993). Some patients developed antibodies (Ab) directed against donor myoblasts and myotubes, even when donors and recipients were matched for class I and class II (DR) histocompatibility antigens (Huard et al., 1992; Roy et al., 1993; Tremblay et al., 1993). In some cases, Ab were capable of fixing complement proteins, thus lyzing the target cells in vitro (Tremblay et al., 1993). Some of the Ab detected in the plasma were directed against donor Dys (Huard et al., 1992; Tremblay et al., 1993). These problems raised the question of the role of the immune system in myoblast transplantations. In the mouse, rejection is very rapid and efficient after major histocompatibility complex (MHC)-incompatible cell transplantation and involves both cellular and humoral immunity (Grounds et al., 1980; Watt et al., 1984; Guérette et al., 1995; Huard et al., 1994*a*; Kinoshita et al., 1994*b*; Vilquin et al., 1994, 1995*a*,*c*). In some studies, the recipients were immunodeficient mice (nude/mdx mice; Partridge et al., 1989; Morgan et al., 1990, 1993, 1994), or tolerized mice (Grounds et al., 1980; Watt et al., 1982). Only partial success has been achieved using H2-compatible mice (Watt et al., 1984; Law et al., 1988*b*; Partridge et al., 1989; Labrecque et al., 1992). Various immunosuppressive agents or combinations have also been employed or compared (Watt et al., 1984; Law et al., 1988*a*; Karpati, 1990; Labrecque et al., 1992; Huard et al., 1994*a*; Kinoshita et al., 1994*a*,*b*; Vilquin et al., 1994, 1995*a*,*c*).

The number and viability of injected myoblasts, together with the injection protocol and pretreatment of the muscle, are important problems to consider in animal models. Immunological reactions, however, have to be understood and controlled because they should compromise myoblast transplantation whatever the protocol. In the present study, we addressed the question of the role of normal Dys itself in graft acceptance or rejection in the mouse. We used fully histocompatible animals. The only difference between these donors and recipients was the presence in donor myoblasts of the full-length normal Dys gene. As normal Dys protein expression would result in the presentation of new antigens to the recipient mouse, we wondered whether this new antigenicity could trigger an efficient immune reaction against fibers formed in part by the donor myoblasts, leading to rejection. We showed evidence of long-term implantation success but also the production of Ab directed against Dys. These Ab were unable to participate in short-term or long-term efficient rejection of the muscle fibers expressing normal Dys. This lack of rejection was attributed to the sequestrated nature of the antigens after fiber formation. These results are relevant to the general aims of normal or genetically modified cell transplantation in experimental animal models.

Materials and Methods

Cell Cultures

Mouse primary myoblast cultures were obtained from newborn skeletal muscle biopsies (Cossu et al., 1980; Vilquin et al., 1992). Briefly, the mice were killed, and the arms and legs skinned and cut into 1 mm^3 fragments, which were dissociated at 37°C under magnetic stirring, initially with collagenase (600 IU/ml for 1 h; Sigma Chemical Co., St. Louis, MO) and then with trypsin (0.1% wt/vol for 30 min; GIBCO BRL, Gaithersburg, MD), dissolved in Ca^{2+}- Mg^{2+}-free HBSS. Each animal yielded 12–15 × 10^6 cells. The cell suspension was grown in 199 medium supplemented with 15% FBS and a mixture of penicillin G (10,000 IU/ml) and streptomycin (10 mg/ml). Cells were harvested at 70% confluence, that is, 2 d after plating, either for immediate grafting or for freezing until grafting. Preliminary experiments indicated that frozen myogenic cells were as good as fresh cultures for transplantation in mice. Freezing was performed in 199 medium containing 15% FBS and 10% DMSO. Different batches of primary cell cultures have been used for this work. Desmin immunoperoxidase labelling indicated that the primary cultures contained, in general, 30–40% myoblasts at the time of injection (personal results). The mice transplanted with different cell batches have been gathered under the letters A, B, C, and D.

Animals

Inbred normal C57BL/10SnJ +/+ and C57BL/10ScSn mdx/mdx mice (mdx) were purchased from Jackson Laboratories (Bar Harbor, ME) and reproduced in our animal facilities. Primary myoblast cultures from newborn +/+ mice were transplanted in 26 male mdx mice, 2–3 mo of age. This work was authorized by Laval University Animal Care Committee and conducted according to the Canadian Council on Animal Care.

Cell Transplantations

3 d before transplantation, the left hind legs of the mdx mice were Cobalt-irradiated (20 Gy). This level of irradiation has been shown to block host myoblast proliferation (Wakeford et al., 1991), thus making the mdx mouse a closer model to DMD patients. 1 d before transplantation, the left tibialis anterior (TA) was exposed and injected with 10 μl of notexin venom (5 μg/ml), which has been shown to trigger muscle fiber degeneration without damaging myoblasts (Harris et al., 1975). On the day of transplantation, the cells were harvested by trypsinization or thawed, washed three times in HBSS, and concentrated as pellets. Cell viability was assessed using trypan blue staining. The left TA muscles were exposed and injected with ~4 × 10^6 viable cells suspended in 10 μl of HBSS. Mice from group A received fresh cultures, and mice from other groups received frozen cells.

Blood and Muscle Collections

2–37 wk after transplantation, the host mice were killed under deep anesthesia by intracardiac perfusion using sodium chloride (0.9%) containing heparin (2 IU/ml; Leo Laboratories, Ajax, ONT, Canada). Plasma were separated by centrifugation from the first 2 ml of perfusates. Grafted and contralateral untreated TA and extensor digitorum longus (EDL) were collected and immersed in a sucrose solution (30% wt/vol) overnight at 4°C. Muscles were embedded in OCT compound, frozen in liquid nitrogen, and serially sectioned at 8 μm using a Zeiss cryostat. Series of sections were separated by 180 μm. Blood was also obtained from some mice at different times after grafting by retro-orbital puncture.

Dystrophin Immunohistochemistry

The endogenous peroxidase activity was first blocked in muscle sections using 1% hydrogen peroxide for 30 min. Nonspecific Ig binding was then blocked with a 10% serum mixture (i.e., containing 3.3% rabbit serum, 3.3% horse serum and 3.3% FCS) in PBS. The R27 polyclonal sheep Ab against a 60-kD Dys antigen (Hoffman et al., 1987; kind gift of Genica Co, Boston, MA) was used 1/1,000 in 1% serum mixture in PBS for 1 h. The second Ab was a peroxidase-conjugated rabbit anti-sheep Ig (Dako, Copenhagen, Denmark). Binding was revealed with DAB (0.5 mg/ml; Sigma Chemical Co.) and 0.015% hydrogen peroxide. Slides were mounted in PBS-glycerol. Dys expression was also confirmed on some sections using the mouse NClDys1 mAb (1/40 for 1 h; NovoCastra, Newcastle upon Tyne, UK) directed against the rod domain of Dys or the mouse NClDys2 mAb (NovoCastra) directed against the COOH-terminal domain. The second Ab was a rabbit anti-mouse Ig conjugated to peroxidase (Dako Corp.). Immunoperoxidase-positive and -negative fibers were counted by microscopic examination for each muscle on the section with apparently the most positive fibers.

Research of Anti-Dys Ab

TA sections from a normal mouse and an mdx mouse were juxtaposed on the same slide. The presence (normal mouse) or absence (mdx mouse) of Dys was demonstrated using R27, NCIDys1, or NCIDys2 as described above. Instead of these Ab, plasma from grafted mice was used on other slides at a dilution 1/40 and/or 1/400. The second Ab was a rabbit anti-mouse Ab conjugated either to biotin or to peroxidase (1/100 for 1 h; Dako Corp.). The peroxidase activity was revealed with DAB. The presence of biotin was detected using FITC-streptavidin (1/200 for 30 min; Caltag, San Francisco, CA). Negative controls were obtained either by omitting the first antibody or plasma or by using the plasma from 12 naive male mdx mice aged 2–10 mo instead of the grafted mouse plasma.

The reaction of some mouse plasma against their own revertant fibers was also analyzed. Serial sections of TA muscles from the contralateral, nongrafted leg of the mouse were incubated with R27, NCIDys1, NCIDys2, or the mouse own plasma using the same protocol as described above. Some clusters of revertant fibers were identified and compared.

Western Blotting and Immunoblotting

To determine the molecular weight of the antigens recognized by Ab formed in recipient mice, Western blotting assays were performed. Protein extracts were obtained from one normal and one mdx, both naive, male mice. Skeletal muscles were solubilized in 0.3% SDS. 20 μg protein samples were solubilized in sample buffer by heating at 100°C for 5 min, and separated on a 6% T-SDS-PAGE gel overlaid with a 3.5% T-stacking gel by applying a current of 40 mA for ~3 h at 4°C. Electrophoretic transfer was performed onto a nitrocellulose membrane using a semi-dry technique (Multiphor II Electrophoresis Unit; Pharmacia LKB, Piscataway, NJ) with a current of 0.8 mA/cm^2 for 90 min. Antibody reactivity was assessed using an immunoblotting technique. Nitrocellulose strips were incubated overnight at 4°C with PBS containing 10% nonfat dried milk, 0.2% Tween 20 and 5% normal horse serum to block nonspecific binding sites. They were then incubated with recipient plasma diluted 1:50 in PBS containing 0.2% Tween 20 and 1% normal horse serum for 90 min under agitation. After several washes in PBS containing 0.05% Tween 20, strips were incubated for 90 min in a 1:500 dilution of horse anti-mouse IgG labeled with biotin (Vector Laboratories, Burlingame, CA). The reaction was followed by another 60-min incubation in the presence of a 1:750 dilution of streptavidin-alkaline phosphatase (Zymed, San Francisco, CA). Alkaline phosphatase was revealed using NBT-BCIP (GIBCO BRL) in Tris buffer (pH 9.5). Reactions were stopped by washing with 20 mM EDTA and the strips were photographed immediately. The presence of Dys in the samples was determined using NCIDys1 mAb and R27 polyclonal Ab.

The fine determination of high molecular weight is not easy in one-dimensional electrophoresis. Thus, in a second set of experiments, the positive plasma from mice B1, B3, B5, B6, B7, B8, C2, D1, D4, and D9 were used in the same electrophoresis preparation of normal mouse muscle extract. Strips for NCIDys1 and R27 were intercalated between the strips prepared for these plasma. This allowed to compare as exactly as possible the position of the bands stained by NCIDys1, R27, and the mice plasma.

Immunocytochemistry

Myogenic cells from C57BL/10SnJ +/+ mice and from C57BL/10ScSn mdx/mdx mice were grown on 2% gelatin-coated plates for 3 d in 199 medium supplemented with 15% FBS. The concentration of FBS was then reduced to 7% to increase myoblast fusion. The 12-well culture plate was divided into two groups: (*a*) six wells were fixed and permeabilized with methanol at −20°C prior to first Ab or plasma incubation; and (*b*) six wells were fixed and permeabilized after such an incubation. Incubations were performed in 199 medium containing 1% blocking serum mixture. The mouse plasma were used at 1/40 dilution. Negative controls and further reaction steps have been described above. The second Ab was FITC-conjugated rabbit anti-mouse IgG (Dako Corp.). Incubations were performed at room temperature to avoid endocytosis by living cells. The mdx myotube cultures do not express Dys and were used as a control for nonspecific Ig binding. A mouse anti-desmin mAb (Dako Corp.) was also used (1/100) on permeabilized and nonpermeabilized mdx myotube cultures to ascertain the efficacy of the protocol used to differentiate intra- and extracellular protein localization.

CD4 Cell, CD8 Cell, Macrophage, and Natural Killer Cell Immunohistochemistry

Monoclonal antibodies were produced as culture supernatants from the following hybridomas: GK1.5 for CD4 (American Type Culture Collection, Rockville, MD), YTS169 for CD8 (kind gift of Dr. Waldmann, Cambridge University, UK), MAC1 for macrophages and natural killer (NK) cells (American Type Culture Collection). These supernatants were used undiluted. The immunohistochemistry protocol has been previously described (Guérette et al., 1995). For each muscle, positively stained cells were counted in 60 microscopic fields randomly selected in six sections (Guérette et al., 1995). Each field represented ~1.5×10^5 μm^2.

Results

Short- and Long-term Dys-positive Fiber Formation after Histocompatible Myoblast Transplantation

All 24 grafted mice gave positive results; that is, they produced 15–82% Dys-positive fibers (Table I). These results were obtained in four different groups of animals (designated A to D) using four different primary cultures. Some differences in the final percentages were noted between batches B, C, and D.

2 wk after transplantation, some small- and medium-diameter Dys-positive fibers were formed (Fig. 1 *A*). These new or hybrid fibers were often associated in clusters that were dispersed in the muscle, probably reflecting the multiplicity of injection sites (Fig. 1 *A*). At this time, some necrotic zones were still visible, probably resulting from notexin injection. The percentage of Dys-positive fibers varied between 15 and 30% (Table I).

1 mo after grafting, the number and size of Dys-positive fibers were increased as compared to results observed 2 wk after grafting (Table I, Fig. 1, *B–D*), and in some cases almost 70% of the fibers were Dys-positive. Most of these fibers had smaller diameters than the original mdx fibers or the remaining dys-negative mdx fibers (Fig. 1, *B* and *D*), suggesting that they arose from the fusion of donor myoblasts and from the small-diameter myofibers and myotubes present 2 wk after grafting. Some Dys-positive fibers, however, had very large diameters, close to the maximum diameter of mdx Dys-negative fibers (Fig. 1 *C*). Most Dys-positive fibers, small or large, were correctly oriented in the muscle section, parallel to Dys-negative fibers.

2, 4, 5, 6, 8, and 9 mo after grafting, the Dys-positive fiber size had increased (Fig. 1, *E–N*) to a maximum that was sometimes greater than 75 μm (Fig. 1 *G*), i. e., the larger fibers described in the TA muscles of mdx mice (Coulton et al., 1988; Louboutin et al., 1993; Pastoret and Sebille, 1993). Normal caliber (i. e., 30–50 μm in TA muscle of this old, Louboutin et al., 1993), however, was the most frequent phenotype. Some necrotic zones were still visible in some muscles 2 mo after grafting but were not observed during the following months (Fig. 1, *E* and *F*). Fibrosis extension was rare (not shown). Large areas of Dys-positive fibers were present (Fig. 1 *G*) and were observed throughout the entire muscle length, but the percentage was higher in the central portion of the muscle (not shown). In these portions, the percentage of Dys-positive fibers reached up to 80% in some mice (Fig. 1 *H*). Some important variations existed, however, from one mouse to another (Fig. 1, *G* and *H*) that may reflect either differences in notexin necrosis or in myoblast implantation be-

Table I. Fiber Formation after Histocompatible Myoblast Transplantation

Mouse number	Days after grafting	Dys +	Dys −	Total	Percent Dys +	Percent Dys −
A1	13	322	1890	2212	14.6	85.4
A2	13	465	970	1435	32.4	67.6
B1	36	1320	1160	2480	53.2	46.8
B2	36	1520	693	2213	68.7	31.3
B3	68	1802	2873	4675	38.6	61.4
B4	68	1909	1771	3680	51.9	48.1
B5	162	2217	565	2782	79.7	20.3
B6	162	830	410	1240	66.9	33.1
B7	239	2354	631	2985	78.8	21.1
B8	239	670	1080	1750	38.3	61.7
Mean B group ± SD		1,578 ± 612	1,148 ± 822	2,725 ± 1086	59.5 ± 16.5	40.5 ± 16.5
C1	55	424	405	829	51.1	48.9
C2	114	883	722	1605	55.1	44.9
C3	114	360	233	593	60.7	39.3
C4	189	315	759	1074	29.3	70.7
C5	189	625	153	778	80.3	19.7
C7	259	1028	473	1501	68.5	31.5
Mean C group ± SD		606 ± 294	457 ± 248	1,063 ± 410	57.5 ± 17.3	42.5 ± 17.3
D1	84	355	870	1225	28.9	71.1
D2	159	388	428	816	47.6	52.4
D3	159	522	1404	1926	27.1	72.9
D5	229	304	551	855	35.6	64.4
D6	229	601	1662	2263	26.6	73.4
D7	229	469	1227	1696	27.7	72.3
D9	229	459	924	1383	33.2	66.8
Mean D group ± SD		443 ± 102	1,009 ± 448	1,452 ± 541	32.4 ± 7.5	67.6 ± 7.5

Mice were sacrificed at the times indicated. Dys histochemistry was done using R27 polyclonal Ab. The numbers of Dys-positive and Dys-negative fibers were counted on the best sections for each mouse. Mice C6 and D8 died accidentaly before muscle biopsy. The muscle from D4 mouse was broken during cryostat sectioning, thus not allowing exact fiber enumerations.

tween each mouse in a given set of experiments. Such differences have been pointed out by others (Morgan et al., 1993). Differences in the total number of muscle fibers in the TA were also observed at given post-grafting intervals. Fiber splitting, a frequent event in mdx muscles, was also observed in some grafted muscles (for example, the diameter of the split fiber in Fig. 1 *K* was ~120 μm). Longitudinal sections showed that Dys-positive fibers were still correctly oriented and that some were more than 750-μm long (Fig. 1 *L*). 8 and 9 mo after grafting, Dys-positive fibers were often grouped as bundles (Fig. 1, *M* and *N*). The size variabilities among Dys-positive fibers, however, was still striking. The percentage of Dys-positive fibers still showed some variations between mice and batches of myoblasts.

In the contralateral nongrafted leg of the same animals, no more than 1% Dys-positive fibers were present (Fig. 1 *O*). These were probably revertant fibers.

Some important bundles of Dys-positive fibers were identified in only one EDL of eight investigated. In three other muscles, few scattered fibers were observed, that did not extend throughout the muscle. Although myoblast migration from transplanted muscles to neighboring muscles (i.e., peroneal or EDL) has been described by others (Morgan et al., 1990, 1993; Watt et al., 1993), our results about possible myoblast migration were not consistent. The best results were observed by these authors either when the permanent C2 cell line was used, or when the neighboring muscles were irradiated or submitted to degeneration–regeneration cycles. In our study, Dys-positive fibers may be due to accidental injection of myoblasts in the EDL during our multiple injections in the TA.

Table II shows that the TA weight of the mice was decreased by more than 50% 5–8 mo after irradiation and notexin necrosis even following myoblast transplantation.

Production of Ab against Normal Muscle Fibers by Grafted Mice

The polyclonal R27 Ab and the NCIDys1 mAb specifically reacted with normal mice muscle fibers but not with the large majority (>99%) of muscle fibers in mdx muscles not injected with normal myoblasts. The plasma from 12 naive, nongrafted mdx male mice, however, did not react with the same normal fibers (Fig. 2, *A–H*). During the course of the experiment, 15 of 26 (58%) grafted mice developed some Ab against the normal muscle fiber membrane, but not against mdx membrane (Table III). The intensity of membrane staining produced by the grafted mice plasma on normal muscle fibers varied from one mouse plasma to another (Fig. 2, *I–O*). In some cases, the mouse reactive plasma gave a stronger staining on normal muscle than that observed with the Ab specific for Dys (Fig. 2, *J–L*). In other cases, staining was weaker (Fig. 2 *I*), but still distinctively stronger than the background staining produced by the same plasma on mdx muscle fibers. The staining produced by even the weakly reactive plasma was also clearly stronger than the background staining obtained on normal muscle fibers when no mouse plasma or the plasma from a naive mouse was used (Fig. 2 *C*). The pattern of labeling around a muscle fiber varied from one transplanted mouse plasma to another: a smooth, regular pattern was observed in some cases (Fig. 2 *I*), a rough, irregular pattern in others (Fig. 2, *L* and *O*).

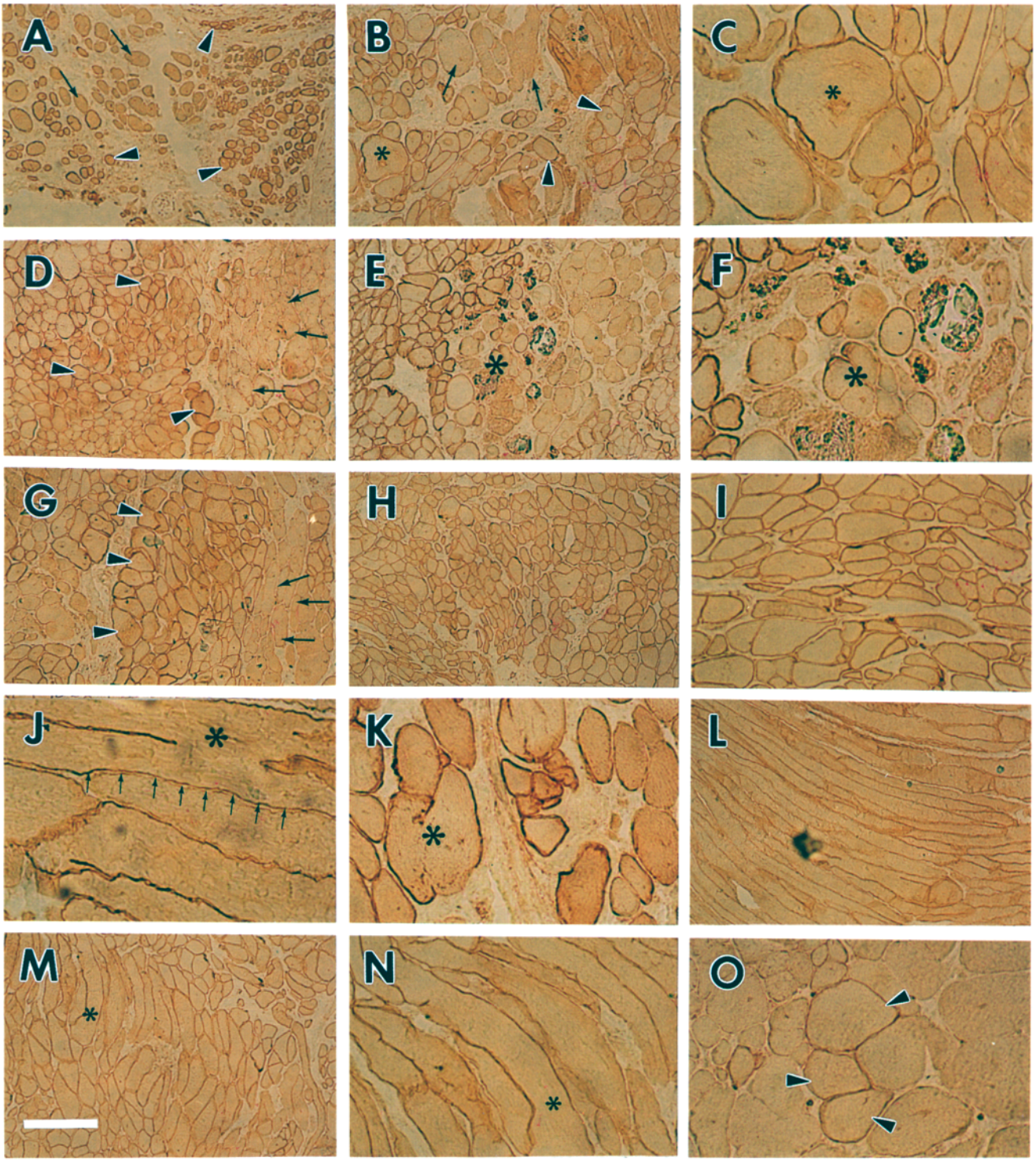

Figure 1. Short- and long-term Dys-positive fiber development after histocompatible myoblast transplantation. Cryostat sections of mouse muscles at various times after grafting and stained using R27 Ab. (*A*) 2 wk; (*B–D*) 1 mo; (*C*) magnification of *B*; the same fiber is indicated (*asterisk*) and probably results from host-donor hybridation. (*E–G*) 2 mo after grafting; one necrotic zone is shown in *E* and magnified in *F* (same area indicated by an *asterisk*); Dys-positive fibers (*arrowheads*) are often surrounded by Dys-negative fibers (*arrows*). (*H–L*) 5 mo after grafting; up to 80% of the fibers were Dys-positive, but size differences were observed between fibers (*I*, magnification of *H*); on longitudinal sections, Dys is expressed over the full-length of the fibers (*J*, *arrows*; *L*); Dys-positive splitted fibers are presented in *J and K* (*asterisks*). (*M–O*) 8 mo after grafting (same mdx mouse); (*M* and *N*) grafted muscle; *N* is a magnification of *M* (same fiber indicated by an *asterisk*); (*O*) nongrafted muscle; one bundle of revertant Dys-positive fibers is shown. Bars: (*A*, *B*, *D*, *E*, *G*, *H*, *L*, and *M*) 190 μm; (*C*, *F*, *I*, *J*, *K*, *N*, and *O*) 75 μm.

Table II. Weight of Some Mice TA

Mouse number	Days after grafting	Control	Treated	Graft/control ratio
		mg	*mg*	
B7	239	90	30	0.33
C4	189	79	23	0.29
C5	189	77	19	0.25
C7	259	51	15	0.29
D2	159	82	29	0.35
D3	159	74	35	0.47
D4	159	66	17	0.26
D5	229	78	28	0.36
D6	229	91	37	0.41
D7	229	61	28	0.46
D9	229	76	24	0.32
Mean ± SD		75 ± 12	26 ± 7	0.34 ± 0.08

Mice were sacrificed at the times indicated. Treated (irradiation, notexin, transplantation) and control (no irradiation, no notexin, no transplantion) TA muscles, were weighed just after biopsy.

Table III displays Ab production in grafted mice at different times after transplantation, when available. After 13 d, only one mouse (A1) of two gave a slightly positive reaction. After 36 d, two mice (B1, B2) of two gave positive reactions but only at a low dilution of 1/40. After 68 days, two mice (B3, B4) of two gave positive reactions, one of which was positive even at dilution of 1/400. Plasma were unfortunately collected from these six mice only once, when they were killed. The following 20 mice were investigated for Ab production at different times after transplantation. In one case (C2), the plasma was positive on day 27, but no preimmune plasma was available. In four cases, the plasma collected 2 (D1, D4, D9) or 44 d (C7) after grafting were negative, but conversion to positivity developed after 75, 101, or 160 d. In nine cases, negative plasma remained negative, whereas plasma that started to be positive at any time remained positive until sacrifice, 34 wk in some cases, demonstrating sustained Ab production over a long time course.

In six of seven cases investigated, the mouse plasma could also react with some revertant fibers which were present in small numbers in the muscle of the naive, male mdx mouse used as a control for Ab staining (Fig. 2 *N*). These revertant fibers were also recognized by R27 Ab or NClDys2 mAb (not shown).

Immune Reactions against Own Revertant Fibers

One to five clusters of revertant fibers were present in the nongrafted muscles. These clusters were identified using R27 polyclonal Ab (Fig. 3, *B*, *E*, *H*, and *K*) and contained one to eight fibers that expressed some Dys-truncated proteins. Some, but not all, of these clusters were also labeled by NClDys1 mAb directed against Dys rod domain (Fig. 3, *A*, *D*, *G*, and *J*) or by NClDys2 directed against the COOH-terminal domain (not shown). The plasma from mice that developed Ab against normal fibers also reacted against their own revertant clusters and recognized more clusters than R27, NClDys1, and NClDys2 (Fig. 3, *C*, *F*, *I*, *L*); for example, the plasma from mouse B8 reacted against four clusters in its own nongrafted TA muscle, whereas R27 detected only three and NClDys1 only two (Fig. 3).

It is noteworthy that back-mutated fibers were often split (Fig. 3, *M–O*). This was already observed by Hoffman et al. (1990). Fiber splitting is frequent in mdx mouse, but it is an aberration in normal mouse muscles (Coulton et al., 1988; Louboutin et al., 1993), and is interpreted as resulting from excessive fiber enlargement or fusion inability.

Immunoblotting

R27 polyclonal Ab and NClDys1 specifically reacted with an antigen whose weight was estimated on Western blots to be ~420 kD. This antigen was expressed only in normal mouse muscles (Fig. 4) and likely corresponds to Dys whose theoretical molecular weight is 427 kD (Koenig et al., 1988). The plasma from 10 of 14 mice reacting against normal muscle membranes in immunohistochemistry also reacted with antigens whose molecular weight was very close or equal to 420 kD. The band intensity varied greatly from one mouse plasma to another (Table IV). Some plasma reacting positively with normal muscle fibers also reacted with other bands of lower molecular weight (Table IV). The high molecular weight antigens stained by NClDys1 and R27 were colocalized on the nitrocellulose strips with bands stained by the 10 positive mouse plasma (not shown). The plasma from grafted mice which were not reactive in immunohistochemistry did not react against the high molecular weight antigens stained by NClDys1 and R27 (Table IV). The control plasma from nongrafted mice served as negative controls (Fig. 4).

Cytochemistry

Normal myotubes express intracellular Dys (Lev et al., 1987). The plasma from naive mice and from mice that did not react with normal muscle fibers did not show any specific binding on myotubes in culture, neither alive nor permeabilized (not shown). Staining was also not observed when living and nonpermeabilized myotubes were exposed to NClDys2 (Fig. 5 *A*) or the positive plasma (Fig. 5, *D* and *G*). On the contrary, the positive plasma from grafted mice showed a preferential localization on myotubes after permeabilization. A fluorescent staining was observed when myotubes were permeabilized with methanol before incubation with NClDys2 mAb (Fig. 5 *B*) or mouse plasma (Fig. 5, *E* and *H*). The intensity of the staining varied among the plasma of different grafted mice. Such staining was never observed when the mouse plasma was omitted from the first step of the reaction (not shown), or when these experiments were performed using mdx myotube cultures, that is, myotubes that do not express Dys (Fig. 5, *C*, *F*, and *I*). The permeabilization protocol really allowed to discriminate between intra- and extracellular antigens, since the intracellular cytoskeletal protein desmin was only stained when cultures were permeabilized (Fig. 5, *J* and *K*). Taken together, these data suggest that most, if not all, of the antigens recognized by the Ab developed by the grafted mice were located inside the myotubes, and not on the extracellular part of the membrane, except in one case (mouse B3) in which a faint myotube labeling was observed on living cells and a stronger labeling inside the myotubes was seen after permeabilization.

Cellular Infiltration

2 and 4 wk after grafting, $CD8^+$ T cells, macrophages, and

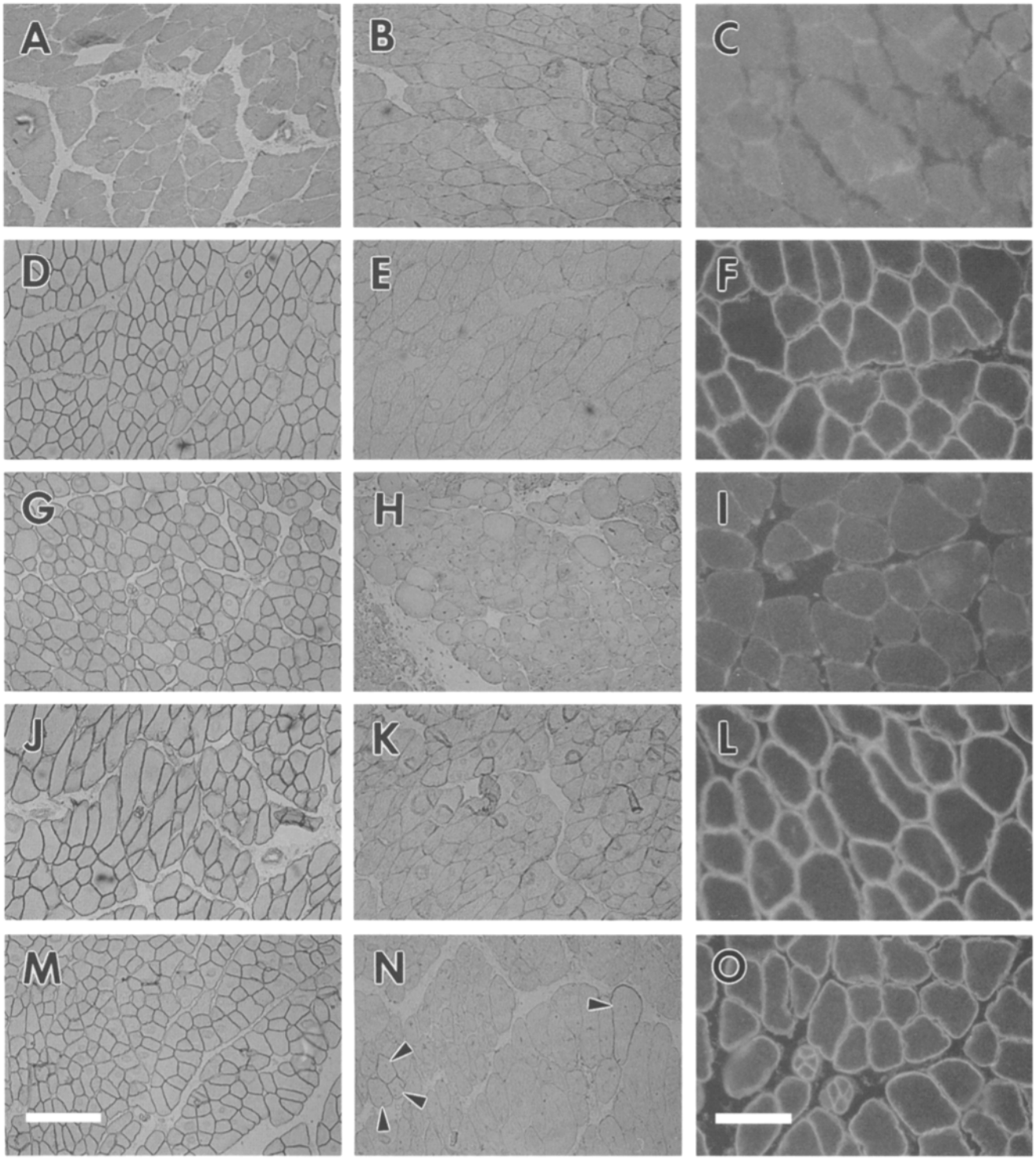

Figure 2. Production of Ab directed against normal muscle membranes. Cryostat sections of normal mouse muscle (*left* and *right*) or mdx muscle (*middle*) were incubated with mice plasma or specific Ab. (*A–C*) Plasma from a naive, nongrafted mouse. Note that mdx muscle background staining is always more important than normal muscle staining. (*D* and *E*) NCIDys1 anti-Dys mAb. (*F*) NCIDys2 anti-Dys mAb. (*G* and *H*) R27 polyclonal anti-Dys Ab. (*I*) plasma from mouse B6 (weakly positive). (*J–L*) Plasma from mouse B8 (strongly positive). (*M–O*) Plasma fom mouse C2 (strongly positive). Note that muscle staining pattern presented some differences from one mouse plasma to another (*I*, *L*, and *O*). The serum from mouse C2 could also react with revertant fibers in the mdx muscle used for the assay (*N*, *arrowheads*). Bars: (*left* and *middle*) 190 μm ; (*right*) 70 μm.

Table III. Reactivity of the Grafted Mice Plasma Against Normal Mouse Muscle Fibers

Mouse number	Days after grafting														
	13														
A1	+ †														
A2	− †														
			36												
B1			+/− †‡												
B2			+/− †												
				68											
B3				+++/++ †											
B4				++/− †											
						75		92			160		239		
B5						++++/++		ND/++			+++†				
B6						+/−		ND			+ †				
B7						++++/++		+++/+++			+++		+++ †		
B8						++++/++		+++/+++			+++		+++ †		
		27		44						101		190		259	
C1		−		− †											
C2		+++		+++/+						+++ †					
C3		−		−						− †					
C4		−		−						−		− †			
C5		−		−						ND		− †			
C6		−		−						− †					
C7		−		−						+		ND		ND †	
	2					75					160		229		
D1	−					+ †									
D2	−					−					− †				
D3	−					−					− †				
D4	−					+++					+ †				
D5	ND					+					ND		+ †		
D6	ND					−					−		ND †		
D7	ND					+					ND		+ †		
D8	−					ND					− †				
D9	−					−					+++		+++ †		

†, Day of mouse sacrifice;
−, negative;
+, weakly positive;
++ to ++++, positive to strongly positive;
‡, Results are given for serum dilution of 1/40. When two results are given, the first was obtained with a 1/40 dilution of the serum and the second with a 1/400 dilution.
ND, not determined.

NK cells were present in the grafted muscles, mainly at the injection sites (not shown). They were more numerous than in a noninjected mouse muscle, but they were less numerous than when MHC-incompatible cells were grafted (Guérette et al., 1995). $CD4^+$ T cells were almost absent from grafted muscles. The values obtained at 2 and 8 mo did not differ from one another (Table V). Some hot spots containing many macrophages or NK cells were located in the immediate vicinity of, or inside, some muscle fibers undergoing degeneration, but this observation is frequent in mdx mouse (not shown) and is the reason for the high standard deviations. Thus, the infiltration of the muscle after histocompatible transplantation was weaker than that observed in previous studies by our group (Guérette et al., 1995; Vilquin et al., 1995*a*) after MHC-incompatible myoblast transplantation. Indeed, once the necrosis caused by notexin and cell injections has been cleared, the number of infiltrating cells was not significantly higher than in the absence of cell injection.

Discussion

Transplantation Results

Using nude/mdx congenic mice, Partridge group demonstrated short- and long-term myoblast transplantation success (Partridge et al., 1989; Morgan et al., 1990, 1993, 1994; Watt et al., 1993). The level of success decreased when H2-compatible M9 mice or tolerized mice were used as recipients (Grounds et al., 1980; Partridge et al., 1989). Rando and Blau (1994) reported good success after injecting retrovirally labeled myoblast clones into isogenic hosts, whereas allogeneic grafts were rejected; the use of cyclosporin A or specific anti-adhesion molecules mAb allowed 4-mo-long acceptance of allogeneic grafts (Pavlath et al., 1994). Huard et al. (1994*a*,*b*) reported 1-mo success after human cell xenografting in immunodeficient nude and SCID mice. Kinoshita reported 1–5-mo-long success following MHC-incompatible myoblast transplantation into mdx mice immunosuppressed with FK506 (Kinoshita et al., 1994*b*; and personal results). Karpati et al. (1989) obtained little, unquantified success following human myoblast xenotransplantation in mdx mice without immunosuppression.

In the present study, 100% of the mice presented short or long-term success after compatible myoblast transplantation, as all the transplanted mdx mice expressed Dys-positive fibers after grafting at any time of analysis. Half of the transplanted mice expressed >50% Dys-positive fibers in their grafted muscles. The total number of Dys-positive

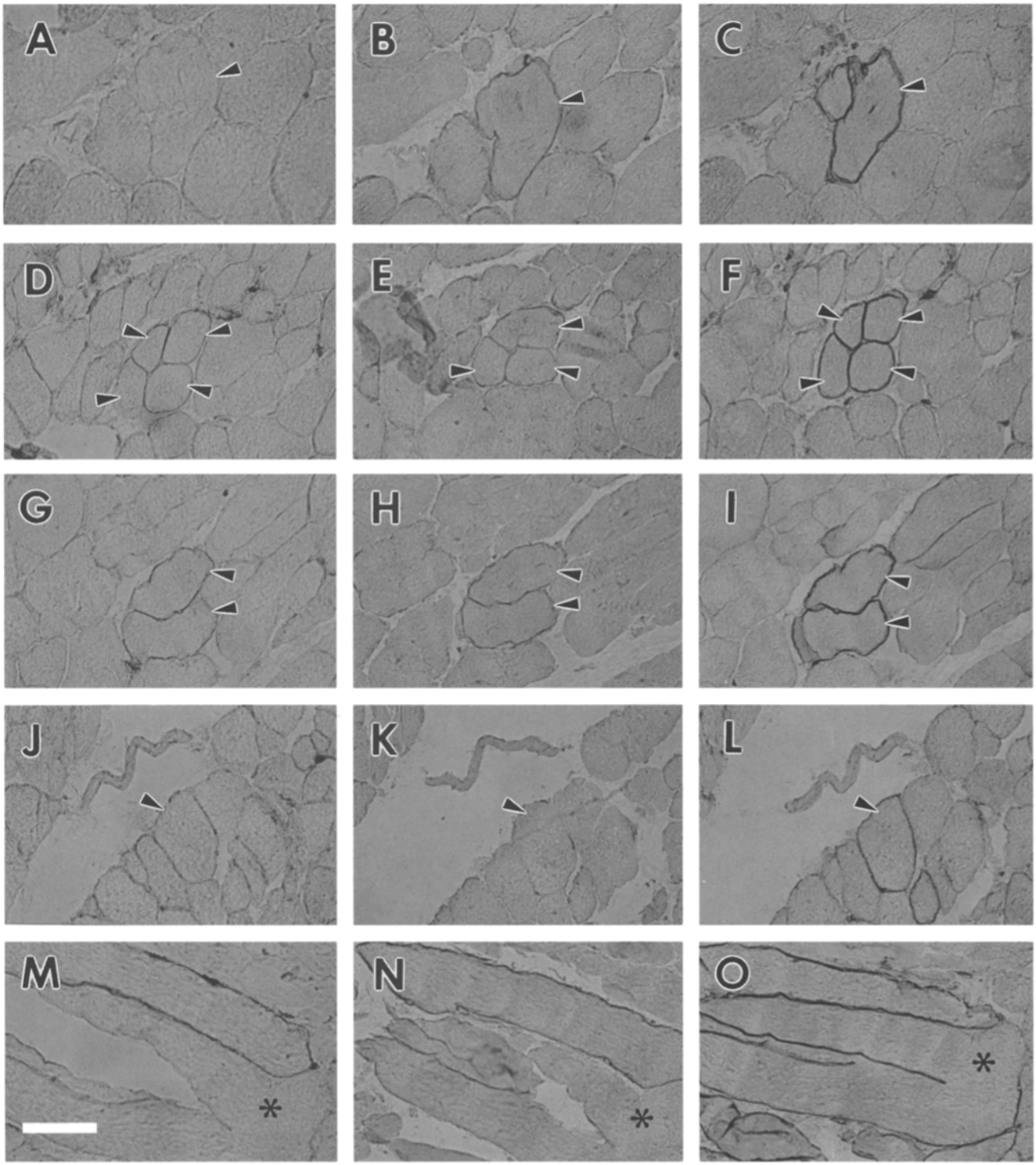

Figure 3. Differential staining of a grafted mouse own revertant fibers by specific anti-Dys Ab or by its own plasma. Serial cryostat sections of the nongrafted TA of mouse B8. *A*, *D*, *G*, and *J* were observed on one muscle section, whereas *M* was observed on another muscle section. NCIDys1 anti-Dys mAb (*left*) labeled two clusters of revertant fibers (*D* and *G*), whereas R27 polyclonal anti-Dys Ab labeled 3 clusters (*B*, *E*, and *H*). The B8 mouse plasma reacted against four clusters (*C*, *F*, *I*, and *L*). The same fibers on serial sections are indicated by arrowheads. Some revertant fibers were split (*M–O*, *asterisk*). Bar, 75 μm.

fibers in the grafted muscle was in some cases >80% of the total fiber number. Myoblast transplantation triggered the formation of hybrid and new Dys-positive muscle fibers. These different fiber categories may be identified by their size 1 mo after grafting. The progressive increase in total number and size of Dys-positive fibers between the first two weeks and the end of the second month indicates that all injected myoblasts do not fuse immediately after injection, but keep proliferating for some weeks. The increase in Dys-positive fibers number may also be due to

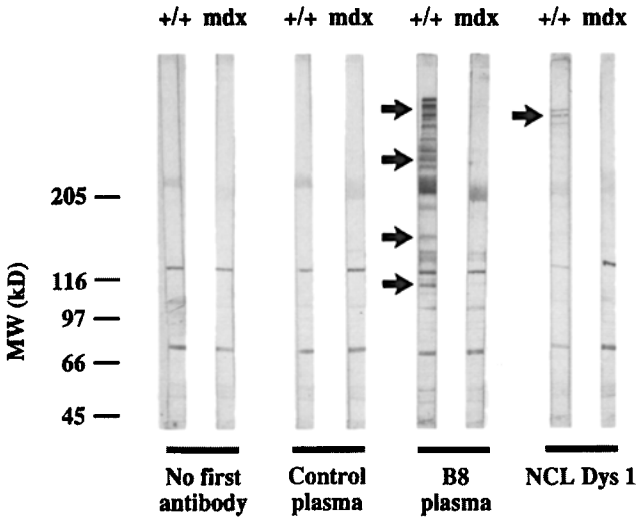

Figure 4. Immunoblot analysis of mouse muscle antigens stained by mouse plasma. NClDys1 mAb specifically reacted with the high molecular weight antigen Dys present in +/+ mouse muscles (*arrow*) and absent from mdx mouse muscles. The plasma from B8 mouse, which strongly reacted in immunohistochemistry, also strongly reacted against some antigens which were present in +/+ muscles and absent from mdx muscles. The molecular weight of these antigens were around 115, 160, 260, and 400 kD (*arrows*). The highest molecular weight antigen migrated to the same place as Dys. The control plasma from a naive, non-transplanted mouse, did not react with these antigens.

Dys accumulation and diffusion. Dys expression, however, is thought to be restricted to nuclear domains (Karpati et al., 1989; Pavlath et al., 1989). Therefore, many injected cells could behave as new satellite cells. Variations in and between groups of transplanted mice are too important to establish whether Dys-positive fibers progressively replaced Dys-negative fibers. Although Dys-positive fibers were obtained with all batches of cell cultures, the D batch produced a consistent lower percentage of Dys-positive cells, indicating some variability in the state of the injected myoblasts.

Rejection reactions may be induced following allotransplantations in both humans and mice by minor histocompatibility antigens (Oppat and Mohanakumar, 1994). In the present study, good transplantation results have likely been obtained because mice compatible for major and minor histocompatibility antigens were used. To short-cut the problem of sex-linked minor histocompatibility antigens (i.e., the HY antigen), male mice were systematically used as recipients, as it is difficult to check exactly the sex of newborn donor mice. Thus, between donor and recipient mice, only full-length Dys was different.

Effect of Irradiation and Notexin on Muscle Regeneration

Except for diaphragm, the skeletal muscles of the young mdx mouse, including the TA muscles under study, do not present the extensive fibrosis and muscle fiber loss observed in Duchenne patients (Louboutin et al., 1993). It has been reported, however, that irradiation and local injection of some snake venoms increased the success of myoblast transplantation in skeletal muscles, probably by suppressing the mitotic activity of host myoblasts and by starting one cycle of degeneration–regeneration in which host myoblast cannot participate (Wakeford et al., 1991; Morgan et al., 1993). Actually, transplantation success was better using irradiation and notexin in mouse than without

Table IV. Molecular Weights of the Normal Muscle Antigens Reacting with Grafted Mice Plasma

Reactivity in histochemistry	Mouse number	Antigens mol wt determination (kD) (1st set of experiments)	Colocalization with Dys (2nd set of experiments)
Positive control	NClDys1	408, 391, 371	yes/R27
	R27	419, 403, 381	yes/NClDys1
Negative	C1	243, 142, 121	ND
	C3	243	ND
	C4	253	ND
	C5	None	ND
	C6	None	ND
	D2	None	ND
	D6	44, 42	ND
Weakly positive	B1	386*, 340, 270, 253	yes
	B2	243	ND
	B4	286, 261, 44, 43	ND
	B6	389, 288, 262, 102, 99, 43, 41	yes
	D1	373, 269, 245, 42	yes
	D5	114	ND
	D7	264, 44	ND
Positive to strongly positive	B3	395, 360*, 260, 243	yes
	B5	389, 379, 321, 238, 103, 43	yes
	B7	405, 389, 330, 300, 273, 251, 238, 42	yes
	B8	437, 414, 386, 377, 361, 283, 268, 162, 114	yes
	C2	424, 403, 377, 271, 53, 45, 43	yes
	D4	379, 277, 251, 238, 42	yes
	D9	400, 389, 273	yes

These plasma were obtained after mouse sacrifice.
*, The bands were very faint on some nitrocellulose strips.
ND, not done.

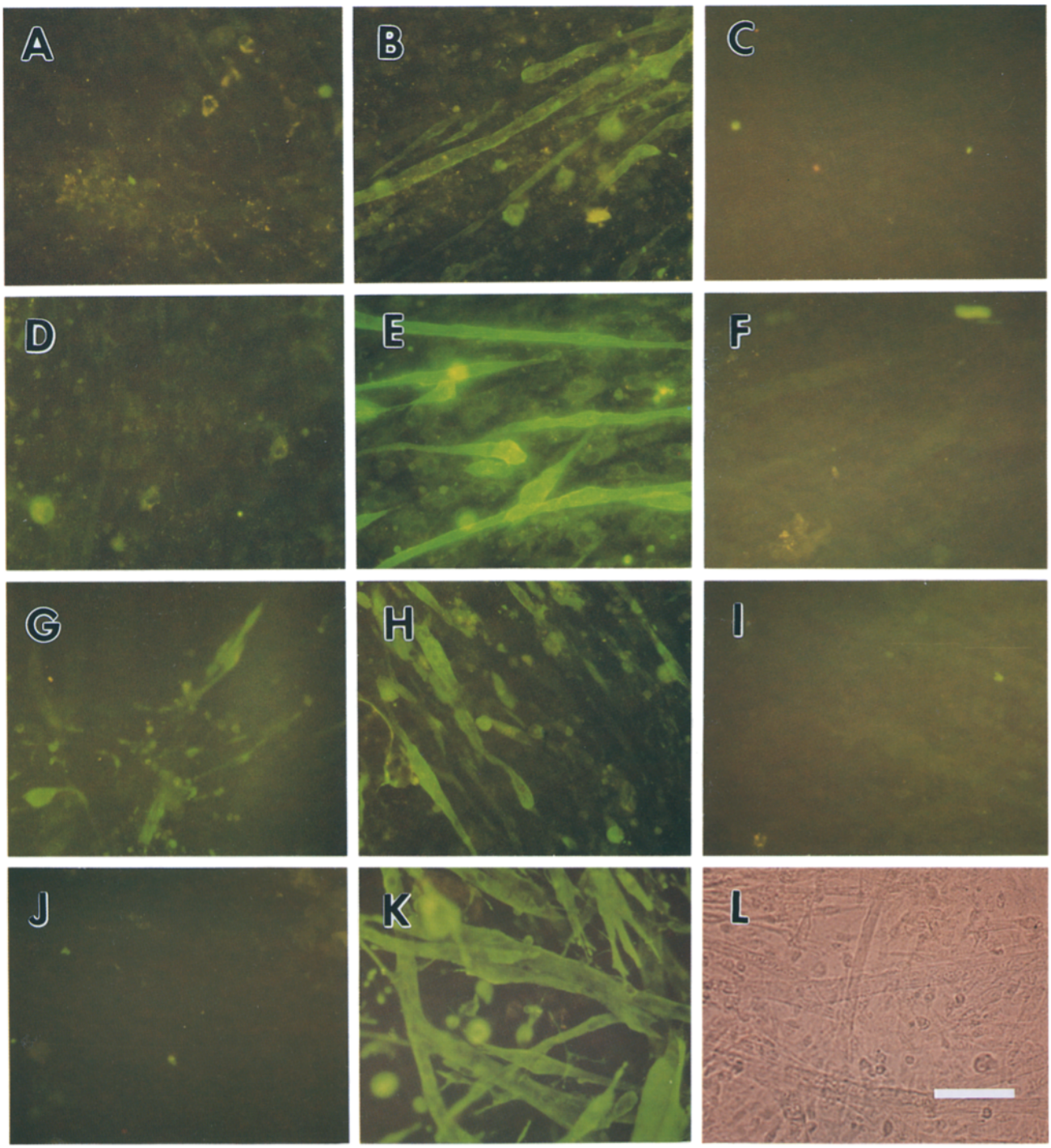

Figure 5. Differential staining of living or permeabilized myotube cultures by specific Ab or mouse plasma. Isogenic myotube cultures were incubated with the specific NCIDys2 mAb (*A–C*), mouse B8 plasma (*D–F*), mouse B3 plasma (*G–I*) or the specific anti-desmin mAb (*J* and *K*). (*Left*) First Ab incubated on living cells; (*middle*) first Ab incubated on permeabilized cells; (*right*) first Ab incubated on permeabilized mdx cells. (*A*, *B*, *D*, *E*, *G*, and *H*) Normal mouse cultures; (*C*, *F*, *I*, *J*, *K*, and *L*) mdx mouse cultures. *L* is a phase contrast image of mdx myotube culture after methanol permeabilization. The specific staining of permeabilized myotubes (*B*, *E*, *H*, and *K*) was not observed when living myotubes were exposed to the Ab (*A*, *D*, *G*, and *J*). In one case (mouse B3), the extracellular part of some myoblasts and myotubes was labeled (*G*), although the intracellular labeling of myotubes was clearly stronger (*H*). Bar, 70 μm.

these pretreatments (personal results). The efficacy of these pretreatments, however, seems to depend on the animal model; for example, notexin seems detrimental in dogs or monkeys (Kinoshita, I., and J.-T. Vilquin, personal results). Even at lower concentrations, notexin necrosis is more extensive in dogs than in mice, where necrosis seems restricted to the injection site (personal results). One may assume that irradiation blocked the regenerative capacities of all the host leg cell types: myoblasts, fibroblasts, chondrocytes, osteoblasts, endothelial, and mesothelial

Table V. Infiltration of Grafted Muscles by Immune Cells

Mouse number	Days after grafting	MAC* (number per field ±SD)	$CD8^+$ cells
B3	68	2.0 ± 2.2	0.15 ± 0.51
B4	68	2.9 ± 2.4	0.17 ± 0.46
B5	162	1.7 ± 1.6	0.23 ± 0.50
B6	162	2.5 ± 4.4	0.18 ± 0.47
B7	239	2.6 ± 3.5	0.18 ± 0.43
C2	101	3.2 ± 4.8	0.07 ± 0.25

*, MAC : MAC1 antigen positive cells. One field represents approximately 1.5×10^5 μm^2.

cells, Schwann cells, and so on. Actually, not only did the TA weight decrease (Table II), but also that of the whole irradiated leg. Thus, in our experimental protocol the result of myoblast transplantation may not be termed muscle regeneration, but rather gene complementation. Some physical or metabolic supports may be lacking for donor cells to sustain full muscle regeneration over a long time course following irradiation.

Anti-Dys Ab Production after Transplantation in Some Mice

In this study, 58% of the mice developed Ab against some antigens present on the membranes of normal muscle fibers but absent from the mdx fibers. These Ab were not produced in some mice and were not detected in the plasma of nongrafted mice, therefore they were allo-Ab, not auto-Ab. The subsequent immunoblot analysis of the antigens recognized by these Ab indicated multiple molecular weights, but in 10 of 14 cases the highest molecular weights corresponded to Dys. It is therefore very likely that these grafted mice developed Ab against Dys. The determination of the nature of the smaller antigens recognized by various plasma would require further investigations.

The Ab appeared slowly after grafting. In some cases, 36 to 75 d were necessary to observe a positive reaction. In the absence of any rejection problem related to major and minor antigens, donor myoblasts slowly colonize part or whole recipient muscle, then they proliferate and/or fuse to form new or hybrid muscle fibers. This process, however, may require at least 1 or 2 wk (Allbrook, 1981; Morgan et al.,1993). As presented in this study, the number of Dys-positive fibers is elevated after only 2 mo. Myoblasts alone do not express Dys to detectable levels (Lev et al., 1987). Dys expression is triggered by muscle differentiation after myoblast fusion (Lev et al., 1987; Scott et al., 1988). Therefore, only myotubes and muscle fibers express the skeletal muscle form of Dys. The immune system of the host may recognize only exogenous Dys after the formation of myotube and/or hybrid fibers, and after they have degenerated once and freed antigens from the inner muscle cells. This degeneration may follow metabolic or mechanic damages. Therefore, it is likely that Dys become fully immunogenic only some weeks after grafting. Then, 3 to 4 wk are necessary before a detectable amount of Ab specific for normal muscle fibers can be seen. Once the reaction is triggered, a low level of continuous stimulation is sufficient to sustain high Ab titers. This stimulation could be due to degeneration–regeneration cycles which certainly take place in grafted animals, because damage in Dys-positive and Dys-negative fibers is a natural phenomenon. The presence of high Ab levels in some cases 8 mo after grafting shows that Dys is continuously presented to the immune system.

Immune Reactions against Autologous Revertant Fibers

Plasma from mice that developed Ab against Dys also reacted against autologous revertant fibers. Mdx mice, depending on their age and breed, present 0.1–1% revertant fibers in their muscles (Danko et al., 1992; Pastoret and Sebille, 1993; Zhao et al., 1993). Revertant, or back-mutated, fibers are believed to result from a punctual, individual somatic mutation of the Dys gene in some myoblasts or satellite cells (Hoffman et al., 1990). The Dys-positive fibers in mdx muscles possess Dys, but sometimes with almost completed size and sometimes with various types of deletions (Zhao et al., 1993). Unfortunately, the polyclonal nature of the mice plasma did not allow to discriminate between the various Dys peptides produced by back-mutation. The presence of Ab directed against revertant fibers could be an immunological paradox. One could assume that a mouse producing back-mutated fibers would become tolerant to Dys and would not produce any Ab against this protein after grafting. The Dys peptides produced after back mutation and presented to the immune system, however, likely do not represent full-length Dys. Second, even if myoblasts can be considered antigen-presenting cells (Goebels et al., 1992), they do not yet express Dys (Lev et al., 1987), and mature muscle fibers do not express class I and class II complexes (Roy et al., 1991; Goebels et al., 1992; Hohlfeld and Engel, 1994). Thus, the production of Ab directed against revertant fibers could be due either to the presentation of irrelevant antigens, or to an absence of antigen presentation.

Absence of Anti-muscle Membrane Ab Production after Transplantation in Some Cases

The lack of Ab directed against normal muscle membranes in 42% of the mice is striking. Dystrophin, even if considered a foreign antigen, is not always capable of triggering Ab formation. If Dys is released in amounts that are too small or in a too discontinuous way, or if the processing or presentation by the antigen presenting cells is inadequate, anti-Dys Ab should not be produced. The parallel between the total numbers of muscle fibers and the production of anti-Dys Ab suggests that an increased number of Dys-positive fibers in the grafted muscle induces a stronger Ab production (Tables I and III). The mean number of Dys-positive fibers in batch B was two to four times higher than that in batches C and D. The strongest Ab reactions were found in group B, which quantitatively expressed more Dys (Table III). Thus, in many cases, the amount of Dys antigens released by the fibers may have been too low to trigger a strong Ab production.

Anti-Dys Ab and the Absence of Rejection

The production of anti-Dys Ab did not lead to rejection of Dys-positive fibers. All the grafts were successful, Dys ex-

pression was sustained over many months, no increase in lymphocyte number was noted, and Dys-positive fibers did not seem to be rejected either at long or short term. Also none of the plasma directed against Dys could induce a complement-dependent lysis of human myotubes in vitro (E. Wagner, personal results).

Dystrophin is an intracytoplasmic, subsarcolemmal protein without transmembrane or extracellular domain (Watkins et al., 1988; Ervasti and Campbell, 1991). Dystrophin is sequestrated in the muscle fibers, whose solid structure is designed to support only a very slow turnover. Thus the absence of Dys-positive fiber destruction could be attributable to a lack of accessibility and thus an absence of recognition of the antigen by the Ab. This hypothesis is confirmed by the differential results obtained in vitro in myotube cultures. When Ab gained access to the cytoskeleton inside the cells following permeabilization, the reaction was clearly positive, whereas the reaction was negative on living non-permeabilized cells, suggesting that the Ab were directed against intracellular antigens. Thus, the recognition of Dys peptides as foreign antigens and the reaction against the intracellular proteins would be two distinct immunological events.

In one clinical trial, human myoblasts compatible for class I and class II (DR) MHC antigens were transplanted in DMD patients (Huard et al., 1992; Tremblay et al., 1993), but histocompatibility was not absolute. Some minor histocompatibility antigens or other immunogenic proteins differences between host and donor could have triggered humoral and/or cellular rejection. In the present experiments, mice were not only histocompatible, but also inbred, and the grafts were not rejected. Thus, we hypothesize that major and minor histocompatibility antigens can trigger rejection following myoblast transplantation, whereas Dys alone cannot.

Concluding Remarks

Histocompatibility, antigen sequestration and slow muscle structure turnover should explain the overall phenomenon reported here. However, access of the immune system to new antigens encoded by normal genomic donor DNA could depend on the availability, location and presentation of these antigens. Immune reactions did not lead to short- or long-term rejection of Dys-containing fibers produced by myoblast-mediated gene complementation in mdx mice, but the situation could be different with other antigens. The data presented in this article should also prove useful in the expanding field of gene therapy where, whatever the vector, the single gene encoding for a non-self-product could lead to immune reactions, with or without associated rejection (Vilquin et al., 1995*b*).

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References

Allbrook, D. 1981. Skeletal muscle regeneration. *Muscle Nerve.* 4:234–245.

Blau, H. M., J. Dhawan, and G. K. Pavlath. 1993. Myoblasts in pattern formation and gene therapy. *Trends Genet.* 9:269–274.

Campion D. R. 1984. The muscle satellite cell: a review. *Int. Rev. Cytol.* 87:225–251.

Cossu, G., B. Zani, M. Coletta, M. Bouche, M. Pacifict, and M. Molinaro. 1980. In vitro differentiation of satellite cells isolated from normal and dystrophic mamalian muscles. A comparison with embryonic myogenic cells. *Cell Differ.* 9:357–365.

Coulton, G. R., J. E. Morgan, T. A. Partridge, and J. C. Sloper. 1988. The mdx mouse skeletal muscle myopathy: I, a histological, morphometric and biochemical investigation. *Neuropath. Appl. Neurobiol.* 14:53–70.

Danko, I., V. Chapman, and J. A. Wolf. 1992. The frequency of revertants in mdx mouse genetic models for Duchenne muscular dystrophy. *Ped. Res.* 32: 128–131.

Ervasti, J. M., and K. P. Campbell. 1991. Membrane organization of the dystrophin-glycoprotein complex. *Cell.* 66:1121–1131.

Goebels, N., D. Michaelis, H. Wekerle, and R. Hohlfeld. 1992. Human myoblasts as antigen-presenting cells. *J. Immunol.* 149:661–667.

Grounds, M., T. A. Partridge, and J. C. Sloper. 1980. The contribution of exogenous cells to regenerating skeletal muscle: an isoenzyme study of muscle allografts in mice. *J. Pathol.* 132:325–341.

Guérette, B., I. Asselin, J. T. Vilquin, I. Kinoshita, R. Roy, and J. P. Tremblay. 1995. Lymphocyte infiltration following allo and xeno myoblast transplantation in mdx mice. *Muscle Nerve.* 18:39–51.

Gussoni, E., G. K. Pavlath, A. M. Lanctot, K. Sharma, R. G. Miller, L. Steinman, and H. M. Blau. 1992. Normal dystrophin transcripts detected in DMD patients after myoblast transplantation. *Nature (Lond.).* 356:435–438.

Harris, J. B., M. A. Johnson, and E. Karlsson. 1975. Pathological responses of rat skeletal muscle to a single subcutaneous injection of a toxin from the venom of the Australian tiger snake, Notechis scutatus scutatus. *Clin. Exp. Pharm. Physiol.* 2:383–404.

Hoffman, E. P., R. H. Brown, and L. M. Kunkel. 1987. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell.* 51:919–928.

Hoffman, E. P., J. E. Morgan, S. C. Watkins, and T. A. Partridge. 1990. Somatic reversion/suppression of the mouse mdx phenotype in vivo. *J. Neurol. Sci.* 99:9–25.

Hohlfeld, R., and A. G. Engel. 1994. The immunobiology of muscle. *Immunol. Today.* 15:269–274.

Huard, J., J. P. Bouchard, R. Roy, C. Labrecque, G. Dansereau, B. Lemieux, and J. P. Tremblay. 1991. Myoblast transplantation produces dystrophin positive muscle fibers in a 16 year old Duchenne patient. *Clin. Sci.* 81:287–288.

Huard, J., J. P. Bouchard, R. Roy, F. Malouin, G. Dansereau, C. Labrecque, N. Albert, C. L. Richards, B. Lemieux, and J. P. Tremblay. 1992. Human myoblast transplantation: preliminary results of four cases. *Muscle Nerve.* 15: 550–560.

Huard, J., R. Roy, B. Guérette, S. Verreault, G. Tremblay, and J. P. Tremblay. 1994*a*. Human myoblast transplantation in immunodeficient and immunosuppressed mice: evidence of rejection. *Muscle Nerve.* 17:224–234.

Huard, J., S. Verreault, R. Roy, and J. P. Tremblay. 1994*b*. High efficacy of muscle regeneration following human myoblast transplantation in SCID mice. *J. Clin. Invest.* 93:586–599.

Karpati, G. 1990. Immunological aspects of histoincompatible myoblast transfer into non-tolerant hosts. *In* Myoblast transfer therapy, R. Griggs and G. Karpati, Eds. Plenum Press, New York. 31–34.

Karpati, G., Y. Pouliot, E. Zubrzycka-Gaarn, S. Carpenter, P. N. Ray, R. G. Worton, and P. Holl. 1989. Dystrophin is expressed in mdx skeletal muscle fibers after normal myoblast implantation. *Am. J. Pathol.* 135:27–32.

Karpati, G., D. Ajdukovic, D. Arnold, R. B. Gledhill, R. Guttmann, P. Holland, P. A. Koch, E. Shoubridge, D. Spence, M. Vanasse, G. V. Watters, M. Abrahamowicz, C. Duff, and R. G. Worton. 1993. Myoblast transfer in Duchenne muscular dystrophy. *Ann. Neurol.* 34:8–17.

Kinoshita, I., J. Huard, and J. P. Tremblay. 1994*a*. Utilization of myoblasts from a transgenic mouse to evaluate the efficacy of myoblast transplantation. *Muscle Nerve.* 17:975–980.

Kinoshita, I., J. T. Vilquin, B. Guérette, I. Asselin, R. Roy, and J. P. Tremblay. 1994*b*. Very efficient myoblast allotransplantation in mice under FK506 immunosuppression. *Muscle Nerve.* 17:1407–1415.

Koenig, M., A. P. Monaco, and L. M. Kunkel. 1988. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell.* 53:219–228.

Labrecque, C., R. Roy, and J. P. Tremblay. 1992. Immune reactions following myoblast transplantation in mouse muscles. *Transplant. Proc.* 24:2889–2892.

Law, P. K., T. G. Goodwin, and H. J. Li. 1988*a*. Histoincompatible myoblast injection improves muscle structure and function of dystrophic mice. *Transplant. Proc.* 20:1114–1119.

Law, P. K., T. G. Goodwin, and M. G. Wang. 1988*b*. Normal myoblast injections provide genetic treatment for murine dystrophy. *Muscle Nerve.* 11:525–533.

Law, P. K., T. E. Bertorini, T. G. Goodwin, M. Chen, Q. Fang, H.-J. Li, D. S. Kirby, J. A. Florendo, H. G. Herrod, and G. S. Golden. 1990. Dystrophin production induced by myoblast transfer therapy in Duchenne muscular dystrophy. *Lancet.* 336:114–115.

Lev, A. A., C. C. Feener, L. M. Kunkel, and R. H. Brown. 1987. Expression of

the Duchenne's muscular dystrophy gene in cultured muscle cells. *J. Biol. Chem.* 262:15817–15820.

Louboutin, J. P., V. Fichter-Gagnepain, E. Thaon, and M. Fardeau. 1993. Morphometric analysis of mdx diaphragm muscle fibres. Comparison with hindlimb muscles. *Neuromusc. Disord.* 3:463–469.

Morgan, J. E., E. P. Hoffman, and T. A. Partridge. 1990. Normal myogenic cells from newborn mice restore normal histology to degenerating muscles of the mdx mouse. *J. Cell Biol.* 111:2437–2449.

Morgan, J. E., C. N. Pagel, T. Sherratt, and T. A. Partridge. 1993. Long-term persistence and migration of myogenic cells injected into pre-irradiated muscles of mdx mice. *J. Neurol. Sci.* 115:191–200.

Morgan, J. E., J. R. Beauchamp, C. N. Pagel, M. Peckham, P. Ataliotis, P. S. Jat, M. D. Noble, K. Farmer, and T. A. Partridge. 1994. Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: a model system for the derivation of tissue-specific and mutation-specific cell lines. *Dev. Biol.* 162:486–498.

Oppat, W. F., and T. Mohanakumar. 1994. Minor histocompatibility antigens. *In* The role of MHC and non-MHC antigens in allograft immunity. T. Mohanakumar, editor. R.G. Landes Company, Austin, TX. 73–111.

Partridge T. A. 1991. Myoblast transfer: a possible therapy for inherited myopathies. *Muscle Nerve.* 14:197–212.

Partridge, T. A., J. E. Morgan, G. R. Coulton, E. P. Hoffman, and L. M. Kunkel. 1989. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature (Lond.).* 337:176–179.

Pastoret, C. and A. Sebille. 1993. Further aspects of muscular dystrophy in mdx mice. *Neuromusc. Disord.* 3:471–475.

Pavlath, G. K., K. Rich, S. G. Webster, and H. M. Blau. 1989. Localization of muscle gene products in nuclear domains. *Nature (Lond.).* 337:570–573.

Pavlath, G. K., T. A. Rando, and H. M. Blau. 1994. Transient immunosuppressive treatment leads to long-term retention of allogeneic myoblasts in hybrid myofibers. *J. Cell Biol.* 127:1923–1932.

Rando, T. A., and H. M. Blau. 1994. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J. Cell Biol.* 125:1275–1287.

Roy, R., G. Dansereau, J. P. Tremblay, M. Belles-Isles, J. Huard, C. Labrecque, and J. P. Bouchard. 1991. Expression of major histocompatibility complex antigens on human myoblasts. *Transplant Proc.* 23:799–801.

Roy, R., J. P. Tremblay, J. Huard, F. Malouin, and J. P. Bouchard. 1993. Antibody formation after myoblast transplantation in Duchenne dystrophic patients, donor HLA compatible. *Transplant Proc.* 25:995–997.

Scott, M., J. E. Sylvester, T. Helman-Paterson, Y. J. Shi, W. Fieles, H. Stedman, A. Burghes, P. Ray, R. Worton, and K. H. Fischbeck. 1988. Duchenne muscular dystrophy gene expression in normal and diseased human muscle. *Science (Wash. DC).* 239:1418–1420.

Sicinski, P., Y. Geng, A. S. Ryder-Cook, E. A. Barnard, M. G. Darkinson, and P. J. Barnard. 1989. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science (Wash. DC).* 244:1578–1580.

Tremblay, J. P., F. Malouin, R. Roy, J. Huard, J. P. Bouchard, A. Satoh, and C. L. Richards. 1993. Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy. *Cell Transplantation.* 2:99–112.

Vilquin, J. T., I. Asselin, B. Guérette, I. Kinoshita, S. Lille, R. Roy, and J. P. Tremblay. 1994. Myoblast allotransplantation in mice: the degree of success varies depending on the efficacy of various immunosuppressive treatments. *Transplant. Proc.* 26:3372–3373.

Vilquin, J. T., I. Asselin, B. Guérette, I. Kinoshita, R. Roy, and J. P. Tremblay. 1995*a*. Successful myoblast allotransplantation in mdx mice using rapamycin. *Transplantation.* 59:422–426.

Vilquin, J. T., S. Braun, P. Labouret, G. Zuber, C. Tranchant, P. Poindron, and J. M. Warter. 1992. Specific effect of corticoids on acetylcholine receptor expression in rat skeletal muscle cell cultures. *J. Neurosci. Res.* 31:285–293.

Vilquin, J. T., B. Guérette, I. Kinoshita, B. Roy, M. Goulet, C. Gravel, R. Roy, and J. P. Tremblay. 1995*b*. FK506 immunosuppression to control the immune reactions triggered by first generation adenovirus-mediated gene transfer. *Hum. Gene Ther.* 6:1391–1401.

Vilquin, J. T., I. Kinoshita, R. Roy, and J. P. Tremblay. 1995*c*. Cyclophosphamide immunosuppression does not permit successful myoblast allotransplantation in mouse. *Neuromusc. Disord.* In press.

Wakeford, S., D. J. Watt, and T. A. Partridge. 1991. X-irradiation improves mdx mouse muscle as a model of myofiber loss in DMD. *Muscle Nerve.* 14: 42–50.

Watkins S. C., E. P. Hoffman, H. S. Slayter, and L. M. Kunkel. 1988. Immunoelectron microscopic localization of dystrophin in myofibres. *Nature (Lond.).* 333:863–866.

Watt, D. J., K. Lambert, J. E. Morgan, T. A. Partridge, and J. C. Sloper. 1982. Incorporation of donor muscle precursor cells into an area of muscle regeneration in the host mouse. *J. Neurol. Sci.* 57:319–331.

Watt, D. J., J. E. Morgan, and T. A. Partridge. 1984. Long term survival of allografted muscle precursor cells following a limited period of treatment with cyclosporin A. *Clin. Exp. Immunol.* 55:419–426.

Watt, D. J., J. Karasinski, and M. A. England. 1993. Migration of lacZ positive cells from the tibialis anterior to the extensor digitorum longus muscle of the X-linked muscular dystrophic (mdx) mouse. *J. Muscle Res. Cell Motil.* 14: 121–132.

Zhao, J. E., K. Yoshioka, T. Miike, and M. Miyatake. 1993. Developmental studies of dystrophin-positive fibers in mdx, and DRP localization. *J. Neurol. Sci.* 114:104–108.