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Successful Transplantation of Genetically Corrected DMD Myoblasts Following *ex Vivo* Transduction with the Dystrophin Minigene[1]

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Myoblast transplantation and gene therapy are two promising therapeutical approaches for the treatment of Duchenne Muscular Dystrophy (DMD). So far, both strategies have met many hurdles, mainly because of immune reactions. In this study, we investigated a third and novel strategy based on the combination of these two basic ones, i.e., transplantation of genetically modified myoblasts. We first derived a primary culture from a muscle biopsy of a young DMD patient (3 years old). Adenoviral-mediated dystrophin gene transfer into these DMD cultures and expression of the dystrophin transgene were achieved *in vitro*. The transduced cultures were then transplanted the same day in immunodeficient SCID mouse muscles. Three weeks following the graft, many human dystrophin-positive fibers were observed throughout sections of the injected muscles. However, many fibers expressed human MHC antigens without expressing human dystrophin due to the low percentage of infected primary muscle cells *in vitro* (even when a high MOI [400] was used) and to a reduction and even to a complete loss of transgene copy number during myoblast replication. From our results, we conclude that, although not at a high proportion, (1) DMD primary myoblast cultures are infectable by adenoviruses; (2) they can be efficiently transplanted back in a muscle, leading to normal fusion of infected myoblasts with the host fibers; and (3) they can correct the dystrophin deficiency in the host fibers by the expression of a mini-dystrophin transgene.

Myoblast transplantation (MT) has been proposed for a number of years as a potential treatment for Duchenne Muscular Dystrophy (DMD). Up to the present time, many research groups have been involved in clinical trials where dystrophin-positive (Dys^+) myoblasts from healthy donors were transplanted in the muscles of dystrophic patients (1-5). Regretfully, all of these studies have had very limited success, even when donor and recipient major histocompatibility complex antigens (MHCs) were identical (3, 5). These poor results have been attributed 1) to immune responses raised against donor myoblasts or hybrid fibers formed by the fusion of donor myoblasts and host fibers, 2) to the limited migration of the myoblasts from the transplantation site (6) and 3) to the massive myoblast death (90%) during the first five days following transplantation (7-9). Nonetheless, immune reactions are thought to be the main obstacle to this strategy. This has been supported by the fact that, although myoblast allotransplantation is not successful in untreated animals, it has been achieved successfully in immunosuppressed animals (10-14). Moreover, it has been shown in a recent study that minor antigens can lead to the slow rejection of fibers formed only in part by the donor myoblasts (15). Indeed, rejection occured in non-immunosuppressed animals even when the donor and recipient MHCs were identical. It thus seems that adequate and sustained immunosuppression is unavoidable to

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Abbreviations used: AAV, adeno-associated virus; CMV, cytomegalovirus; DMD, Duchenne Muscular Dystrophy; Dys, dystrophin; HLA, human leukocyte antigen; MHC, major histocompatibility complex; mini-Dys, dystrophin minigene; MT, myoblast transplantation; MOI, multiplicity of infection; SCID, severe combined immunodeficiency; TA, tibialis anterior.

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obtain efficient MT when myoblasts are obtained from a healthy donor. On the other hand, the use of long-term immunosuppression has been shown to have considerable side-effects like nephro- and neurotoxicity (16) combined with a high risk of infection. Gene therapy has also been investigated as experimental approach for the treatment of DMD (17-21). Although genetic engineering has greatly improved the vectors used to transduce eukaryotic cells *in vivo*, it hardly could be used efficiently for the treatment of DMD for the following reasons. First, muscles fibers are not directly infected with viral vectors because the viral receptors are blocked by the basal membrane that surrounds muscle fibers (22). Viral vectors thus have to infect myoblasts which then fuse with the muscle fibers to introduce the transgene. Secondly, DMD patients which are older than 5 or 6 years-old have a greatly reduced myoblast pool due to extensive muscle fiber degeneration/regeneration cycles. Consequently, even if all the myoblasts were transduced *in vivo*, their relatively small number would be insufficient to increase the patient strength and alleviate the disease. A feasible treatment could, however, combine both gene therapy and autologous MT by correcting the dystrophin deficiency in myoblasts *ex vivo* and by transplanting them back to the patient. The use of autologous MT could bypass the immune reactions against major and minor antigens on myoblasts (23). Hence, a protocol must be defined to collect the patient myoblasts, expand them in culture, efficiently transduce them *in vitro* and lastly, transplant the cells back in the patient dystrophic muscle. This short article reports on (1) efficient transduction of a dystrophin minigene construct in DMD myoblasts by a first-generation adenoviral infection, (2) successful transplantation of the modified myoblasts in immunodeficient SCID mice and (3) normal dystrophin expression pattern *in vivo*.

MATERIALS AND METHODS

Cell culture. A muscle biopsy was obtained from a 3 year-old DMD patient and enzymatically-treated with collagenase and trypsin (24). The resulting primary cells were cultured in MCDB 120 medium containing 15% Donor Calf Serum (Gibco-BRL) and 20 ng/ml basic fibroblast growth factor (bFGF). The ΔE1-ΔE3 adenoviral vector AdCMV-Dys (containing a human mini-dystrophin gene (25)) was produced and purified as described elsewhere (26). A previously frozen adenovirus stock solution of 10^{10} pfu/ml was used to infect low-passage cultures at a MOI of 400. The cells were transplanted 2 hours following the infection.

Transplantations. Three months-old BALB/c SCID mice were purchased at Jackson Laboratories. (The left hind legs of the SCID mice were irradiated (20 Gy) three days before transplantation. This dose of irradiation has been already shown to inhibit the proliferation and thus reduce competition of the host myoblasts (27-29). The day before transplantation, fiber necrosis was induced in the left tibialis anterior (TA) with 7 μl of notexin (5 μg/ml), injected directly in the muscle after surgical exposure (30). The next day, 3×10^6 infected cells were collected, pelleted and resuspended in approximately 15 μl of HBSS. This volume was then injected in approximately 20 sites of the left TA in 3 SCID mice.

Dystrophin, HLA class I and Spectrin immunocytofluorescence. Dystrophin was detected in cultured myoblasts by NCLDys3, a specific antibody for human dystrophin (1:6; Novocastra Laboratories) followed by a secondary anti-mouse antibody coupled to FITC (1:100; Dako). Dystrophin was immunolabelled in the mouse muscle with the same primary antibody followed by an anti-mouse antibody conjugated with horseradish peroxidase (1:100; Dako). To detect specifically the presence of human cells within our grafts, we used antibodies specific for HLA class I and human spectrin. HLA class I was detected by the W6/32 mouse monoclonal antibody (1:100; ATCC) followed by a biotinylated anti-mouse antibody (1:100; Dako) and Streptavidin-Cy3 (1:700; Sigma). The labelling of human spectrin was done with NCL-SPEC-1 antibody (1:100; Novocastra) and a FITC coupled anti-mouse antibody (1:100; Dako). To evaluate the amount of myoblasts/myotubes in the DMD primary muscle cultures, we immunostained the cells for desmin with the monoclonal DE-R-11 antibody (1:40; Dako) followed by a biotinylated anti-mouse antibody (1:100) and Streptavidin-Cy3 (1:700).

Dystrophin and β-actin RT-PCR amplifications. Mini-Dys mRNA was monitored in culture by reverse transcriptase (RT)-PCR. The cells were lysed in culture and the total RNA was isolated in TRIZol (Gibco-BRL). Two primer sets were used to amplify in the same reaction β-actin and dystrophin mRNAs. The dystrophin 5′ primer (5′-CCAAACTAGAAATGCCATCTTC-3′) corresponds to nucleotides 7578 to 7599 of the normal human dystrophin cDNA (GenBank accession no. M18533, M17154, M18026). The dystrophin 3′ primer (5′-TCTGAATTCTTTCAATTCGAT-3′) corresponds to nucleotides 7863 to 7883 in normal human dystrophin cDNA. Primers for β-actin amplification (5′-GTGGGCCGCTCTAGGCACCAA-3′) and (5′-CTCTTTGATGTCACGCACGATTTC-3′) were described elsewhere (31). 200 ng of RNA was first reverse-transcribed by MoMLV RT and further amplified by Tth polymerase (Boehringer Mannheim).

RESULTS AND DISCUSSION

DMD primary cultures were immunostained for desmin expression to evaluate the amount of myogenic cells (Fig. 1A). Approximately 90% of the culture was found to be desmin-positive indicating a high myogenic potential. These cells were infected *in vitro* with the adenoviral vector (Ad-CMV-Dys) at a MOI of 400. After two days, the cells reached confluency and started to form small myotubes. Dystrophin was detected by immunofluorescence 5 days post-infection, when a sufficient number of myotubes was seen in the culture. As illustrated (Fig. 1C), approximately 20% of the cells in the cultures were Dys^+, while no Dys^+ cells were observed in the non-infected controls (Fig 1B). Surprisingly, many mononuclear cells expressing dystrophin were observed (Fig. 1C), a phenomenon that was not observed when primary mouse cells were infected with the same adenoviral vector, i.e., only mouse myotubes were positively stained (Moisset et al., submitted). The amount of dystrophin mRNA expressed in control and infected cultures was quantified by RT-PCR (Fig. 2A) using β-actin mRNA as a normalizing PCR product. A marked overexpression (approximately 8 times) of dystrophin mRNA was measured in the infected cul-

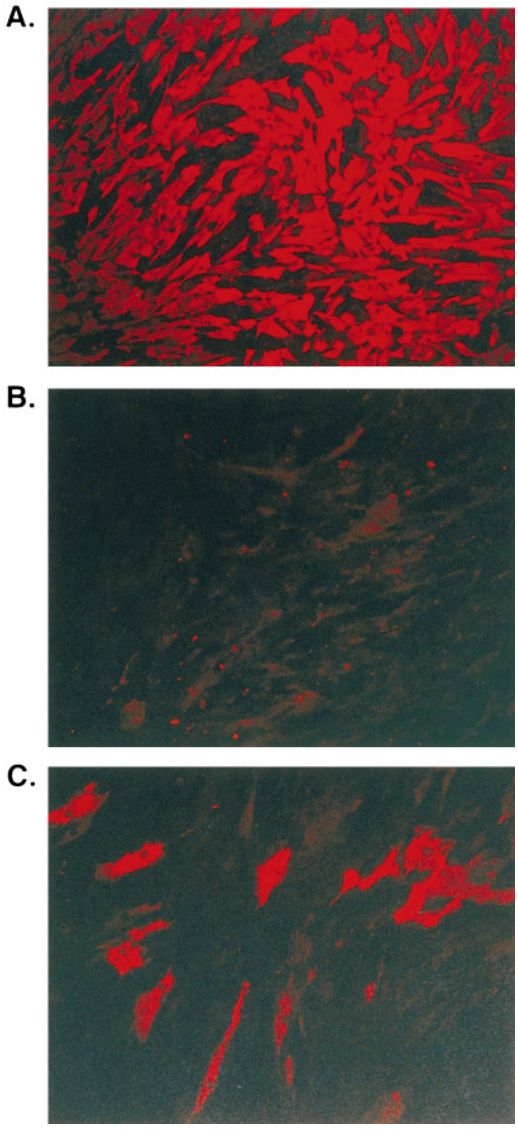

FIG. 1. DMD myoblasts in culture. The high percentage of myogenic cells in the DMD culture is shown in (A) by desmin immunostaining. Dystrophin immunofluorescence in control (B) and infected DMD (C) cultures 5 days after differentiation medium was added.

tures (Fig. 2B) compared to the low level expression of the control cultures.

Control and infected cultures were transplanted as described above in SCID mice. Three weeks after transplantation of the infected DMD cultures, many clusters of fibers expressing human dystrophin were observed throughout the muscles (Fig. 3B). Nevertheless, many fibers expressing the HLA class I antigen without expressing human dystrophin were observed close to clusters of human Dys^+ fibers (Fig. 3D). This is probably due to the fact that only 20% of the myoblasts/myotubes observed in culture were human Dys^+. Moreover, many mononuclear cells of human origin (human Dys^-, HLA I^+, human $spectrin^-$) were also seen surrounding the clusters of fibers expressing human dystrophin, indicating the survival of at least some non-fusing cells in the mouse muscle (Fig. 3F). No human Dys^+ fibers were found in the muscles grafted with control DMD cultures (Fig. 3A). However, clusters of HLA^+ and human $spectrin^+$ fibers were found indicating that fusion of Dys^- DMD myoblasts still occurs *in vivo* (Fig. 3 C, E). Furthermore, fibers expressing HLA and human spectrin were found in similar numbers in muscles transplanted with control or infected myoblasts arguing against a toxic effect of the adenoviral infection (Fig. 3 C-F).

Taken together, the above results show that transplantation of *ex vivo* modified myoblasts is feasible. However, it has to be underlined that the experiments were made in the best possible conditions (irradiation, notexin and immunodeficient animals). This technique

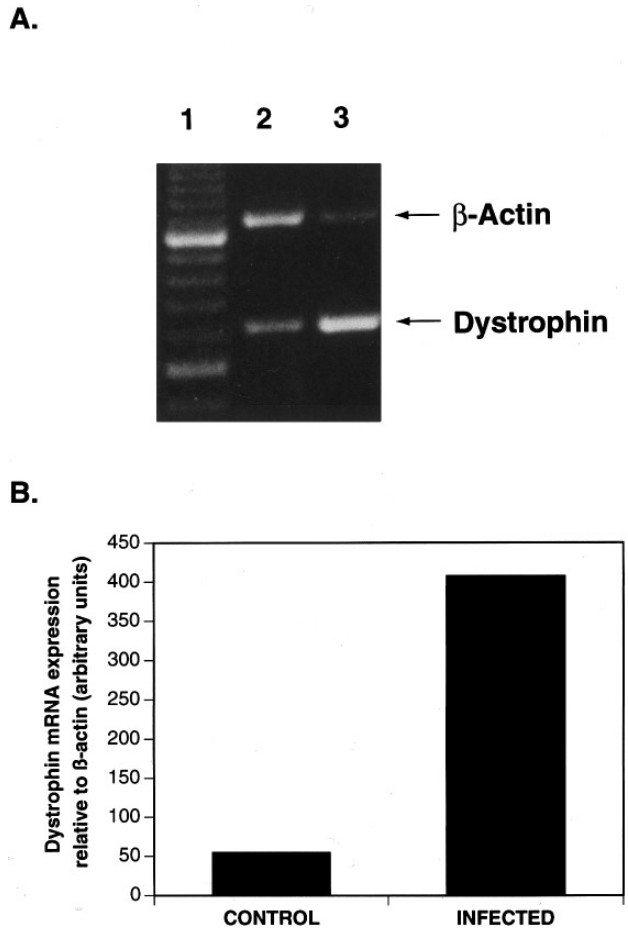

FIG. 2. RT-PCR detection of dystrophin mRNA in differentiated control and infected DMD cultures. (A) Lane 1, molecular weight marker; lane 2, control DMD culture; lane 3, infected DMD culture. (B) Relative densitometric levels of dystrophin mRNA in control and infected cultures (normalized with β-actin).

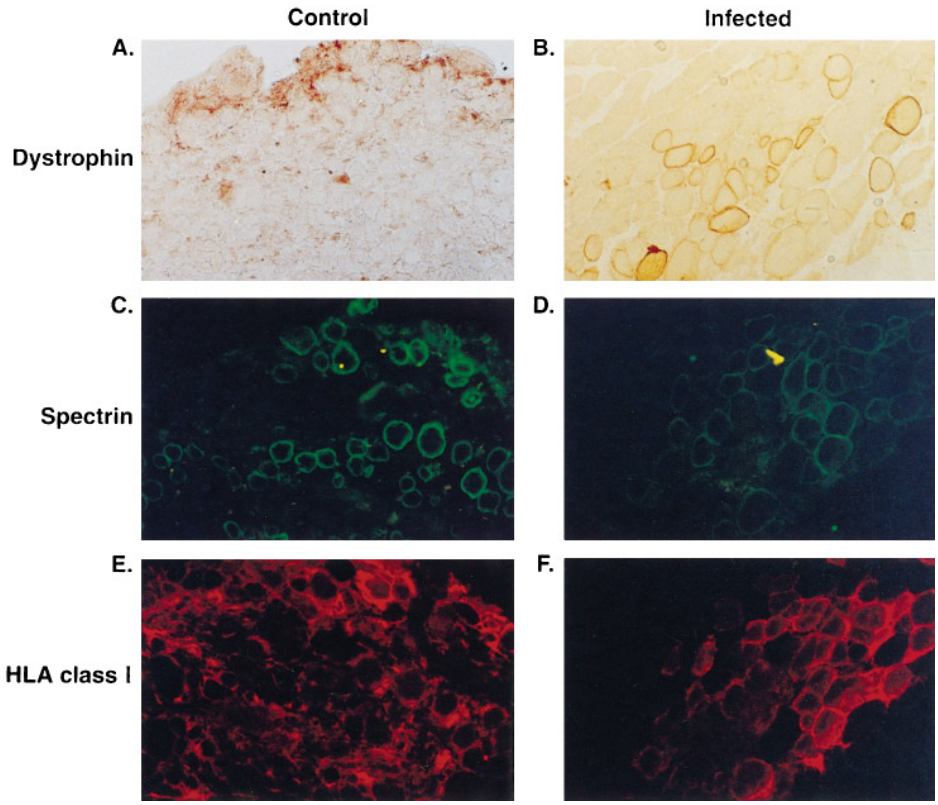

FIG. 3. Transplantation results in SCID mice. Human dystrophin (A, B), human spectrin (C, D), and HLA class I (E, F) expression patterns were observed in mice grafted with control (A, C, E) and infected (B, D , F) DMD myoblasts 3 weeks posttransplantation.

could hardly be directly transposed to immunocompetent animals without immunosuppression, mainly because of the use of first-generation adenoviral vectors which express low levels of early and late adenoviral antigens (32-34). Promising new vectors containing no viral sequences (gutless adenoviruses) or having a reduced expression of viral genes (ΔE1/ΔE4) have been described in many studies (19, 20, 35). Those vectors will probably allow simultaneously an efficient delivery of transgenes and a significantly reduced immune reaction to the transduced cells. Recent data in *ex vivo* gene tranfer has been published using gutless adenoviruses (third-generation adenoviruses) in immunocompetent animals (36). Although the latter study has shown successful transduction of full-length dystrophin in mdx muscles, the amount of dystrophin-positive fibers was considerably reduced 20 days later. Moreover, the presence of immune cells around the transplantation site indicated an ongoing rejection of the infected cells/fibers possibly due to the presence of helper virus in the gutless virus preparation. Thus, problems still arise even with third-generation viruses for *in vivo* or *ex vivo* gene transfer. Increasing interest has also been directed on recombinant adeno-associated viruses (AAV) as they can infect and stably express the transgene in both post-mitotic and dividing cells with high efficiency (37). However, their small size limit the length of the insertable transgene (maximum of 4.5 Kb; (38)). To insert a DNA sequence that could complement the dystrophin deficiency in AAV, one would have to either modify the capsid of AAV vectors to insert larger DNA fragments or to engineer a novel mini-dystrophin gene that would be even shorter. An efficient therapeutical procedure for DMD patients, one that does not involve long-term use of immunosuppressive drugs, will probably combine the basic protocol used in this paper and these new generation viral vectors.

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