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Dystrophin Expression in Myofibers of Duchenne Muscular Dystrophy Patients Following Intramuscular Injections of Normal Myogenic Cells

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Three Duchenne muscular dystrophy (DMD) patients received injections of myogenic cells obtained from skeletal muscle biopsies of normal donors. The cells (30×10^6) were injected in 1 cm^3 of the tibialis anterior by 25 parallel injections. We performed similar patterns of saline injections in the contralateral muscles as controls. The patients received tacrolimus for immunosuppression. Muscle biopsies were performed at the injected sites 4 weeks later. We observed dystrophin-positive myofibers in the cell-grafted sites amounting to 9 (patient 1), 6.8 (patient 2), and 11% (patient 3). Since patients 1 and 2 had identified dystrophin-gene deletions these results were obtained using monoclonal antibodies specific to epitopes coded by the deleted exons. Donor dystrophin was absent in the control sites. Patient 3 had exon duplication and thus specific donor-dystrophin detection was not possible. However, there were fourfold more dystrophin-positive myofibers in the cell-grafted than in the control site. Donor-dystrophin transcripts were detected by RT-PCR (using primers reacting with a sequence in the deleted exons) only in the cell-grafted sites in patients 1 and 2. Dystrophin transcripts were more abundant in the cell-grafted than in the control site in patient 3. Therefore, significant dystrophin expression can be obtained in the skeletal muscles of DMD patients following specific conditions of cell delivery and immunosuppression.

Key Words: cell transplantation, Duchenne muscular dystrophy, dystrophin, myogenic cell, skeletal muscle, tacrolimus

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked genetic myopathy characterized by progressive skeletal-muscle degeneration, leading to muscle weakness in childhood, severe paresis in adolescence, and death in the 20s. The only treatments available being palliative, the prognosis of this disease remains unchanged. Since the molecular origin of the disease is a severe deficiency of dystrophin, a subsarcolemmal protein that seems to accomplish a protective role in membrane integrity [1], dystrophin restoration in myofibers is the objective of most experimental strategies addressed to its potential treatment, by pharmacological-, gene-, and cell-based approaches [2]. While some animal experiments in the past years have been considered promising, none of the protocols tested in DMD patients proved to produce systematically significant levels of dystrophin expression [3–8]. In the present study, we report the systematic increase in dystrophin expression in skeletal muscles of three DMD patients who participated in a current clinical trial of myogenic-cell allotransplantation.

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RESULTS AND DISCUSSION

Three DMD patients, with identified mutations in the dystrophin gene (Table 1), received injections of myogenic cells obtained from a tissue culture of a skeletal muscle biopsy of a normal donor (the patient's father in each case). We injected 30×10^6 cells in less than 1 cm^3 of the left tibialis anterior muscle (TA) in about 25 parallel injections. As a control, we injected the right TA in a similar way but using a saline solution instead of a cell suspension. The three patients received tacrolimus for immunosuppression. Patients 1 and 2 were previously taking deflazacort to delay the progression of the disease and we maintained this treatment during the study. Four weeks after the cell implantation, we performed muscle biopsies in the cell/saline-injected sites, which were analyzed by histology (general structure, dystrophin immunodetection, evidence of acute rejection) and RT-PCR (dystrophin-transcript detection). We performed this study with the informed consent of the patient's parents and the assent of the patients, in accordance with a protocol approved by Health Canada and the Ethical Committee for the Clinical Research of the Laval University Hospital Center.

The histological analysis of the biopsies showed in all the cases skeletal muscle exhibiting features of a dystrophic process: myofibers with a great increase in diameter variation, rounded profiles, splitting, necrosis, and regeneration, together with moderate fibrosis and fat infiltration. We used the NCL-DYS3 antibody to detect dystrophin in all the biopsies; this antibody recognizes an epitope near the N-terminal portion of the molecule. All the biopsies exhibited NCL-DYS3-positive myofibers, both in saline-injected and in cell-grafted sites; however, their relative number was much higher in the cell-grafted sites.

In the biopsies of patient 1, we were able to identify specifically the donor dystrophin with the MANEX45A antibody, which reacts with an epitope of dystrophin coded by exon 45 (deleted in this patient). Many MANEX45A-positive myofibers were present in the cell-grafted site (Figs. 1a and 1b), while they were absent from the saline-injected site (Fig. 1c). The percentages of MANEX45A-positive myofibers are indicated in Table 1.

In the biopsies of patient 2, we were able to identify specifically the donor dystrophin using the MANEX50 antibody, which reacts with a dystrophin epitope coded by exon 50 (deleted in this patient). Many MANEX50-positive myofibers were present in the cell-grafted site (Figs. 1d and 1e), while they were absent from the saline-injected site (Fig. 1f). The percentages of MANEX50-positive myofibers are indicated in Table 1. The use of these specific antibodies confirmed that the few NCL-DYS3-positive myofibers observed in the saline-injected sites (1% in patient 1 and 0.4% in patient 2) corresponded to the expression of ''revertant'' dystrophin, i.e., the truncated but functional dystrophin expressed in rare myofibers of DMD patients due to a second occasional mutation that restores the in-frame translation of the protein [9].

Since the dystrophin-gene mutation responsible for the dystrophin deficiency in patient 3 was an exon duplication, specific immunodetection of a donor-dys-

TABLE 1: Brief clinical data of the patients and results

Patient	1	2	3
Age	10 years	16 years	8 years
Dystrophin-gene mutation	Exon 45 deletion	Exon 50 deletion	Exons 45–50 duplication
Functional status	Still ambulant	Wheel-chair dependent	Still ambulant
HLA haploidentity (patient)	A2, A33, B44, C5, Dr1, Dr4, Dq5, Dq7	A1, A29, B8, C7	A2, A31, B50, B51, Dr4, Dr7, Dq2, Dq7
HLA haploidentity (donor)	A2, A29, B44, C5, Dr4, Dr7, Dq2, Dq3	A1, A11, B8, C7	A29, A31, B51, B56, Dr4, Dr8, Dq4, Dq7
NKH-1-positive cells among the donor cells (two batches tested)	82–93%	79.5–83%	89–97%
Corticoid treatment	Deflazacort	Deflazacort	None
Range of tacrolimus blood concentrations (μg/L)	8.3–16.2	11.1–26.4	7.9–12.9
Percentage of dystrophin-positive myofibers in the cell-grafted site[a]	MANEX45A: 9% (242/2698)	MANEX50: 6.8% (306/4500)	DYS3: 11% (662/6050)
Percentage of dystrophin-positive myofibers in the saline-injected site[a]	MANEX45A: 0%	MANEX50: 0%	DYS3: 2.8% (69/2373)
Number of dystrophin-positive small myofibers or myotubes not included into fascicles[b]	MANEX45A: 510	MANEX50: 155	No
Histological evidence of acute rejection	Some lymphocyte accumulation	Rare lymphocyte accumulation	No
Antibodies against donor cells	No	No	No

[a] The numbers included in parentheses correspond to dystrophin-positive myofibers versus total myofibers.
[b] Not considered for the percentage count.

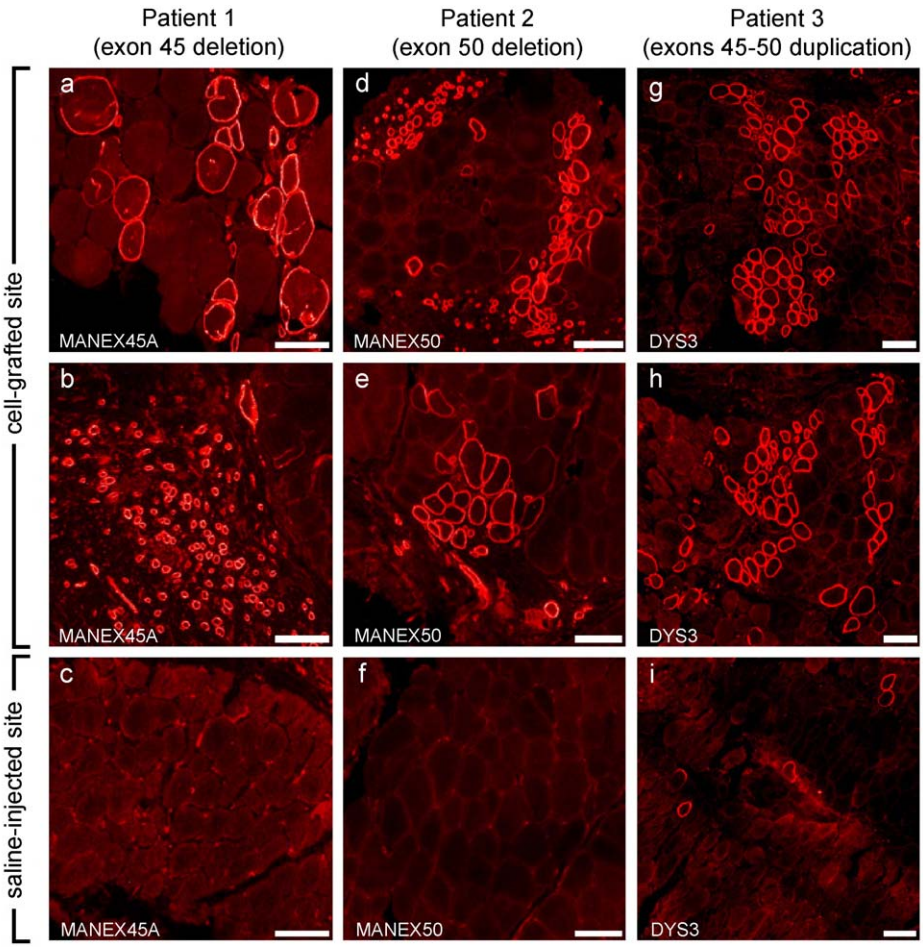

FIG. 1. Dystrophin immunodetection in the muscle biopsies of the DMD patients 4 weeks after the intramuscular injection of normal myogenic cells. The results corresponding to each patient are represented by column. In patient 1, we used the MANEX45A antibody (a–c) to detect specifically an epitope coded by exon 45, which was deleted in this patient and present in the donor. In the cell-grafted site, donor-dystrophin expression is observed both (a) in many large myofibers inside the muscle fascicles and (b) in many small-size myofibers or myotubes forming accumulations between the muscle fascicles. (c) No MANEX45A-positive myofibers are observed in the saline-injected site. In patient 2, we used the MANEX50 antibody (d–f) to detect specifically an epitope coded by exon 50, which was deleted in this patient and present in the donor. (d, e) In the cell-grafted site, donor-dystrophin expression is observed both in many large myofibers inside the muscle fascicles and in some small-size myofibers or myotubes. (f) No MANEX50-positive myofibers are observed in the saline-injected site. Since patient 3 had exon duplication instead of deletion, specific donor-dystrophin detection was not possible and only the NCL-DYS3 antibody was used to detect dystrophin (g–i). (g, h) In the cell-grafted site, donor-dystrophin expression is observed in abundant large myofibers inside the muscle fascicles. (i) Scarce revertant DYS3-positive myofibers were observed in the saline-injected site. The bars in the histological plates correspond to 100 μm.

trophin epitope was not possible. However the percentage of NCL-DYS3-positive myofibers in this case was fourfold higher in the cell-transplanted (Figs. 1g and 1h, Table 1) than in the saline-injected site (Fig. 1i, Table 1). This large difference in the percentages of dystrophin-positive myofibers, together with the pattern of their distribution (along parallel tracks correlating with the trajectories of cell injections), supports that most of them were produced by the fusion of the injected cells with the host myofibers reached by the injections. This pattern of donor-protein-positive myofiber distribution was similar to that previously reported following myogenic-cell injections in nonhuman primates [10,11].

It must be noted that we observed two different populations of dystrophin-positive myofibers in patients 1 and 2: (a) large myofibers (i.e., with a mean diameter similar to that of the dystrophin-negative myofibers) integrated into the fascicles (Figs. 1a, 1e, 1g, and 1h) and (b) very small myofibers, generally dispersed into a connective tissue independent of the muscle fascicles (Figs. 1b and 1d, quantified in Table 1). Based on previous experiments in animal models, the most likely hypothesis to explain this observation is that the first group corresponds to fusion of donor myogenic cells with preexisting myofibers, while the second corresponds to fusion of the donor myogenic cells among

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themselves. Only the first group was taken into account to determine the percentage of dystrophin-positive myofibers, because the functional importance of the second group is uncertain.

We also performed immunodetection of α-sarcoglycan as an attempt to confirm whether this *"de novo"* dystrophin expression was associated with restoration of proteins from the dystrophin-associated complex. We observed α-sarcoglycan expression in all the grafted sites, following the same pattern of distribution as dystrophin-positive myofibers (Fig. 2).

In addition to the immunodetection of dystrophin, we did RT-PCR tests to detect the presence of dystrophin transcripts in the muscle biopsies. In the cases of patients 1 and 2, specific detection of the donor-dystrophin transcripts was possible because one of the primers used in each case corresponded to the deleted exon in the patient (Figs. 3a and 3d). With this strategy, we detected specific donor-dystrophin transcripts in the cell-grafted sites of patients 1 and 2, while they were absent from the saline-injected sites (Figs. 3b, 3c, 3e, and 3f). The presence of exon duplication in patient 3 did not allow the specific detection of a donor-dystrophin transcript (Fig. 3g). Despite this, dystrophin transcripts were barely detectable in the saline-injected site, while they were more abundant in the cell grafted site (Figs. 3h and 3i). Therefore, the detection of dystrophin transcripts by RT-PCR was coincident with the immunohistological observations. The sizes of the amplified products for the cell-grafted sites corresponded exactly to the product sizes obtained from the donor and to a dystrophin control plasmid (Figs. 3b, 3e, and 3h).

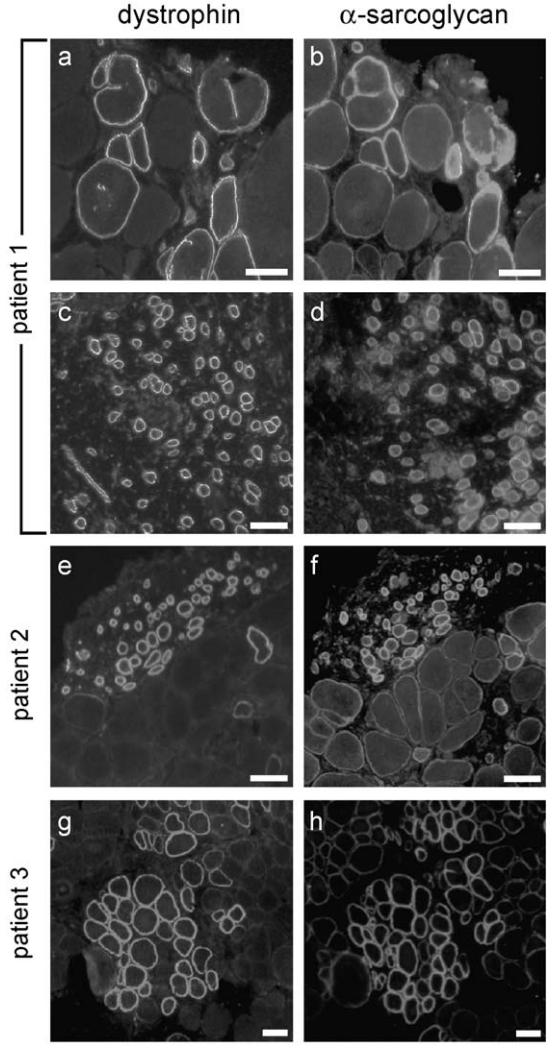

FIG. 2. (a, c, e, g) Immunodetection of dystrophin in the cell-grafted sites is correlated with (b, d, f, g) α-sarcoglycan expression. Monoclonal first antibodies were (a, c) MANEX45A, (e) MANEX50, (g) NCL-DYS3, and (b, d, f, h) NCL-a-SARC. The bars in the histological plates correspond to 50 μm.

Since our protocol to induce the expression of dystrophin in the DMD skeletal muscles was based on a cell allotransplantation, an evaluation of the effectiveness of the immunosuppression to control the acute rejection of the donor cells is of importance. The histological sections of the cell-injected site exhibited some small pockets of lymphocyte infiltration composed by $CD4^+$ and $CD8^+$ cells in patient 1, scarce and small $CD4^+$ and $CD8^+$ cell accumulations in patient 2, and no lymphocyte accumulations in patient 3. Detection of antibodies against the donor cells was negative in the three cases. The only potential side effect of the immunosuppression was some weight increase in patient 3.

Therefore, our study confirms that specific conditions of cell delivery and immunosuppression can produce donor-dystrophin expression in DMD skeletal muscles. These conditions were not used in the unsuccessful former clinical trials of myogenic cell transplantation [3–6,12,13], of which only one study unequivocally detected significant levels of donor dystrophin (10% of the myofibers in the muscle biopsy) in 1 DMD patient of 12 injected with normal myogenic cells [4]. Our homogeneous positive results can be attributed to a better control of acute rejection than in the previous studies (using tacrolimus-based immunosuppression) and/or a better protocol of cell injection (combining closer cell injections with higher number of donor cells by muscle volume).

We used tacrolimus in the present study because previous experiments showed that this was the best immunosuppressant for myogenic-cell implantation in mice [14] and monkeys [10,11,15]. The advantage of tacrolimus over cyclosporin (used for immunosuppression in many of the previous myogenic-cell transplantation clinical trials [4–6]) could be supported by the observation that cyclosporin inhibits the fusion and differentiation of myogenic cells and induces apoptosis when they start to differentiate [16,17]. Cyclophosphamide was also used in a clinical trial [3], but it was

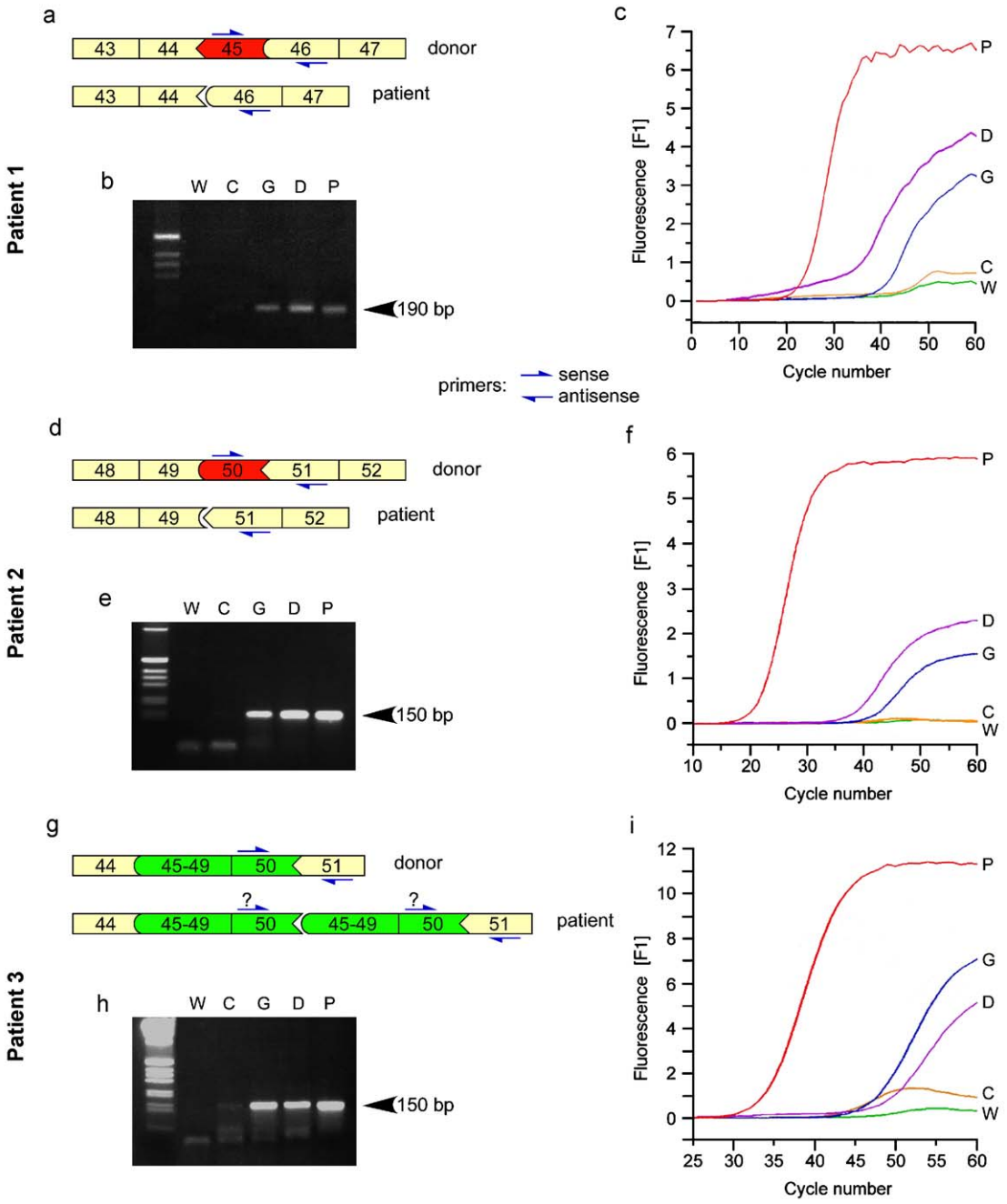

FIG. 3. Analysis of dystrophin transcripts in the muscle biopsies of the DMD patients 4 weeks after the intramuscular injection of normal myogenic cells. The abbreviations are as follows: P, plasmid (a plasmid containing the full-length dystrophin, positive control); D, donor (muscle cells from the donor); G, graft (cell-grafted site in the patient); C, control (saline-injected site in the patient); and W, water (negative control). (a, d, g) Schematic representations of the dystrophin transcripts for the donors and patients with the positions of the sense and antisense primers indicated by blue arrows illustrate the strategy used in each case to detect the donor dystrophin transcripts by RT-PCR. Question marks are placed on the sense primers corresponding to patient 3 (g) because we do not know the organization of the primary transcript after the splicing of this gene duplication. In patient 1, exon 45 was detected in the mRNA extracted from the cell-grafted site but not in the saline-injected site as observed by (b) electrophoresis and (c) real-time RT-PCR. In patient 2, exon 50 was detected in the mRNA extracted from the cell-grafted site but not in the saline-injected site as observed by (e) electrophoresis and (f) real-time RT-PCR. In patient 3 a dystrophin transcript was detected more abundantly in the mRNA extracted from the cell-grafted site than in the saline-injected site as observed by (h) electrophoresis and (i) real-time RT-PCR.

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inefficient in mice experiments, and it was suggested that this antiproliferative drug killed the transplanted cells because they proliferated after transplantation [18].

The presence of many donor-dystrophin-positive myofibers in the absence of serum antibodies against the donor cells and absence of histological evidence of acute rejection in patients 2 and 3 and only some lymphocyte accumulation in patient 1 indicate that the immunosuppression was quite appropriate. It must be noted that patients 1 and 2 were receiving chronic corticosteroid therapy (deflazacort) to delay the time course of the DMD; thus an additive effect of this treatment on the tacrolimus-based immunosuppression is perhaps possible. The long-term benefit of tacrolimus-based immunosuppression for myogenic-cell transplantation in DMD patients remains to be tested in future studies. In these future experiments, the transplantation of myogenic cells throughout the whole muscle will also permit the investigation of the physiological consequence of this intervention. However, our preclinical nonhuman primate experiments showed that an appropriate tacrolimus-based immunosuppression provides good myogenic-cell transplantation results up to 1 year following transplantation (the longest period studied) [10,11].

We used intramuscular injections as the method to deliver donor cells, because it is the only one that has repeatedly proved to ensure a good uptake of satellite-cell-derived myoblasts in skeletal muscles, the intravascular route being inefficient (for a review of donor-cell delivery to skeletal muscles see [19]). The specific sampling of the cell-grafted area in the present study was thus of importance, based on our previous observations in nonhuman primates that donor cells fuse mainly with the myofibers near the injection trajectories [11]. In fact, we observed here that the distribution pattern of donor-dystrophin expression followed mainly the trajectories of the cell injections, similar to that observed in monkeys [11]. That implies that intramuscular injections of cells must be very close to each other to reach a clinically significant percentage of dystrophin-positive myofibers in a muscle. The interinjection distances that were used in the previous clinical trials (e.g., 5 mm [3,4,6]) were too large to produce good results. Still more obvious is the impossibility of obtaining any clinical result by performing only two to eight myoblast injections in limb skeletal muscles [20,21]. Although a protocol for efficient myogenic-cell delivery by close intramuscular injections remains a challenge, some potential solutions were envisioned [22]. Obviously, this may be appropriate for limb muscles (looking to preserve autonomy and life quality in DMD patients) but not for a respiratory muscle such as the diaphragm. Nevertheless, accessory respiratory muscles, which are useful for breathing [23] and coughing [24] in quadriplegic patients, could be injected in this way.

Finally, a brief discussion must be made in reference to the problem of the early survival of the donor cells, since the early donor-cell death that follows transplantation is frequently referred as a ''limiting'' factor to myogenic-cell transplantation. Our mice studies have demonstrated that although 75 to 80% of the donor cells die during the first 3 days following transplantation; the surviving cells proliferate sufficiently to compensate for the donor-cell death [25]. Although the problem of the early donor-cell survival remains to be completely understood, our experimental results in mice suggested that this death could be due to an acute inflammatory reaction [26,27]. This problem has not been studied in nonhuman primates or in humans, but our preclinical experiments in monkeys showed that excellent myogenic-cell transplantation results can be obtained despite this early donor-cell death [10,11,28]. Whether this is produced because donor cells survive better and/or proliferate well in primates or because we inject sufficient quantities of cells to compensate any donor cell death remains to be resolved.

In conclusion, this is the first report of a protocol producing systematically an increase in dystrophin expression in skeletal muscles of DMD patients. The objective of the present study was only to verify whether a cell-delivery method and an immunosuppression protocol, based on our previous studies on nonhuman primates [10,11], were adequate for a cell-based treatment in DMD patients. For ethical reasons, at this phase we performed the injections in a small volume of muscle (less than 1 cm^3), thus a functional effect was not tested nor expected to occur. Nevertheless, the present observations are important to progress later in designing myogenic-cell implantation strategies through whole muscles, to test, at that time, for a physiologic effect.

MATERIALS AND METHODS

Patient and donor selection. Three DMD patients with identified mutations in the dystrophin gene participated as receivers for myogenic-cell allotransplantations. We determined the haplotype of both parents to verify whether one was a better MHC histocompatible donor. We tested the selected donors (the fathers) for cytomegalovirus, HIV, Epstein–Barr, hepatitis B and C, and syphilis, before performing a muscle biopsy. We performed this study with the informed consent of the patient's parents and the assent of the patients, in accordance with a protocol approved by Health Canada and the Ethical Committee for the Clinical Research of the Laval University Hospital Center.

Cell culture. We obtained the donor cells from biopsies performed in one deltoid of each donor. The muscle fragments were digested with collagenase and trypsin and cultured for 4 weeks in modified MCDB120 medium with 15% FBS and 10 ng/ml bFGF. We assessed the myogenicity of the cultured cells by the expression of NKH-1. Myogenic-cell purification by preplating [29] was planned in case the percentage of NKH-1-positive cells in the culture was lower than 80%. Preplatings were not done because the percentages of NKH-1 for all patients were always above this lower limit (Table 1). The capacity of the cells to fuse and to form myotubes and

myofibers was tested *in vitro* and by transplantation in SCID mice as previously described [30]. We excluded the tumorigenic potential of these cells by transplantation in SCID mice and by an *in vitro* test as described [31]. The presence of mycoplasma and bacteria in the cell culture was tested. We retested the donors for HIV and hepatitis before the cell transplantation, 6 months after the biopsies.

Cell implantation. Donor cells for transplantation were at passage 2 of the culture. We detached the cells from the flasks with trypsin–EDTA and washed them in HBSS. The cells (30×10^6) were resuspended in HBSS and three 100-μl Hamilton syringes with 25-gauge needles were filled with this suspension. Similar syringes were filled with saline (HBSS). Roughly 25 parallel injections were done in less than 1 cm^3 of the TAs either with cells (left leg) or with saline (right leg) under local anesthesia. We identified both sites by drawing a square on the skin, instructing patients and parents to preserve this label until the end of the trial.

Immunosuppression. Tacrolimus (a generous gift from Fujisawa Canada, Inc., Markham, Canada) administration started 7 days before the cell transplantation and was maintained until the biopsies. The initial dose was 0.3 mg/kg/day, being adjusted later to maintain a blood concentration between 8 and 20 μg/L (Table 1). We determined this blood concentration with the IMx Tacrolimus II test (Abbott, Wiesbaden, Germany). Periodic monitoring of the patients included weight, hemogram, glycemia, lipid profile, and serum levels of creatinine, urea, sodium, potassium, and chlorides.

Sampling. We performed muscle biopsies in the cell/saline-injected sites 4 weeks after the cell implantation, using local anesthesia. The muscle samples were mounted in OCT medium and frozen in liquid nitrogen. Serial sections of 10–12 μm were made in a cryostat and collected alternatively onto microscopic slides and microtubes.

Immunohistochemistry. We stained some muscle sections with hematoxylin–eosin. The antibodies used for the immunodetection of dystrophin were NCL-DYS3 (Novocastra, Newcastle upon Tyne, UK) and MANEX45A and MANEX50 (kindly supplied by Dr. Glenn E. Morris, North East Wales Institute, Wrexham, UK). The NCL-a-SARC monoclonal antibody (Novocastra) was used to detect α-sarcoglycan. Mouse anti-human CD8 and CD4 antibodies (Biosciences, Mississauga, Canada) were used to detect T lymphocytes. Incubations were 1 h with the primary antibody, 30 min in a biotinylated anti-mouse IgG antibody (Dako, Copenhagen, Denmark), and 30 min in streptavidin–Cy3 (Sigma, Oakville, Canada).

RT-PCR. We extracted the total RNA from the muscle sections and, as a positive control, from myoblasts and myotubes of the donors. Medium containing water instead of RNA was the negative control. Reverse transcription was done with Superscript II RNase H (Invitrogen, Burlington, Canada) in the presence of dystrophin-specific oligonucleotides. The primers for reverse transcription were 5′-CCAGTAACTTGACTTGCTCAAGCT-3′ (patient 1) and 5′-GTCACCCACCATCACCCTCTG-3′ (patients 2 and 3). Sense and antisense primers for real-time PCR were 5′-ACAGGAAAAATTGGGAAGCC-3′ and 5′-TTGCTGCTCTTTTCCAGGTT-3′ (patient 1) and 5′-AGGAAGTTAGAAGATCTGAGCTCT-3′ and 5′-GTAACCACAGGTTGTGTCACCA-3′ (patients 2 and 3). Reverse transcriptions were done at 42°C for 50 min. We performed real-time PCR using a LightCycler. Reactions were set up with 0.5 μM each specific primer to exons 45, 46, 50, and 51; 3 mM $MgCl_2$; 1× SYBR Green Master Mix (Roche, Laval, Canada); and 2 μl of cDNA. Cycling conditions were denaturation (95°C for 5 min), amplification, and quantification (95°C for 10 s, 60 (patients 1 and 2) or 65°C (patient 3) for 5 s, and 72°C for 10 s, with single fluorescence measurement at 80 (patients 1 and 2) or at 82°C (patient 3) at the end of the 72°C for 5 s segment) repeated 60 times; a melting curve program (72–95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement); and a cooling step to 40°C. We included a plasmid containing the human full-length dystrophin cDNA as an index of the expected size of the amplified product. We analyzed the amplified products on 1.8% agarose gel containing 1× TBE.

Cytofluorometry. We detected the presence of antibodies against the donor cells in blood samples taken before and at days 14, 28, and 56 after cell transplantation. Cells from the same batch as those transplanted were cultured in the presence of human interferon-γ and then with serum from the transplanted patient, followed by incubation with an anti-human IgG conjugated to FITC (Medicorp, Montreal, Canada). The percentage of labeled cells was measured in a flowcytometer at 488 nm.

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