ABSTRACT: We evaluated myoblast implantation in 10 boys with Duchenne muscular dystrophy (DMD) and absent dystrophin (age 5–10 years) who were implanted with 100 million myoblasts in the anterior tibial muscle of one leg and placebo in the other. Cyclosporine (5 mg/kg/day) was administered for 7 months. Pre- and postimplantation (after 1 and 6 months) muscle biopsies were analyzed. Force generation (tetanic tension and maximum voluntary contraction) was measured monthly in a double-blind design. There was increased force generation in both legs of all boys, probably due to cyclosporine. Using the polymerase chain reaction, evidence of myoblast survival and dystrophin mRNA expression was obtained in 3 patients after 1 month and in 1 patient after 6 months. These studies suggest a palatable effect of cyclosporine upon muscular force generation in Duchenne muscular dystrophy; however, myoblast implantation was not effective in replacing clinically significant amounts of dystrophin in DMD muscle.

Key words: Duchenne muscular dystrophy; myoblasts; cyclosporine; cell transplantation; gene therapy

MYOBLAST IMPLANTATION IN DUCHENNE MUSCULAR DYSTROPHY: THE SAN FRANCISCO STUDY

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Duchenne muscular dystrophy is a relentlessly progressive disease of skeletal and cardiac muscle resulting from dystrophin deficiency. The genetic defect has been localized to the short arm of the X chromosome and the gene has been cloned. In dystrophin-deficient (mdx) mice, myoblast implantation has resulted in conversion of up to 30–40% of muscle fibers from dystrophin-negative to dystrophin-positive in some immune-compromised mice. Encouraged by these results, a few clinical trials have been undertaken in boys with Duchenne muscular dystrophy (DMD) to evaluate the feasibility and safety of this approach.

Prior to initiating this clinical trial of myoblast implantation, we evaluated the effect of cyclosporine A (CsA), the immunosuppressant drug, to be used upon force generation in DMD in view of the beneficial effects of prednisone on strength in DMD. There was increased force generation in DMD during 2 months of CsA treatment. We previously showed by polymerase chain reaction (PCR) that donor myoblast derived dystrophin transcripts could be detected in 3 of 10 boys 1 month posttransplantation. We now report the clinical results from our double-blind placebo-controlled 6-month study of 10 boys with DMD, each of whom received donor myoblasts from a brother or father, and immune suppression with cyclosporine. This was a phase I trial

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designed to evaluate safety and feasibility of myoblast transplantation in patients with a lethal muscle wasting disease.

**METHODS**

**Participants.** Ten boys with Duchenne muscular dystrophy participated in this study (mean age 7.8 years, range 6–10 years). Two boys were wheelchair dependent (patients 1 and 2, Table 1) but were included because of a near perfect match in histocompatibility leukocyte antigen (HLA) typing with the male related donor. Each patient fulfilled clinical criteria for the diagnosis of Duchenne muscular dystrophy, and each had dystrophin deficiency by immunofluorescence or immunoblot of muscle biopsy tissue using the 30-kd antibody. Anterior tibial muscle strength ranged from 3 to 8 by manual muscle testing on the modified Medical Research Council scale. In most cases, the donors were the biological father, but in two instances a male sibling provided muscle biopsy tissue (patients 3 and 10, Table 1). HLA tissue typing was carried out on each patient and each prospective male donor. Four patients were perfectly matched for major HLA (class I and II) antigens with their donor, while a 50% match was obtained in the other 6. Informed consent, approved by the committees on Human Research and Experimentation at the University of California at San Francisco, Stanford University Medical Center, and the California Pacific Medical Center (formerly Children’s Hospital of San Francisco) was obtained prior to enrollment in the study. The experimental nature of the study was carefully underscored and the risks were fully elaborated. The study was conducted double blind, such that neither the members of the evaluating team nor the patients nor their families were aware of which leg received injected myoblasts and which received placebo. Cyclosporine A (CsA) administered orally as gelatin capsules, 5 mg/kg/day in divided doses after meals, was started 2 weeks prior to implantation. Eight of the 10 patients had deletions in the dystrophin gene by lymphocyte DNA analysis determined by multiplex PCR assay, which was confirmed in the complementary DNA (cDNA) of preimplant muscle biopsies taken from these patients.

**Laboratory Evaluation.** A standard serology screen for the presence of infectious disease was obtained on each patient and each male donor prior to implantation. This included: cytomegalovirus, human immunodeficiency virus, hepatitis B surface and core antigens, hepatitis C virus, human T-cell lymphotropic virus, and venereal disease. Blood urea nitrogen and creatinine were obtained along with CsA levels biweekly for 6 weeks, and then monthly during the 6 months of drug administration. Serum CsA levels were assessed 4–6 h after the last dose using radioimmunoassay. Compliance was measured by counting the pills remaining. The CsA dose was adjusted: (1) if the creatinine concentration rose to twice the baseline value in two consecutive visits or if it exceeded 0.7 mg/dL; (2) if the diastolic pressure rose more than 10 mmHg above the baseline value; (3) if systolic pressure rose more than 15 mmHg above the baseline value; (4) if the trough CsA blood

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Boldface type indicates in whom dystrophin was detected in the myoblast-injected leg using PCR or immunofluorescence. n.d., not determined.

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<th>*Percentage of cultured cells that were myoblasts at time of implantation by fluorescence-activated cell sorter analysis using monoclonal antibody 5.1 HII.45</th>
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§Patients with no detectable deletion. Thus PCR analysis was not possible.
level rose above 300 μg/mL or fell below 75 μg/mL; (5) if new clinical symptoms occurred; or (6) if infection appeared. All of the subjects avoided taking medications which are known to influence CsA metabolism. Side effects, weight, and blood pressure were monitored at 2 weeks, 4 weeks, 6 weeks, and then monthly after the introduction of CsA therapy. Each parent and subject was questioned about the occurrence of headache, gastrointestinal symptoms (nausea, vomiting, dyspepsia, abdominal discomfort, diarrhea), infection, hirsutism, fatigue, gum hypertrophy, cramps, edema, paresthesias, and tremor.

**Physiological Testing.** Physiologic studies were performed on the anterior tibial muscle bilaterally as previously described. Leg skin temperature was maintained above 32°C. Subjects were comfortably seated with the knee flexed and foot supported on an adjustable shoe-shaped platform and strapped in a fixed position at 120° plantar flexion. An adjustable strap across the metatarsal heads held the plantar surface of the foot against the platform. The distance between the anterior edge of the strap and the medial malleolus was the same for both feet at each visit (to control the length of the lever arm as a variable in force generation). The foot platform was attached to a force transducer beneath the foot plate (Gould Inc., Statham Instruments Div., Oxnard, CA). A Velcro strap across the thigh was used to restrain the knees and prevent thigh muscles from contributing force during maximum voluntary contraction of the anterior tibial muscle. To record electromyographic activity from the muscle, surface electrodes (circular disks 10 mm in diameter) were applied to the skin with conducting gel over the belly and tendon of the anterior tibial muscle. A ground electrode was taped to the skin between the recording and stimulating electrodes.

Supramaximal stimulation (stimulus intensity 30% more than that necessary to produce a maximum compound muscle action potential; 0.2 ms duration; TECA Corporation, Pleasantville, NY) of the anterior tibial muscle was accomplished through surface electrodes placed over the peroneal nerve just below the fibular head. The compound muscle action potential from the anterior tibial muscle was amplified (TECA, AA6MKIII; band pass filter settings 1.6 Hz–16 kHz) and recorded (sweep speed of 2 or 5 ms/cm) on photographic paper.

The force of the maximum voluntary isometric contraction (MVC) was determined by asking each subject to maximally dorsiflex the foot for 3 s. The signal from the force transducer was amplified by a direct current amplifier (TECA, AD6M) and fed into a calibrated analog voltmeter to provide visual feedback to the subjects and also recorded on photographic paper. Three recordings of MVC at 2-min intervals were obtained. In addition a superimposed, supramaximal, tetanic stimulus (50 Hz, 240 ms) was given during the third MVC trial to evaluate the degree of maximum voluntary activation of the anterior tibial muscle. The best of three attempts with less than 10% variation was selected as the MVC. Two recordings of isometric twitch tension were obtained with the ankle in 120 ± 5° plantar flexion, a position which evoked maximum twitch tension, to ensure reproducibility. Supramaximal stimulation of the peroneal nerve at 50 Hz for 240 ms was applied to the resting muscle and the resultant isometric tetanic force was recorded on photographic paper.

All subjects had isometric force measurements of twitch tension, MVC, tetanic force (TF), and compound muscle action potential from both anterior tibial muscles at monthly intervals for at least 4 months prior to starting CsA (natural history phase). Prior to the natural history phase, data were obtained for 2 months, but these were considered practice sessions and the data were discarded to avoid any learning effects. After implantation, all measurements were obtained monthly.

**Statistical Methods.** A paired t-test was used to determine whether force measures differed in the myoblast versus the placebo-treated leg. For each person, the difference between legs, myoblast minus placebo, was calculated to form the t-statistic. A paired t-test was also used to determine whether cyclosporin had an effect on MVC or TF. In this test, the treated and untreated leg measurements for each visit were added together. The paired differences were formed by subtracting the sum for the fourth visit from each subsequent visit for each person. The fourth visit was the last before cyclosporin administration and was considered to be the baseline (untreated) measurement. The sample size of 10 patients provided 90% power to detect an 18% increase in MVC and a 35% increase in TF over baseline values.

**Magnetic Resonance Imaging (MRI) Scans.** An MRI scan of both legs, to evaluate muscle cross-sectional area and fat content and to search for signs of immune rejection, was carried out on each subject before and at 1 and 6 months after myoblast implantation. In 1 normal volunteer adult subject, saline was injected in a site of the anterior tibial muscle in a fashion identical to that used for myoblast implantation. The saline was uniformly distributed through the entire thickness of the muscle in a narrow track,
using MRI monitoring. Following repeated strong muscular contractions, the fluid dispersed more broadly through the muscle, but none extravasated into extramuscular spaces.

**The Protocol.** Preimplantation muscle biopsies and MRI scans were obtained of the anterior tibial muscle bilaterally. A series of 20 maximal, lengthening (eccentric) contractions of the anterior tibial muscle was carried out against examiner resistance in each leg 2–3 days prior to myoblast implantation in an attempt to activate satellite cells in the patient’s muscle and enhance fusion with donor myoblasts.\(^{21,54,37,44}\) For the same purpose and at the same sitting, 30 needle (22 ga) insertions to a depth of 30 mm, in sites 5 mm apart, were performed under local anesthesia. Four to six weeks prior to implantation, a muscle biopsy (approximately 0.5 cm\(^3\)) was obtained from the biceps brachii of a brother or father.

The biopsy was transported in Ham’s F10 and 50 µg/mL gentamycin to a quality controlled commercial facility (Somatix). Here, the muscle tissue was dissociated, and myoblasts were purified by fluorescence-activated cell sorting and grown from 10\(^4\) to 10\(^8\) cells as described.\(^{2,3,45}\) Briefly, after tissue dissociation the cells were grown in medium [Ham’s F10, 10% fetal bovine serum, 5% calf serum (defined, supplemented with iron; Hyclone), 1 ng/mL basic fibroblast growth factor, 50 µg/mL gentamycin] conditioned on MRC-5 fibroblasts grown in the same medium (American Tissue Type Collection) for 24 h, filtered through a 0.45-µm filter, and diluted 1:1 with fresh medium for 2–5 days until the cultures were approximately 40% confluent. The cells were then routinely cultured without the use of conditioned medium using an antibody to a muscle-specific isoform of neural cell adhesion molecule.\(^{45}\) After 2 weeks, myoblasts were purified by fluorescence-activated cell sorting. Purified myoblasts were grown for an additional 2 weeks to yield 1–2 × 10\(^8\) cells. Myoblasts were tested for bacterial, viral, and fungal contamination prior to implantation into patients. One million myoblasts in a 50-µL volume of saline and 0.5% human serum albumin were injected into the anterior tibial muscle in each of 80–100 sites, 5 mm apart, in a rectangular grid (4 × 6 cm) centered over the belly of the anterior tibial muscle. A total of approximately 80–100 million myoblasts were injected. Placebo, consisting of saline and 0.5% human serum albumin, was injected in a similar distribution in the contralateral leg. The total volume injected into each muscle ranged between 4 and 5 mL and was well tolerated with no evidence of a compartment syndrome. Patients were given 500 mg chloral hydrate orally 45 min before the procedure, 50 µg intramuscular fentanyl 20 min prior to the implantation, and subcutaneous lidocaine for local anesthesia. All patients were awake during the procedure, and all injections were administered through a 25-gauge needle that was 3 cm long. In patients #7–10, additional injections of cells were placed at 2.5-mm intervals at the lower central portion of the injected area with a tattoo mark in the skin for later demarcation of biopsy. In patient #1, the preimplantation biopsy was done immediately prior to myoblast implantation, but in subsequent patients the preimplantation biopsy was performed between 48 and 72 h prior to the myoblast implantation. The myoblast implantation was well tolerated and produced no more than moderate discomfort. Patients #1–5 were allowed to walk immediately after the procedure with no limitation on activity, whereas patients #6–10 were kept immobile until the next day. No patient had soreness or pain the next day, and normal activities were resumed. The immobilization was a precaution to prevent possible mechanical extravasation of the suspended cells in the first hours after implantation (see below).

In each patient, 1 and 6 months after implantation, two muscle biopsies, one superficial and one deep within the muscle, were obtained from each tibialis anterior and analyzed for the presence of dystrophin using both immunofluorescence and the PCR technique.\(^{12}\) Biopsies were analyzed double blind with no knowledge of which was the placebo-injected and which was the myoblast-injected muscle. Hematoxylin and eosin stained cryostat sections of pre- and postimplant biopsies were prepared in standard fashion. Biopsies (0.1 g) were flash frozen in isopentane cooled in liquid nitrogen, coded, and stored at −80°C until analysis. In addition, serum was collected 1 month after implantation, and antibodies against donor myoblasts were evaluated by cytotoxicity as previously described.\(^{43}\) CsA was continued until 6 months after implantation and then it was stopped after the 6-month biopsy was taken. No therapeutic exercise was undertaken by patients during the study.

**Dystrophin Analysis.** Immunofluorescence analyses of frozen muscle sections were performed in two different laboratories (Pittsburgh and Stanford) using either polyclonal antibodies to dystrophin directed against the amino-terminal portion of the rod domain (60 kd\(^{15,26}\)) and the carboxyl-terminal cysteine-rich domain (d10\(^{17,19}\)), or monoclonal anti-
bodies to dystrophin directed against the rod domain and the carboxyl terminal domain. One to 10 cryosections of each biopsy were scored for the total number of fibers and the dystrophin immunostaining pattern. Cross sections of biopsies contained between 275 and 2200 myofibers, and the number of dystrophin-positive or partially dystrophin-positive fibers was compared to placebo-injected biopsies.

PCR was performed on messenger RNA (mRNA) extracted from the patient’s biopsies and subjected to reverse transcription. Two sets of primers specific for the N-terminal or C-terminal regions of the dystrophin molecule were used for amplification. One set of primers, amplifying a nondeleted region of the gene in the patient, served as a positive control for the PCR reaction and cDNA integrity; the other primer set, recognizing a sequence missing in the patient’s gene but not in the donor’s, was used to check for the survival of implanted myoblasts. The details of both the immunofluorescence and PCR analysis were as previously reported.

RESULTS

Safety and Toxicity. We found no pain, redness, or muscle swelling after implantation nor any other clinical evidence of rejection of the implanted cells. The MRI scans and muscle biopsies are discussed in more detail below.

There were no serious adverse effects from cyclosporine. One boy had fever and cough which resolved in 3 days without change in CsA schedule, and 1 boy had diarrhea, nausea, vomiting, and abdominal pain for 2 days which resolved spontaneously. CsA was withheld until symptoms improved (2 and 3 days, respectively) and then continued without recurrent difficulty. Hirsutism was noted in 1 boy and a transient perioral rash in another. The mean (±SE) serum level of CsA, obtained 4–6 h after the last dose, was 146 ± 11 μg/mL. The blood urea nitrogen did not rise significantly during CsA treatment (10.4 ± 0.9 pretreatment and 12.4 ± 0.5 mg, 6 months posttreatment). However, the serum creatinine, which was very low in all patients, increased significantly from 0.25 ± 0.02 mg to 0.31 ± 0.03 mg (P < 0.01). During 6 months of CsA treatment there was no significant change in blood pressure. The mean systolic blood pressure rose minimally, from 82.4 ± 2.3 to 82.8 ± 2.8 mmHg, and mean diastolic pressure fell from 54.0 ± 1.6 to 52 ± 2.4 mmHg. Body weight did not change significantly during the study (26 ± 3.9 kg pretreatment and 26.9 ± 4.4 kg 6 months later).

Thus there were no serious ill effects observed in this study from myoblast implantation or immune suppression with cyclosporine.

Dystrophin RNA and Protein. Dystrophin was detected by immunofluorescence staining and subjectively quantitated in both preimplantation and post-implantation biopsies. The examiners were blind to the status of each biopsy (placebo vs. myoblast injected). Myoblast implanted and placebo biopsies were compared and the results of dystrophin-positive fibers correlated between the Pittsburgh and Stanford laboratories.

In 3 patients (#5, 7, and 10), the PCR analysis of muscle biopsies taken 1 month after implantation indicated the presence of donor-derived transcripts in the myoblast-injected leg (Table 1), and in biopsies taken 6 months after implantation, donor-derived transcripts were detected in the myoblast-injected leg of 1 of these 3 patients. Immunofluorescence analyses of dystrophin protein in the biopsies were performed in the two independent laboratories using two different sets of antibodies (see Methods). There was some modest variability in the number of dystrophin-positive fibers between the two protein testing laboratories. This difference may in part be due to the subjective nature of dystrophin protein quantitation by immunofluorescence and in part to the different antibodies used by the two laboratories. However, in both laboratories, all patients demonstrated a low number of dystrophin-positive fibers in both placebo- and myoblast-injected legs possibly due to the presence of reversion events. Revertants may result from expression of dystrophin due either to second site mutations or exon skipping resulting in the production of a truncated protein. Currently, full-length dystrophin protein cannot be distinguished from truncated protein due to lack of the necessary antibodies. Biopsies from 2 of the 3 patients with detectable transcripts by PCR at 1 month and in 1 patient with detectable transcripts at 6 months showed evidence of a small increase in the number of dystrophin-positive fibers by immunofluorescence. The frequency of positive fibers in the plane of a single section was approximately 1%. Thus mRNA and protein analysis correlated well.

Sera obtained 1 month after implantation contained no antibodies against donor myoblasts by cytofluorometry.

Imaging. MRI scans prior to implantation disclosed significant fatty infiltration of muscle, along with increased connective tissue. No significant changes in
composition or cross-sectional area of the tibialis anterior muscle were observed at 1 and 6 months after myoblast implantation.

**Histopathology.** Histologic features seen in all biopsies were those of a chronic myopathic process with varying degrees of severity (Fig. 1). These changes included variation in fiber size with rounded atrophic fibers, hypertrophied fibers, split fibers, increased numbers of central nuclei, degeneration with myophagocytosis, regeneration, and endomyosial and perimysial fibrosis with focal fatty change. Biopsies from 6- and 7-year-old boys showed less variation in fiber size, fewer split fibers and central nuclei, and less fibrosis in both pre- and posttransplant biopsies than biopsies from 9- and 10-year-old boys. Three patients showed focal endomyosial, mononuclear cell infiltrates, which ranged from mild to marked, unrelated to fibers undergoing phagocytosis in posttransplant (1 month) biopsies, from both placebo- and myoblast-injected muscles. One of the patients also showed inflammation in the muscle biopsy at 6 months. Only 1 patient showed endomyosial mononuclear cell infiltrates unrelated to myophagocytosis on the pretransplant biopsy. There were no other distinguishing features between pre- and posttransplant biopsies. There were no distinctions between deep and superficial or myoblast- versus placebo-injected muscle biopsies, nor were there significant differences between 1- and 6-month posttransplant specimens. Thus there was no histologic evidence of rejection phenomena.

**Muscle Force Generation.** Both maximum voluntary contraction and the tetanic force increased steadily in both limbs after institution of cyclosporine therapy and myoblast implantation (Fig. 2). There were no significant differences between the myoblast- and placebo-treated leg muscles by any measure. During the 4 months of the natural history phase there was a decline in MVC (Fig. 2A) by $11.5 \pm 3.1\%$ (myoblast implanted) and $14.0 \pm 4.0\%$ (placebo). After 2 weeks of CsA treatment there was an increase in MVC of $9.5 \pm 7.5\%$ (myoblast) and $12.4 \pm 7.8\%$ (placebo) and a maximum increase of $25.5 \pm 20.6\%$ (myoblast) and $18.5 \pm 19.2\%$ (placebo) after 4 weeks of CsA (all values significantly greater than baseline, visit 4, $P < 0.02$). All 10 patients showed increases, ranging from $5\%$ to $47\%$ at 2 weeks and from $5\%$ to $116\%$ at 4 weeks of CsA. Similar changes were observed in the tetanic force (Fig. 2B). During the natural history phase there was a decline in tetanic force by $25.2 \pm 2.7\%$ (myoblast) and $20.9 \pm 3.5\%$ (placebo). After 2 weeks of CsA treatment tetanic force increased by $11.9 \pm 9.2\%$ (myoblast) and $16.7 \pm 7.9\%$ (placebo) and the maximum increment was $21 \pm 12.2\%$ after 4–6 weeks (all values significantly higher than baseline, visit 4, $P < 0.02$). Eight of 10 patients showed increases, ranging from $0.1\%$ to $86\%$ at 2 weeks and from $4\%$ to $145\%$ at 4 weeks of CsA. At the end of the washout period there was a decline in tetanic force of $13.7 \pm 6.9\%$ (myoblast) and $37.6 \pm 18\%$ (placebo). The decline in MVC was $28.8 \pm 8.5\%$ (myoblast) and $37.6 \pm 9.9\%$ (placebo). The data for MVC from myoblast- and placebo-treated legs were added together and found to be significantly higher than baseline (visit 4) at visits 5–11 ($P < 0.05$). Similarly, the data for tetanic force were pooled for both legs and found to be significantly higher than baseline (visit 4) at visits 5–8 ($P < 0.05$).

Twitch responses were more variable, but the trends were similar to those observed for MVC and tetanic force. During the natural history phase, twitch tension decreased by $12.4 \pm 9.1\%$ (myoblast) and $13.7 \pm 7.9\%$ (placebo). The maximal increase in twitch tension was $34.3 \pm 13.9\%$ after 4 weeks. During the washout period the decline in twitch tension was $12.6 \pm 18.5\%$ (myoblast) and $18.8 \pm 12.6\%$ (placebo) after 3 months.

During the natural history phase the mean compound muscle action potential amplitude decreased by $10.6 \pm 4.5\%$ (myoblasts) and $13.7 \pm 7.9\%$ (placebo). Subsequently, the muscle action potential in-
creased by 3.2 ± 8.9% (implanted) and 12.4 ± 13.0% (placebo).

Thus, there was a definite and significant rise in
tory generation within 2 weeks of instituting CsA
treatment. The nearly identical response in both the
myoblast- and placebo-injected muscles suggests a
drug effect.

**DISCUSSION**

There are three major findings from this study. First,
myoblast implantation as well as immunosuppression
with CsA appear to be safe in patients with Duchenne
muscular dystrophy. Second, small numbers of im-
planted donor cells survived and produced normal
dystrophin transcripts in some patients with Du-
chene muscular dystrophy but did so with a very
low efficiency which did not alter muscular strength.12
Third, cyclosporine treatment appears to increase
force generation in Duchenne muscular dystrophy.42

**Safety Issues.** More than 50 patients have been im-
planted with myoblasts at four centers in North
America and no instances of serious complications
have been encountered to date.12,21,28,43 This experi-
ence is reassuring with respect to the safety of future
studies. The myoblast implantation itself has been
performed under both general anesthesia25 and local
anesthesia.28,43 Our experience with local anesthesia
and modest sedation, implanting myoblasts in awake
patients, has been quite favorable and safe. Different
types of immune suppression have also been uti-
лизed in other centers (prednisone and cyclophos-
phamide), and neither these medications nor cyclosporine have produced any serious ill effect.

**Dystrophin Production.** PCR analyses indicate that human myoblasts can survive and produce normal transcripts in boys with DMD up to 6 months after implantation, albeit in a lower number of cases. The yield of individual dystrophin-positive fibers is low and must be increased before immunofluorescence analyses can be used to screen for the success of myoblast transfer. Immunofluorescence results are difficult to interpret using currently available antibodies, which cannot distinguish between donor- and patient-derived dystrophin. Furthermore, the yield of dystrophin-positive myofibers must be increased before this technique can be expected to produce any clinical change in strength. Recently, gene therapy at conception eliminated both the mechanical and histologic abnormalities in a mouse model. This however is not an approach that can be used in man. Tests of force improvement have not been carried out in mice treated with myoblasts postnatally. Thus, the number of positive fibers needed to produce clinically significant improvement in force generation is not known. Studies of women with dystrophinopathy suggest that weakness may only be mild or moderate if 30–40% of fibers are dystrophin positive. Thus, a substantial increase in the efficiency of this system must be achieved before it can be clinically useful in alleviating the muscular weakness of DMD.

It is not clear which factors are responsible for the low efficiency of myoblast transfer in DMD patients. To investigate the possibility that these cells were extravasated due to mechanical factors, we injected saline in a normal subject in a fashion identical to the myoblast implantation (see Methods). The fluid was nicely dispersed near the injection track and no extravasation was noted after vigorous muscle exercise. Thus, a physical redistribution of the cells outside the muscle seems unlikely. The age of the patient may be important, since the 3 patients with detectable dystrophin were among the youngest, with the least muscle fibrosis and fatty replacement. The increased connective tissue in these dystrophic muscles may represent a barrier to myoblast migration and fusion. The degree of HLA match between donor and patient (Table 1) did not seem to be a major factor, but this point cannot be settled on the basis of the present study. The possibility of a subclinical rejection of the myoblasts must be considered in view of the recent report of serum antibody formation against donor myoblasts and myotubes. The finding of uniform expression of class I major histocompatibility complex (MHC) determinants on the surface of many nonregenerating muscle fibers after myoblast implantation in another study is also consistent with some immunologic response on the host. However, two recent studies document poor myoblast survival beyond 10–14 days even in animals with virtually absent immune responses, although in another study significantly better survival was observed. Whether cyclosporine inhibits myoblast fusion is another unsettled concern.

**Increased Muscular Strength with Cyclosporine.** All muscles became stronger with a time sequence that is similar both to that reported with prednisone as well as to a previous study conducted in this laboratory which also demonstrated that cyclosporine increases force generation in boys with Duchenne muscular dystrophy. Our previous trial involved 8 weeks of CsA treatment, so that the present 6-month study extends and confirms our earlier results. The same time course of improved strength was observed in this study in the first 8 weeks of CsA treatment as in the prior trial, and there was still increased strength after 6 months of treatment compared with the baseline measurements. The increment in force became apparent within 2–4 weeks of instituting CsA therapy, and reached a peak 4–7 weeks later with a subsequent further decline. Although it is possible that the observed increase in force generation was placebo effect since there was no control for the CsA, this seems unlikely since both electrically stimulated (tetanic) and voluntarily activated force followed a similar pattern. Further, the pattern of response in this study follows a time course that is virtually identical to that observed in placebo-controlled trials of prednisone. However, these results will still require further confirmation in view of the absence of a similar effect in another recent study of CsA, which may have been due to smaller sample size.

The mechanism of increased force generation in DMD patients treated with CsA is unclear. CsA and prednisone may interfere with the production of various cytokines which prevent damaged muscle fibers from regenerating. Alternatively, CsA might promote expression of the gene encoding dystrophin in view of a recent study demonstrating that another immunosuppressive agent methylprednisolone in-
creases dystrophin expression in cultured myoblasts, albeit by a different molecular mechanism. Although prednisone appeared to increase muscle mass in boys with Duchenne muscular dystrophy, there is as yet no evidence that CsA has a similar effect. Finally, CsA may exert a direct effect upon skeletal muscle that involves increased release of calcium from intracellular stores, which is a well-documented effect of the drug in vascular smooth muscle that leads to hypertension. If CsA increases the calcium available for release from the sarcoplasmic reticulum of skeletal muscle in patients with Duchenne muscular dystrophy, this might account for the increased force generation. Whatever the mechanism, this apparent beneficial effect of the drug may point in a new direction in developing therapeutic approaches to this disease in the future.

The poor efficiency of myoblast implantation in our study is similar to that in other human trials. Even with monthly myoblast injections for 6 months the results have been disappointing. It has been suggested that both irradiation and inducing muscle damage (e.g., maracane, notexin, etc.) have been key factors in the higher yields reported in some animal studies. However, in other cases such invasive techniques have not been essential for extensive myoblast incorporation into muscle of mice. Much more investigation in animal models is needed to understand the factors which influence survival of myoblasts and the efficiency of myoblast transfer.

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