

## Myoblast Transplantation in Monkeys: Control of Immune Response by FK506

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**Abstract.** Myoblasts were grown from monkey muscle biopsies and infected *in vitro* with a defective retroviral vector containing a cytoplasmic  $\beta$ -galactosidase ( $\beta$ -gal) gene. These myoblasts were then transplanted to 14 different monkeys, 6 of which were immunosuppressed with FK506. Without immunosuppression, only a few myoblasts and myotubes expressing  $\beta$ -gal were observed 1 week after the transplantation, but no cells expressing  $\beta$ -gal were observed after 4 weeks. This result was attributed to immune responses since infiltration by CD4+ or CD8+ lymphocytes was abundant 1 week after transplantation but not after 4 weeks. The expression of interleukin 6 (IL-6), interleukin 2 (IL-2), granulocyte/macrophage colony stimulating factor (GM-CSF), transforming growth factor-beta (TGF- $\beta$ ) and granzyme B mRNAs was increased in the myoblast-injected muscle indicating that the infiltrating lymphocytes were activated. Moreover, antibodies against the donor myoblasts were detected in 3 out of 6 cases. When the monkeys were immunosuppressed with FK506, muscle fibers expressing beta-galactosidase ( $\beta$ -gal) were present 1, 4 and 12 weeks after the transplantation. There was neither significant infiltration by CD4 or CD8 lymphocytes, nor antibodies detected. The mRNA expression of most cytokines was significantly reduced as compared to the nonimmunosuppressed monkeys. These results indicate that FK506 is effective in controlling short-term immune reactions following myoblast transplantation in monkeys and suggest that it may prove useful for myoblast transplantation in Duchenne Muscular Dystrophy patients.

**Key Words:** FK 506; Immunosuppression; Monkeys; Myoblast; Transplantation.

### INTRODUCTION

Myoblast transplantation is a potential treatment for several recessive myopathies (1-14). Since normal myoblasts contain all the normal genes expressed in muscle fibers, their fusion with the patient's own myoblasts or muscle fibers can restore the expression of the gene whose absence is responsible for the disease. The restoration of the missing gene product has been first demonstrated for dystrophin in nude/mdx mice, an animal model for Duchenne Muscular Dystrophy (DMD) (2). This observation rapidly led to several clinical trials of myoblast transplantations in DMD patients (5, 7, 10-14). These clinical trials produced at best very limited results. Our research group reported the expression of dystrophin in the muscles of 6 patients as well as transient increases of strength in 3 out of 9 patients (5, 7). Similarly, Law et al reported the presence of dystrophin and some increase of strength in his patients, but these results raised some controversy (8, 10, 11, 15-18). Gussoni et al (12) observed the presence of the donor dystrophin RNA by polymerase chain reaction (PCR), but this observation

may be due to the presence of only a few donor cells. More recently Mendell et al (14) reported their results of myoblast transplantation in 6 patients immunosuppressed with cyclosporine. Only one of them, who had received myoblasts from a donor compatible for 5 out of 6 MHC loci, had 10.3% of muscle fibers expressing donor-derived dystrophin in biopsies obtained after myoblast transfer. These dystrophin-positive fibers were identified with an antibody reacting specifically with a segment of dystrophin coded by a region of the dystrophin gene absent in the patient gene. Moreover, these dystrophin-positive fibers were distributed equally throughout the superficial and deep regions of the biceps muscle. This is so far the best proof of successful myoblast transplantation in humans. The results in this recent trial by Dr Mendell were probably not good in the other patients because cyclosporine, which was used in only half of the patients, is not sufficiently effective to prevent rejection of the transplanted myoblasts, especially when the donors and the recipients are not compatible at 1 to 3 MHC loci. The other patients did not received any immunosuppression even when they were not perfectly histocompatible.

Huard et al (5) and Tremblay et al (7) attributed the limited success of most clinical trials to immune reactions against the donor cells. They observed in their own clinical trials the presence of antibodies reacting with the donor myoblasts and myotubes in 5 out of 9 patients despite the selection of MHC-compatible donors. In a subset of these patients, some of the antibodies were directed against dystrophin; expression was partly restored by the myoblast transplantation. The presence of antibodies against dystrophin following myoblast transplantation also proves that at least some muscle fibers expressing dystrophin were formed after myoblast

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transplantation. During the last four years, the Tremblay research team has demonstrated that mouse muscles injected with myoblasts were rapidly infiltrated by macrophages, T-helper lymphocytes (CD4+) and cytotoxic T lymphocytes (CD8+) (19). The peak of these infiltrations was about 1 week post myoblast transplantation. These cellular infiltrations were recently confirmed by Irintchev et al (20). Moreover, these infiltrating lymphocytes were activated since they expressed the IL-2 receptor, the mRNAs of serine protease (granzyme B) and of the lymphokine Interferon-gamma (IFN- $\gamma$ ) (21, 22). Such cellular immune reaction may have been responsible for the lack of success of myoblast transplantation in patients for which no humoral reaction was detected.

The limited success of myoblast transplantation can also be explained by at least two other types of problems. The first one identified by Karpati et al (13) is the limited mobility of the myoblasts injected in the muscle due to the extracellular matrix and the basal lamina. The second one identified by Beauchamp et al (23) and Huard et al (24) is that 95% to 99% of the injected myoblasts die within the first five days after transplantation. These two problems may be responsible for the limited increase in strength that our team observed following myoblast transplantation between two identical twin girls, the recipient a symptomatic carrier and the donor an asymptomatic carrier (25).

Various immunosuppressive treatments such as cyclosporine (26–31), cyclophosphamide (13), rapamycin (32) and FK506 (33) have been investigated for myoblast transplantation in mice and in humans. Our group has observed that the degree of success of myoblast transplantation in mice varied with the efficacy of the immunological treatment (29). The best results in mice were obtained with FK506 immunosuppression (33). These latter results suggested that perhaps the immunosuppressive treatments used in clinical trials were not sufficiently effective especially when the donor and the recipients were not MHC compatible, which was the case in most of the clinical trials. The choice of an effective immunosuppressive treatment is important. For example, our research group has recently shown in experiments in mice that cyclophosphamide, an antitumoral agent which kills rapidly proliferating cells (34), rapidly killed all of the injected myoblasts. The importance of selecting the right immunosuppressive treatment for myoblast transplantation in clinical trials led to the present investigation of FK506 immunosuppression for myoblast transplantation in nonhuman primates.

## MATERIALS AND METHODS

### Animals

Fourteen nonhuman primates (12 *Macaca mulata* and 2 African green monkeys [*Sarcopithecus eathiots sabaus*]) between

5 to 10 years old were used as recipients for this experiment. This work was authorized and supervised by the Laval University Animal Care Committee and was conducted according to the guidelines set out by the Canadian Council of Animal Care.

### Myoblast Culture and Transfection

Myoblasts were grown in culture from muscle biopsies of 9 different monkeys (7 *Macaca mulata* monkeys and 2 African green monkeys). The muscles were dissociated with collagenase and trypsin according to a modification of the technique of Yasin et al (35), and the dissociated cells were grown in MCDB 120 medium (36) with 15% FSC and 10 ng/ml basic fibroblast growth factor. The primary myoblasts were infected twice in vitro with a retroviral vector LNPOZ (gift from Dr C Cepko, Harvard University, Boston, USA) encoding the bacterial lacZ gene. The myoblasts were exposed to the virus during 4 hours and expanded during 7 days before transplantation. The percentage of infected cells at the time of injection varied between 10 to 40%.

### Myoblast Transplantation

About 7 days after infection, the myoblasts were detached from the flasks with 0.1% trypsin followed by three suspensions in Hank's balanced salt solution (HBSS) and centrifugations (6500 RPM, 5 minutes). The final concentration was about 50 million myoblasts in 0.1 ml.

The 14 monkeys were divided into 2 groups: 8 monkeys received no immunosuppressive drug; 6 monkeys received FK506 as an immunosuppressive treatment (Fujisawa Pharmaceutical Co Ltd, Osaka, Japan). FK506 was administered at 10 mg/kg/day orally starting on the transplantation day. None of the monkeys were irradiated before myoblast transplantation. In only one case (monkey #13, table 2) the recipient muscle was damaged by injection of notexin (50  $\mu$ l, 1  $\mu$ g/ml) before myoblast transplantation. On the day of transplantation, several monkeys received myoblast injections in two or three different muscles to permit muscle biopsies at different intervals during the follow-up period.

The monkeys were anesthetized with 0.5 ml of a solution containing 13 mg/ml of xylazine and 87 mg/ml of ketamine. The skin was opened to expose the biceps brachii or tibialis anterior muscles. The myoblast suspension was taken up into a glass micropipette with 10  $\mu$ m tip (Drummond Scientific Company, Broomall, PE, USA). Each muscle was injected at about 20 to 30 sites located within a one cm<sup>3</sup> region with a total of about 7 to 42 million cells. Unabsorbable suture points were placed on each side of the injection region to identify it at the time of biopsy. The skin was then closed with sutures.

### Muscle Examination

The histological results were observed in a muscle biopsy of about 0.5 to 1 cm<sup>3</sup> made at the site of myoblast injection. One, 3, 4, or 12 weeks after myoblast transplantation, the grafted muscles were biopsied under deep anesthesia. Most muscle biopsies were divided in two pieces: one for histology and one for mRNA extraction. The latter was quickly frozen in liquid nitrogen, whereas the sample for histology was immersed in a 30% sucrose solution at 4°C for 4 to 5 hours, embedded in OCT

(Miles Inc, Elkhart, In, USA) and frozen in liquid nitrogen. Serial cryostat sections (8 and 20  $\mu\text{m}$ ) of the muscles were thawed on gelatin coated slides. The 20  $\mu\text{m}$  sections were fixed in 0.25% glutaraldehyde and stained in 0.4 mM of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal, Boehringer Mannheim Canada, Laval, Canada) in a dark box overnight at room temperature to detect the muscle fibers containing  $\beta$ -galactosidase. The 8  $\mu\text{m}$  sections were used to detect lymphocyte infiltrations. Nonspecific binding sites were blocked with normal horse serum (5% in PBS, 20 minutes). The tissue was then incubated with a mouse antibody directed against human CD4 or CD8 cells (dilution of 1 in 200, Exalpha, Boston, Ma, USA) during 60 minutes. These antibodies against human CD4 and CD8 lymphocytes were found in preliminary experiments to react with monkey lymphocytes. Following this first incubation, the endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide for 15 minutes. The sections were then incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Copenhagen, Denmark) for 30 minutes and the peroxidase activity was revealed with 3,3'-diaminobenzidine (1 mg/ml) and hydrogen peroxide (0.03%).

### Cytofluorometry

A blood sample of the transplanted monkeys was taken at the time of each muscle biopsy to detect the presence of antibodies against the donor myoblasts. Samples of the grafted myoblast cultures were proliferated for three or four days. The day of the cytofluorometric analysis, these myoblasts were trypsinized and washed by centrifugation and resuspended in RPMI with 10% fetal calf serum. This myoblast suspension (40  $\mu\text{l}$ ) was first incubated 60 minutes at room temperature with 20  $\mu\text{l}$  of the serum of the transplanted monkey. The cells were then washed in 4 ml of cold PBS and incubated for 15 minutes at 4°C with 200  $\mu\text{l}$  of a 1/40 dilution of a goat anti-monkey antibody conjugated to FITC (Immunoconjugate, Tilburg, The Netherlands). Following several rinses in PBS, the percentage of labeled myoblasts was determined with a flow cytometer (FAC Sort, Beckton Dickinson) operating at 488 nm.

### RNA Extraction

Total cellular RNA was isolated from snap-frozen muscle biopsies by a modification of the guanidium isothiocyanate/phenol-chloroform isolation method as described by Chomczynski and Sacchi (37). Briefly, muscle biopsies were homogenized in 1 ml of guanidine thiocyanate buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M  $\beta$ -2-mercaptoethanol) and then divided into two 500  $\mu\text{l}$  samples. A 0.1 volume of 2 M sodium acetate pH 4.0, 1 volume of phenol (BRL, Burlington, Ontario, Canada) and 0.2 volume of chloroform/isoamyl alcohol (49:1) were added to each sample. The aqueous layer containing RNA was collected and precipitated one hour at  $-20^{\circ}\text{C}$  with 1 ml of cold 100% ethanol. The pellets were resuspended in 150  $\mu\text{l}$  of the guanidine thiocyanate buffer and the two samples of each muscle were pooled. The RNA was reprecipitated with 21  $\mu\text{l}$  of 3 M sodium acetate pH 5.2 and 780  $\mu\text{l}$  of cold 100% ethanol for one hour at  $-20^{\circ}\text{C}$ , pelleted and then washed with cold 70% ethanol. The isolated RNA was air-dried and resuspended in 200  $\mu\text{l}$  of

TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) until DNase.

### DNase

The RNA (200  $\mu\text{l}$ ) was incubated with 1.2  $\mu\text{l}$  of 1 M  $\text{MgCl}_2$ , 0.8  $\mu\text{l}$  of 2.5 M NaCl and 10 U of DNase (Promega-Fisher, Quebec, Canada) for one hour at 37°C. The RNA was then extracted with 100  $\mu\text{l}$  of phenol and 100  $\mu\text{l}$  of chloroform/isoamyl alcohol (24:1). The upper layer was collected and a second extraction was performed with 200  $\mu\text{l}$  of chloroform/isoamyl alcohol (24:1). The aqueous layer containing RNA was precipitated by adding 0.1 volume of 3 M sodium acetate pH 5.2 and 2.2 volume of cold 100% ethanol for 10 minutes on dry ice. The pellets were air-dried and resuspended in 10 to 30  $\mu\text{l}$  of TE (10 mM Tris-HCl pH 7.4, 0.1 mM EDTA). RNA extracts were kept at  $-80^{\circ}\text{C}$  until cDNA synthesis.

### Reverse Transcription (RT)

First strand cDNA synthesis was performed in a 20  $\mu\text{l}$  reaction volume containing 400 or 800 ng of the isolated RNA with 200 ng of oligo dT, 26 U of RNase inhibitor (Promega-Fisher, Quebec, Canada), 0.5 mM of each dNTP and 200 U of MMLV reverse transcriptase (Gibco-BRL, Burlington, Ontario, Canada) as described by the manufacturer. For samples to be directly compared, cDNA was synthesized using a master mix of reagents. Negative controls (without MMLV) for the presence of genomic DNA contaminant were performed on all samples, as well as on water.

### PCR Analysis

PCR amplification of cDNA was performed with human primers as previously published for transforming growth factor-beta (TGF- $\beta$ ) (38), granzyme B (GrB) (39), interleukin 2 (IL-2), interleukin 6 (IL-6) and granulocyte/macrophage colony stimulating factor (GM-CSF) (40) (Table 1). cDNA (1.5  $\mu\text{l}$ ) was amplified in a 50  $\mu\text{l}$  reaction volume containing buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl; 1.5 mM  $\text{MgCl}_2$ ; 1 mM  $\text{MgCl}_2$  with GM-CSF primers), 0.2 mM of each dNTP, 1.5 U Taq Polymerase (Promega) and primers. All reagents were added as a master mix to samples. All samples for each treatment were analyzed in a single PCR amplification for each cytokine. Glyceraldehyde phosphate dehydrogenase (GAPDH) primers were used concomitantly with the cytokine primers, first to rule out failure of the RT reaction and PCR amplification and secondly to detect gross variation in cDNA quantity among samples. Concentrations of primers were: 0.5  $\mu\text{M}$  IL-6 primers + 0.06  $\mu\text{M}$  GAPDH primers; 0.5  $\mu\text{M}$  IL-2 primers + 0.06  $\mu\text{M}$  GAPDH primers; 0.15  $\mu\text{M}$  TGF- $\beta$  primers + 0.08  $\mu\text{M}$  GAPDH primers (580 bp); 0.25  $\mu\text{M}$  GM-CSF primers + 0.06  $\mu\text{M}$  GAPDH primers; 0.25  $\mu\text{M}$  Gr B primers + 0.10  $\mu\text{M}$  GAPDH primers. Note that a 580 bp amplicon of GAPDH was used to standardize the TGF- $\beta$  mRNA amplification, and a 307 bp amplicon of GAPDH was used to standardize the other cytokine mRNAs (Table 1). The samples were amplified in a 9600 Perkin Elmer Cycler with the following parameters: denaturation 3 minutes 94°C; amplification cycle: 1 minute 94°C, 1 minute 58°C (55°C for IL-6), 1 minute 72°C, 30 cycles (TGF- $\beta$ ), 32 cycles (GrB), 35 cycles (IL-6), 38 cycles (GM-CSF, IL-2); extension 5 minutes 72°C. Optimization of conditions was carried

TABLE 1  
Primer Sequences and Concentrations for RT-PCR

Gene	Primer	Product size	$\mu\text{M}$	GAPDH $\mu\text{M}$
IL-2	Forward	5'ATGTACAGGATGCAACTCCTGTCTT3'	0.50	0.06
	Reverse	5'GTTAGTGTGAGATGATGCTTTGAC3'		
IL-6	Forward	5'ATGAATCCTTCTCCACAAGCGC3'	0.50	0.06
	Reverse	5'GAAGAGCCCTCAGGCTGGACTG3'		
GM-CSF	Forward	5'ATGTGGCTGCAGAGCCTGCTGC3'	0.25	0.06
	Reverse	5'CTGGCTCCCAGCAGTCAAAGGG3'		
Gr B	Forward	5'GACTTCGTGCTGACAGCTGCTCAC3'	0.25	0.10
	Reverse	5'CGTCCATAGGAGACAATGCCCTGG3'		
TGF- $\beta$	Forward	5'GCCCTGGACACCAACTATTGCT3'	0.15	0.08
	Reverse	5'AGGCTCCAAATGTAGGGGCAGG3'		
GAPDH	Forward	5'CATCACCATCTCCAGGAGC3'	—	—
	Reverse	5'CATGAGTCCTTCCACGATACC3'		
GAPDH	Forward	5'ACCAGCGCTGCTTTTAACTCTG3'	—	—
	Reverse	5'CAGTAGAGGCAGGGATGATGTTCTG3'		

out for each primer with and without GAPDH to avoid false appearance of uniformity of product. All measurements of cytokine mRNAs were done at least twice during the exponential phase of the PCR amplification and negative controls were performed in all experiments. GAPDH primers were designed using Genbank sequence information and Genework Software.

#### Semiquantitative Analysis

Amplified products (10  $\mu\text{l}$ ) and DNA markers (500 ng pGEM, Promega-Fisher, Quebec, Canada) were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide. The bands were photographed with a Polaroid 667 type film and the negatives were analyzed by "Bioimage-Visage 110S" (Millipore Corp., Ann Arbor, Michigan). When needed, cDNA volumes used for PCR were adjusted to obtain similar GAPDH sample signals. To correct for further possible variations in mRNA concentrations in each sample, the densitometry value for each cytokine was divided by the corresponding GAPDH value.

#### Statistical Analysis

The significance of the results was evaluated with an ANOVA (Stat View 4.0.1, Abacus Concept Inc, Berkeley, CA). A Fisher a posteriori test was used to determine the level of significance of differences between subgroups.

### RESULTS

#### 1) Allotransplantation Without Immunosuppression

Myoblast allotransplantations were done in 8 monkeys without any immunosuppressive treatment. The results of these transplantations have been observed at 1 week (8 monkeys) as well as at 4 weeks (6 monkeys) posttransplantation.

a) *Presence of Myoblasts, Myotubes and Muscle Fibers:* The myoblasts were labeled in culture with a retrovirus containing the  $\beta$ -galactosidase ( $\beta$ -Gal) gene before transplantation to facilitate the identification of donor cells in the muscle injected with myoblasts. However,

since only 10 to 40% of the myoblasts were labeled in culture by the retrovirus, the number of cells of donor origin in the muscle biopsy is underestimated. In monkeys, without immunosuppressive treatment, only a few myoblasts and myotubes expressing  $\beta$ -gal were usually observed one week after transplantation (Table 2, Figs. 1A, B). Four weeks after myoblast transplantation, no myoblasts, no myotubes and no muscle fibers expressing the  $\beta$ -gal reporter gene were observed (Fig. 1C).

b) *Cellular Infiltration:* Without immunosuppressive treatment, infiltration by CD4+ (T helper) and CD8+ (T cytotoxic) lymphocytes was always present one week after myoblast injections (Figs. 1D, 1E; Table 2). No infiltration was observed in muscle biopsies of the same monkeys performed at a site not injected with myoblasts. This cellular infiltration was always reduced and sometimes completely absent 4 weeks after transplantation (Fig. 1F).

c) *Antibodies Against the Donor Myoblasts:* In non-immunosuppressed monkeys, antibodies against the donor myoblasts were never detected by cytofluorometry in the preimmune serum of the recipients as well as 1 week after myoblast transplantation. Such antibodies were, however, detected in 3 out of 6 cases 4 weeks after myoblast transplantation (Table 2).

d) *RT-PCR Results:* RT-PCR amplification was also used to detect the mRNAs of serine protease (granzyme B) and of several cytokines. The results of the amplifications are illustrated in Figure 2. The amplifications were normalized from one muscle biopsy to another by amplifying simultaneously the mRNA of GAPDH, a housekeeping gene. Presence of granzyme B, IL-6, IL-2, GM-CSF and of TGF- $\beta$  mRNAs at concentrations above those observed in the control (i.e. muscle biopsy obtained before any myoblast transplantation) were always observed 1 week after transplantation (Figs. 2, 3). Four weeks after myoblast transplantation, all cytokines and

TABLE 2  
Results of Myoblast Transplantation in Monkeys

	Duration after MT (Weeks)	Treatment	Number of myoblasts (% of infected cells)	Expression of $\beta$ -galactosidase		Anti-donor myoblast Ab	Infiltrating cells	
				Myoblasts/Myotubes	Muscle fibers (number)		CD4	CD8
1MM	1	None	24 × 10 <sup>6</sup> (40)	++	2	neg	+	+
	4	None	24 × 10 <sup>6</sup> (40)	-	0	pos	-	-
2MM	1	None	24 × 10 <sup>6</sup> (40)	+	0	neg	+++	+++
	4	None	24 × 10 <sup>6</sup> (40)	-	0	pos	-	-
3MM	1	None	24 × 10 <sup>6</sup> (40)	+	0	neg	+	+
	4	None	24 × 10 <sup>6</sup> (40)	-	0	neg	+	+
4MM	1	None	24 × 10 <sup>6</sup> (30)	-	0	neg	++	++
	4	None	24 × 10 <sup>6</sup> (30)	-	0	pos	+	-
5MM	1	None	24 × 10 <sup>6</sup> (30)	-	0	neg	+	+
	4	None	24 × 10 <sup>6</sup> (30)	-	0	neg	-	+
6MM	1	None	13 × 10 <sup>6</sup> (20)	+	3	n.d.	+	+
7MM	1	None	13 × 10 <sup>6</sup> (20)	+	3	n.d.	+	+
8MM	1	None	7 × 10 <sup>6</sup> (40)	+++	12	neg	++	++
	4	None	7 × 10 <sup>6</sup> (40)	-	0	neg	-	-
9MM	1	FK506	27 × 10 <sup>6</sup> (10)	+	3	neg	-	-
	4	FK506	27 × 10 <sup>6</sup> (10)	+	3	neg	-	-
10MM	4	FK506	42 × 10 <sup>6</sup> (7)	+	60	neg	-	-
11AG	1	FK506	16 × 10 <sup>6</sup> (10)	+	2	neg	-	-
	4	FK506	16 × 10 <sup>6</sup> (10)	+	90	pos	-	-
12AG	12	FK506	16 × 10 <sup>6</sup> (10)	+	40	neg	-	-
	1	FK506	14 × 10 <sup>6</sup> (10)	+	3	neg	-	-
12AG	4	FK506	14 × 10 <sup>6</sup> (10)	+	100	neg	-	-
	12	FK506	14 × 10 <sup>6</sup> (10)	+	60	neg	-	-
*13MM	3	FK506	47 × 10 <sup>6</sup> (8)	+	332*	neg	-	-
14MM	4	FK506	21 × 10 <sup>6</sup> (7)	+	72	neg	-	-

\* = The muscle of this monkey was damaged by notexin injection before myoblast transplantation.

+++ = More than 50 cells/section of 10 mm<sup>2</sup>.

++ = Between 10 to 50 cells/section of 10 mm<sup>2</sup>.

+ = Less than 10 cells/section of 10 mm<sup>2</sup>.

- = Less than 1 cell/section of 10 mm<sup>2</sup>.

Neg = Negative.

Pos = Positive.

n.d. = Not done.

MM = *Macaca mulata* recipient and donor.

AG = African green recipient and donor.

MT = Myoblast transplantation.

granzyme B were back towards normal values (Figs. 2, 3).

## 2) Transplantation Results in Monkeys Immunosuppressed with FK506

Myoblasts labeled with the  $\beta$ -gal reporter gene were also transplanted in 6 monkeys immunosuppressed with FK506. The results of these transplantations were observed at 1, 3, 4, and 12 weeks posttransplantation.

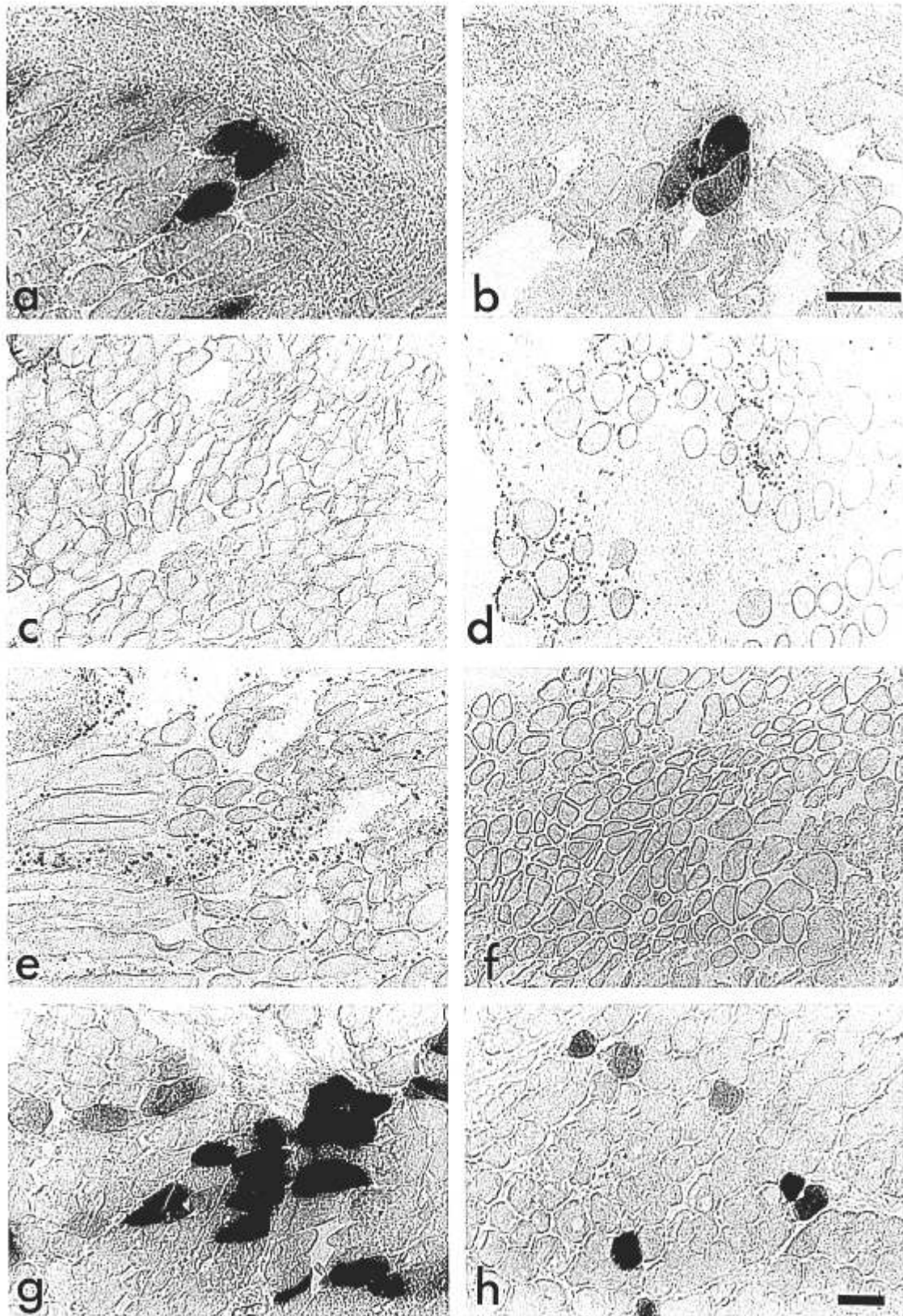
a) *Presence of Myoblasts, Myotubes and Muscle Fibers*: Myoblasts and myotubes expressing the  $\beta$ -gal reporter gene were always observed in the injected muscles after either 1, 4, or 12 weeks posttransplantation. Moreover, although only a few  $\beta$ -gal muscle fibers were observed 1 week after transplantation (Table 2), many

muscle fibers expressing  $\beta$ -gal were observed at 4 (Fig. 1G) and 12 (Fig. 1H) weeks posttransplantation (Table 2).

b) *Cellular Infiltration*: There was essentially no significant CD4+ or CD8+ lymphocyte infiltration of the muscle injected with myoblasts. Occasionally a few lymphocytes were observed but they were no more abundant than in normal muscles not injected with myoblasts (Table 2).

c) *Antibodies Against Donor Myoblasts*: Antibodies reacting with the donor myoblasts were usually not observed by cytofluorometry in the recipient serum at any interval after myoblast transplantation (Table 2).

d) *RT-PCR Results*: The mRNA of granzyme B was significantly lower in animals immunosuppressed with



**Fig. 1.** Monkey muscle cross sections obtained one week (a, b) and four weeks (c) after myoblast transplantation without immunosuppression. There are only a few  $\beta$ -gal-positive cells one week after the transplantation, but none were observed at 4 weeks. Without immunosuppression there was infiltration at 1 week by CD4<sup>+</sup> lymphocytes (T helper cells) (d) and CD8<sup>+</sup>

FK506 than in those not immunosuppressed (Figs. 2, 3). At four weeks posttransplantation, granzyme B was not significantly different between the immunosuppressed and the nonimmunosuppressed monkeys. Granzyme B mRNA was, however, significantly increased at 4 weeks posttransplantation compared to control values (muscles not transplanted with myoblasts). The increase was, however, less than that observed at one week posttransplantation in monkeys not immunosuppressed. In the immunosuppressed monkeys, no increase of the mRNAs of IL-2 and GM-CSF was observed either at one or four weeks posttransplantation. TGF- $\beta$  expression was, however, significantly increased 1 week but not 4 weeks after transplantation in the immunosuppressed animals (Figs. 2, 3). The increase at 1 week was, however, significantly less than that observed in nonimmunosuppressed monkeys. The FK506 immunosuppression thus controlled partially or completely the increased expression of these cytokines as observed in nonimmunosuppressed transplantation. The immunosuppression with FK506 did not, however, prevent an increase of the mRNA of IL-6 one week after the transplantation.

#### DISCUSSION

The present study in primates shows that allogenic myoblast transplantations are rapidly rejected in the absence of immunosuppressive treatments. One week after myoblast transplantation, the presence of a cellular immune reaction was suggested by infiltration of CD4+ or CD8+ T lymphocytes. Expression of cytokine genes (i.e. IL-2, IL-6, TGF- $\beta$  and GM-CSF) was increased after one week but was reduced to normal levels at four weeks. Moreover, the same tendency was noted for the cellular cytotoxicity activity as measured by an increase of the mRNA for granzyme B. Cytokine mRNA profiles, especially those linked to CD4+ helper T cells, have been shown in different experimental transplantations to be associated with events of allogenic reactions. In particular, IL-2 and IL-6 gene transcription, which primarily mediate cellular immunity and antibody responses by activation of T helper-1 and T helper-2, have been shown to be associated with rejection in different kinds of organ transplantation (41, 42). Furthermore, IL-6, TGF- $\beta$  and GM-CSF produced by alloreactive T cells, NK cells, or resulting from nonspecific inflammatory process following activation of granulocytes and macrophages were demonstrated to correlate with histological rejection of heart or kidney transplants (43, 44).

The presence of alloreactive cells, as revealed by specific patterns of cytokine gene expression and enzyme

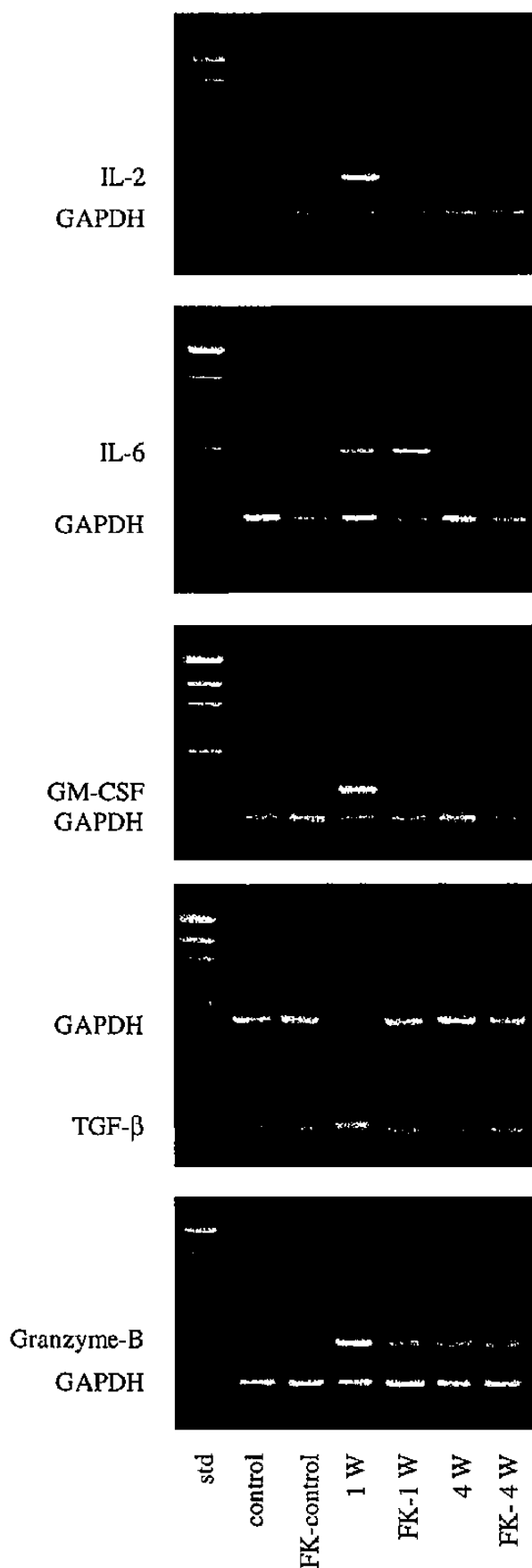
reactivity of granzyme A and B, have also been shown to correlate with cellular immune rejection in different types of organ transplantation (45–51). Recently we have shown an increase of granzyme B gene expression and interferon gamma production during rejection of myoblast transplantation in mice (21). In monkeys, the allogenic reaction seems to be induced early after transplantation. Since monkeys have an immunological system very similar to humans, our observations in monkeys may account for the poor success of previous studies of myoblast transplantation in dystrophic patients (5, 7, 10–14).

The presence of antibodies in the serum of the recipients reacting specifically with the myoblasts of the donor also indicates that there was an humoral immune reaction in about half of the recipients following these allotransplantations. As in mice, this humoral reaction was delayed relative to the cellular immune reaction (19). The presence in the serum of antibodies reacting with the donor myoblasts was also observed in about half of the cases following myoblast transplantation in DMD patients (5, 7). It is remarkable that as in primates, this humoral immune reaction was also observed in only half of the subjects. This observation sustains our hypothesis that in DMD patients in which no transplantation success was obtained and yet no humoral reaction was detected, the lack of success was due in part to a rapid cellular immune reaction not detected in muscle biopsies of the patients because they were done too late (13).

In monkeys, the cellular infiltration of the myoblast-injected muscles by helper and cytotoxic T lymphocytes is remarkably reduced when FK506 immunosuppression is used. FK506 is a relatively new immunosuppressant which is now on the market in Japan and which is used in phase III clinical trials in the United States. This drug inhibits the calcium dependent phosphatase, calcineurin dependent signal transduction. It suppresses IL-2 synthesis and downregulates the IL-2 receptor (52). A similar reduction of cellular infiltration was also observed following myoblast transplantation and immunosuppression with FK506 in mice (33), but this phenomenon is clearer in monkeys. In the present study with immunosuppression, the significant reduction of lymphocyte infiltration is also confirmed by the reduction of mRNA expression of granzyme B, as well as of several cytokines. The FK506 treatment seems efficient in controlling the cellular immune reaction one week after the transplantation as indicated by the reduced expression of IL-2, TGF- $\beta$  and GM-CSF. As shown in other studies, this treatment has no effect on proinflammatory cytokine such as IL-6

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lymphocytes (T cytotoxic cells) (e) but not at 4 weeks (f).  $\beta$ -gal-labeled muscle fibers were present at 4 weeks (g) and at 12 weeks (h) posttransplantation in animals treated with FK506.



(53). Moreover no antibodies against the donor myoblasts were detected in the animals treated with FK506. This last observation indicates that this drug also controls the humoral immune reaction. This absence of humoral reaction was also observed following myoblast transplantation in mice treated with FK506 (33). The long-term effects of FK506 immunosuppression will have to be investigated further. Indeed at four weeks posttransplantation, there seems to be some residual granzyme B activity, suggesting that the cellular immune reaction is not completely controlled by FK506. This may be due to the activity of NK cells. The results of myoblast transplantation were examined at 12 weeks in 2 monkeys and many muscle fiber expressing the  $\beta$ -gal reporter gene were observed, suggesting that the FK506 immunosuppression is still adequate. Unfortunately, no muscle samples for PCR investigation were obtained from these monkeys.

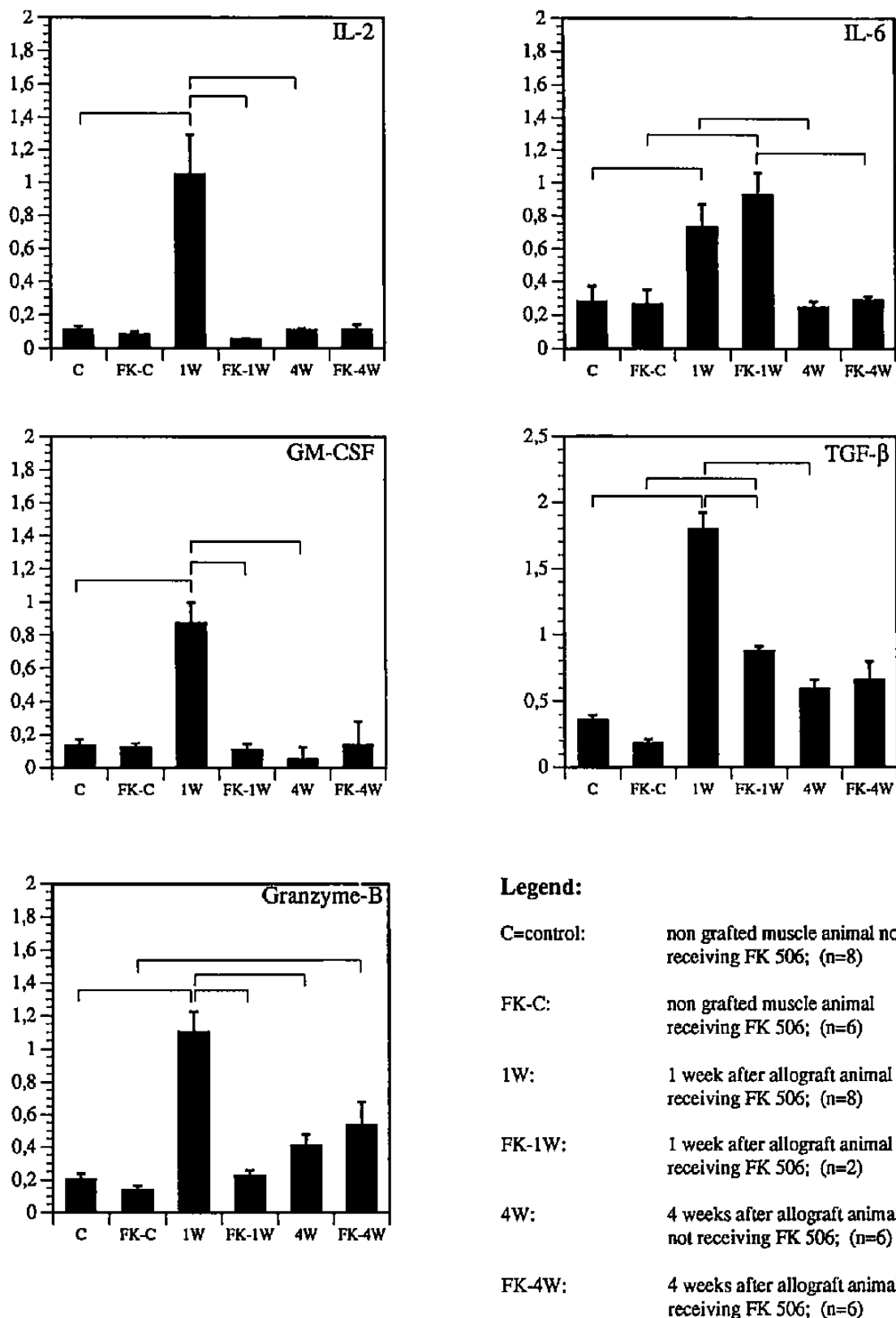
FK506 was previously shown to be a good immunosuppressive treatment for myoblast transplantation in mice (33). The results obtained in the present series of experiments also indicate that FK506 is an effective short-term immunosuppressor for myoblast transplantation in primates. The possibility of using FK506 for myoblast transplantation in DMD patients is also supported by the excellent transplantation results obtained with that drug in heart, liver, lung, and intestinal transplantations in children (54–56). Although muscle fibers expressing the  $\beta$ -gal reporter gene were obtained in the present study, the number of labeled muscle fibers was rather low considering that 15 to 20 million myoblasts were injected in a region of only one  $\text{cm}^3$  for each muscle. This problem may be due to a rapid death of more than 95% of the myoblasts during the first five days following their transplantation. This problem has indeed been reported in mice by Beauchamps et al (23) and by Huard et al (24). We have not yet confirmed the existence of a similar problem in monkeys. A second problem which may be responsible for the limited number of labeled muscle fibers following myoblast transplantation is the difficulty of motion of the myoblasts through the connective tissue and the basal lamina. Both of these problems will have to be resolved before a new clinical trial can be undertaken. Some progress has recently been made in our laboratory and the mortality of injected myoblasts has been reduced to 15% in mice (manuscript in preparation). We have also recently observed that damaging monkey muscles with notexin before myoblast transplantation increased the number of muscle fibers expressing the  $\beta$ -gal

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Fig. 2. Examples of PCR amplification of cytokine and granzyme B mRNAs. GAPDH mRNA was always coamplified with each cytokine. (Abbreviations—same as in figure 3).



CYTOKINE / GAPDH



**Fig. 3.** This figure summarizes the results of PCR amplifications of various cytokine and granzyme B mRNAs. The intensity of the cytokine (or granzyme B) band is normalized by dividing it by the intensity of the GAPDH band. The brackets indicate significant differences ( $p < 0.05$ ) between the groups using an analysis of variance and a Fisher a posteriori test.

reporter gene by several folds (see monkey #13 in the present study). Notexin is a drug which damages muscle fibers but does not affect myoblasts (57). This drug triggered a muscle degeneration/regeneration cycle which favored the incorporation in muscle fibers of the injected myoblasts. Muscle damage may thus somehow overcome the diffusion barrier for myoblasts. Muscle damage with notexin is not acceptable in DMD patients because of the high toxicity of this drug; however, other less toxic pharmacological agents may reproduce this effect. Such treatment may not be necessary in young DMD patients since they have ongoing degeneration which is not present in the normal monkeys used in this study.

In the present study, no evaluation of the effect of myoblast transplantation on the muscle strength of the monkey was done for two reasons. First, the experiments were not made with dystrophic monkeys (none have been identified so far); therefore, these monkeys had normal strength and strength improvement was not expected. Second, myoblasts were injected only in a small portion (1cc) of the muscle to limit the number of myoblasts necessary for the experiment and yet to maximize the probability of visualizing the resulting muscle fibers one month later.

Although the myoblast transplantation is limited to a small portion of one muscle, our results illustrate the importance of immunological problems for the survival of hybrid muscle fibers. The present results therefore support the eventual use of FK506 for a new clinical trial of myoblast transplantation in DMD patients when other problems limiting the efficacy of such transplantation are resolved.

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